

**Evaluation of the Anticancer Potential of
Plants Used as Traditional Medicines by
Aboriginal People**

by

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STATEMENT OF ORIGINALITY

I certify that, unless explicitly stated otherwise, the research work described in this thesis was my own. The ideas for experiments were mostly mine and I planned the experiments and carried them out by myself. My supervisors, Associate Professor Baker and Dr Phillip Oates also contributed their ideas, offered advice and guidance and helped me to refine the manuscript. The collection of plant material, extractions and chemical analyses were carried out by others, as acknowledged elsewhere. Any published work I cited has been referenced according to the APA system.

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ABSTRACT

Chapter 1.

Aboriginal Australians maintain the oldest continuous culture on earth, and their wealth of phytochemical knowledge is largely unknown to the Western world. This study sought to tap into that knowledge, not only in the hope of finding a compound that may be of therapeutic use to cancer patients outside the indigenous communities, but also to empower the communities themselves.

This introductory chapter encompasses why it is important to find new compounds to combat cancer and the various strategies employed to do so. The importance of natural products in current chemotherapies is emphasised. Advantages of the ethnopharmacological approach to screening plants for bioactive compounds are highlighted, leading into a rationale for the current project.

Chapter 2.

This chapter described the various materials and methods employed in the investigations.

Chapter 3.

The study began by justifying the choice of cell lines and optimising their culture conditions. Next, the validity of the MTT assay as a measure of cytotoxicity was demonstrated and its refinement was described. Finally, relevant controls for these bioassays were determined.

Chapter 4.

This chapter examined the ethnopharmacological approach to drug discovery, whereby plants potentially containing suitable drug candidates were selected based on traditional

medical knowledge of two Aboriginal desert communities. These plants were screened for bioactivity against a panel of cell lines representative of the five most common types of cancer. Bioactivity was determined by demonstrating a reduction in cancer cell proliferation via the MTT assay. Using this assay, it was shown that more than half of the methanolic extracts of plants identified as having medicinal properties displayed cytotoxic or cytostatic effects against human cancer cell lines. Some of these plant extracts were more potent than others and showed selectivity for different cancer cell types.

Chapter 5.

From these initial screening studies, four different species were chosen for further evaluation. These were *Euphorbia drummondii*, *Eremophila sturtii*, *Eremophila duttonii* and *Acacia tetragonophylla*. Fresh specimens of these were collected and fractionated into methanolic, ethyl acetate and aqueous extracts, which also demonstrated a range of cytotoxic and cytostatic effects on cancer cells. The cytotoxicity of several of these extracts appeared to be via a specific mechanism, as opposed to non-specific general toxicity, as brine shrimp were not sensitive to the same treatment. Additionally, the chemical profiles of the various extracts were compared via HPLC and GC-MS analyses.

Chapter 6.

Based on the results of Chapter 5, the most promising extract, the ethyl acetate extract of *E. duttonii* (EA3), was chosen for characterisation. The effects of this extract on cancer cells were shown to be cytotoxic rather than cytostatic. Additionally, the cytotoxic effects of EA3 were shown to be associated with controlled Ca^{2+} entry into the cytosol and not a rapid influx of Ca^{2+} indicative of necrosis. Overall, the experiments suggested that EA3-induced cytotoxicity may be due to apoptosis.

The major constituents of EA3 were identified by LC-MS to be the flavonoids rutin, quercetin, luteolin, apigenin and naringenin. Several of these flavonoids are known to have cytotoxic activity *in vitro* and antitumour effects *in vivo*. However, while quercetin and luteolin alone displayed cytotoxic or cytostatic effects against the panel of cancer cell lines used in this study, they did not display bioactivity at the concentrations present in EA3. Nevertheless, due to various unknown synergistic and antagonistic interactions, it is still possible that, in combination, these known compounds were responsible for the observed cytotoxicity/cytostaticity. While there were many other compounds present in EA3 that could have explained its inhibitory effects, time constraints precluded their evaluation as the active constituent/s. Future studies will ascertain whether the observed cytotoxic effects of EA3 are due to a known or a novel compound.

Chapter 7.

A plant sample (*Haemodorum spicatum*), chosen for its purported antitumour activity, and an unexamined marine sponge, selected randomly, were screened for bioactivity. Using these examples, the ethnopharmacological approach was shown to be a relevant, but not necessarily better, strategy to selecting plants for initial screening studies.

Chapter 8.

This chapter is an overview of the various sections, commenting on some issues that were raised and discussing possible future directions. It was concluded that, even though a novel cytotoxic compound was not identified from this research, the bioactivity of plants used by Aboriginal communities as phytomedicines was verified. This may help these communities and perhaps even lead to marketable herbal products. Additionally, the results of these experiments may inspire future collaborative studies using traditional knowledge in the ongoing search for drugs to combat cancer and other diseases.

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List of Abbreviations and Acronyms

4P	4-Parameter
5FU	5-Fluorouracil
AACR	Australasian Association of Cancer Registries
ABA	Access and Benefit Sharing Agreement
ABC	ATP-binding cassette
ABS	Australian Bureau of Statistics
ACNTA	Aboriginal Communities of the Northern Territory of Australia
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
AIDS	Acquired immune deficiency syndrome
AIHW	Australian Institute of Health and Welfare
ANOVA	One-way analyses of variance
ASW	Artificial sea water
ATCC	American Type Culture Collection
ATP	Adenosine tri-phosphate
B	Bottom-fixed
BCA	Bicinchoninic acid
BSLA	Brine shrimp lethality assay
BSS	Balanced salts solution
CAM	Complementary and alternative medicine
CBD	Convention on Biological Diversity
CC	Combinatorial chemistry
CCWA	Chemistry Centre of Western Australia
CI	Confidence interval
CMFS	Calcium and magnesium free salts
COX	Cyclooxygenase

CPT	Camptothecin
CS	Cellscreen
DDW	Double deionised water
DFO	Deferoxamine mesylate
DKCRC	Desert Knowledge Cooperative Research Centre
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ETO	Etoposide
FCS	Foetal calf serum
FDA	Food and Drug Administration
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HPMA	N-(2-Hydroxypropyl) methacrylamide
HTS	High-throughput screening
I3A	Ingenol 3-angelate
IP	Intellectual Property
JBAC	Jarlmadangah Burru Aboriginal Community
L1	Deferriprone
LC-MS	Liquid Chromatography-Mass Spectrometry
mAb	Monoclonal antibody
MDR	Multidrug resistance

MEM	Minimum Essential Medium (Eagle)
MS	Mass Spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NCE	New chemical entity
NCI	National Cancer Institute
NE	Non-essential
NF- κ B	Nuclear factor kappa light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
NMSC	Non-melanocytic skin cancer
NP	Natural product
NSW	New South Wales
NT	Northern Territory
O/N	Overnight
P4P	Plants for People
PAC	Paclitaxel
PBS	Phosphate buffered saline
PRS	Physiological rodent saline
QIMR	Queensland Institute of Medical Research
Qld	Queensland
Rb	Retinoblastoma
Ro5	Rule of five
RT	Room temperature
SA	South Australia
SDS	Sodium dodecyl sulfate
SE	Standard error of the mean
SEM	Scanning electron microscopy
SNK	Student Newman-Keuls

T	Top-fixed
TB	Top- and bottom-fixed
T/CAM	Traditional, complementary and alternative medicine
TAM	Tamoxifen citrate
TEM	Transmission electron microscopy
TK	Traditional knowledge
TNE	Tris, NaCl and EDTA (buffer)
TNF	Tumour necrosis factor
TRIPS	Trade-related aspects of intellectual property rights
TTP	Time to peak
TUNEL	Terminal deoxynucleotidyl transferase-dUTP nick end labelling
TX-100	Triton X-100, t-octylphenoxypolyethoxyethanol
UK	United Kingdom
UN	United Nations
USA	United States of America
UV	Ultraviolet
UWA	University of Western Australia
Vic	Victoria
VIN	Vinblastine sulfate
VIS	Visible
WA	Western Australia
WIPO	World Intellectual Property Organization
WTO	World Trade Organization

List of Symbols

$[\text{Ca}^{2+}]_i$	Concentration of intracellular calcium ions
$[\text{Ca}^{2+}]_o$	Concentration of extracellular calcium ions
EC_{50}	50% maximal effective concentration
F_{340}	Fluorescence intensity at 340 nm
F_{380}	Fluorescence intensity at 380 nm
IC_{50}	50% maximal inhibitory concentration
K_d	Dissociation constant for Ca^{2+}
LD_{50}	50% maximal lethal dose
OD	Optical density
R	Measured F_{340}/F_{380} ratio at a particular point
$R_{\max}$	F_{340}/F_{380} ratio in the presence of 1 mM Ca^{2+}
$R_{\min}$	F_{340}/F_{380} ratio in the absence of Ca^{2+}
r^2	Squared correlation coefficient
SG	Specific gravity
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
β	Ratio at 380 nm excitation for zero Ca^{2+} and saturating Ca^{2+}
γ	Interaction index
λ	Wavelength

List of Units

bp	Base pair
°C	Degree Celsius
cm	Centimetre
Da	Dalton
g	Gram
h	Hour
km	Kilometre
kPa	Kilopascal
L	Litre
μg	Microgram
μL	Microlitre
μm	Micrometre
μM	Micromolar
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar
mosm/kg	Milliosmole per kilogram
min	Minute
mg	Milligram
mL	Millilitre
M	Molar
nm	Nanometre
nM	Nanomolar
pg	Picogram
rpm	Revolutions per minute
s	Second

I would like to dedicate this thesis to my wonderful family.

I don't say it enough, but you all mean the world to me.

Losing Greg suddenly last year has driven home just how important
it is to appreciate how vital we all are to each others lives
and to make the most of the time we have together.....

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Chapter 1: General Introduction

1.1 INTRODUCTION

The goal of this project was to use traditional Aboriginal knowledge of plant medicines as a guide to identifying compounds that may be of use in the treatment of cancer. The first section of this introductory chapter is a brief review of the literature, focusing on what we know about cancers in general and providing some illustration of what drugs have been developed to combat this notorious disease. An overview of the various approaches to drug discovery follows. Advantages of the ethnopharmacological strategy to biodiscovery are then discussed, setting the scene for the rationale of this PhD project. Finally, the scope of this thesis is addressed, in which the specific aims and main outcomes of the study are summarised.

1.2 CANCER

1.2.1 Significance

Cancer is one of the world's greatest killers and figures show its incidence is increasing. In the year 2000, 10 million new cases of cancer were diagnosed worldwide and 6 million people died from an illness related to cancer, representing a 22% increase in both incidence and mortality in ten years (Sikora *et al.*, 1999; Schwartzmann *et al.*, 2002). Moreover, it is estimated that by 2020, these figures will rise to 20 million and 10 million per year, respectively. In Australia alone, at current rates, one in three men and one in four women will be directly affected by cancer before the age of 75. Each year more than 100,000 new cancer cases are diagnosed and over 40,000 people die from cancer related illnesses (AIHW & AACR, 2008). In Australia, cancer is now the leading cause of all deaths, ahead of cardiovascular disease (ABS, 2007). However, if diagnosed early, many cancer patients can

live a normal life span when treated appropriately with surgery, radiotherapy, cytotoxic drugs and endocrine therapies (Sikora *et al.*, 1999).

To find a cure for cancer is not only a major objective of drug companies but, arguably, like finding the holy grail of science itself (Valleley, 2006). One major problem is that cancer is not a single disease, but a variety of diseases caused by numerous factors and affecting various target organs (Fearon, 1997; Dewick, 2002). In fact, there are over 200 types of cancer, with variable responses to current chemotherapeutic drugs (Sikora *et al.*, 1999). Furthermore, conventional chemotherapy can be very toxic to patients, and multi-drug resistance can also be a major obstacle (Altinoğlu & Adair, 2009; Zhu *et al.*, 2010). Hence, more targeted strategies are being developed, but this can result in the treatment being very specific for only a certain type of cancer (Segota & Bukowski, 2004). Since a complete cure for cancer is unlikely to come from any specific therapeutic agents, combined treatment with conventional chemotherapy might have to be the compromise (Sandal, 2002; Eichhorn *et al.*, 2007). This PhD project was concerned with finding new knowledge to add to the arsenal of existing anticancer treatments.

1.2.2 Cancer biology

Cell specialisation is the great advantage of multicellularity, but it necessitates that proliferation of the various specialised cell types is separately controlled. Terminal differentiation into the mature phenotype shuts down cell cycling, whereas arrest of progress in differentiation causes uncontrolled cell proliferation (von Wangenheim & Peterson, 1998). Normal cells only proliferate when compelled to do so by developmental or other mitogenic signals in response to tissue growth requirements (Deshpande *et al.*, 2005). In adult tissues, the rate of cell death must equal the rate of cell division, otherwise tissues would grow or shrink.

Excess cells undergo programmed cell death, or apoptosis, the selective removal of aging, damaged or otherwise unwanted cells (see Appendix A3). In contrast to cells that die as a result of acute injury via necrosis, cells that undergo apoptosis die neatly, without bursting and leaking their potentially inflammatory-inducing cell contents over neighbouring cells (Kerr *et al.*, 1972; Al-Lamki *et al.*, 1998). Cell death by apoptosis also occurs during embryogenesis and normal development, altering the structures the cells form (e.g. a tadpole's tail) and during the regulation of the immune response (Maddika *et al.*, 2007). Both the processes of proliferation and deletion are tightly regulated by complex signalling pathways (see Appendix A2 and A3). However, if the normal cell cycle controls are altered in any way, for example through mutation to key regulatory genes, the cell becomes vulnerable to deranged proliferation, the main hallmark of cancer (Sandal, 2002). In the interests of brevity, it was not possible to provide a discussion of what goes wrong in a cancer cell as part of this chapter. The reader is directed to Appendix A for an overview of what mechanisms are in place to prevent uncontrolled cell division.

Cancer is basically a disease caused by abnormal and uncontrolled cell cycling and division resulting in tumours which may spread throughout the body (Alberts *et al.*, 2004). Cancer cells have two main heritable characteristics: they (and their progeny) all reproduce in defiance of the normal cell cycle restraints on cell division and they invade and colonise other tissues. If an isolated abnormal cell proliferates no more than its usual neighbours, it does no significant damage, regardless of what other undesirable features it may have. However, if it proliferates uncontrolled, a tumour, or neoplasm, will arise. Neoplastic cells that remain packed together in a single mass are known as benign tumours and can usually be removed by surgery, resulting in a complete cure. On the other hand, if the neoplastic cells acquire the ability to invade surrounding tissue, the tumour is said to be malignant and is then truly considered a cancer.

Invasiveness usually means neoplastic cells detach from the parent tumour and enter the bloodstream or lymphatic system, forming secondary tumours known as metastases. The more widely a cancer metastasises, the more difficult it is to eradicate surgically or by localised irradiation, and the more deadly it is (Fidler, 1978).

Carcinogenesis (the generation of cancer) can be spontaneous or due to external agents, or carcinogens. Chemical carcinogens (e.g. asbestos and tobacco) typically cause simple local changes in the nucleotide sequence, ionising radiation (e.g. X-rays) causes chromosome breakages and translocations and viruses introduce foreign DNA into the cell. Spontaneous carcinogenesis is linked to age, as discussed Appendix A5.

As described further in Appendix A6, as well as additional DNA damage caused by exposure to radiation (e.g. UV rays) or carcinogens (e.g. tobacco), a low level of DNA damage occurs in the life of any cell. However, damaged DNA is only passed on to a cell's progeny if the DNA damage checkpoints are not functioning properly. The long-term accumulation of damaged genes in cells without checkpoints leads to an increase in the frequency of mutations and, hence, the likelihood of cancer (Sandal, 2002).

In general, there are some key properties that enable cells to grow as cancers. Six aberrant behaviours of cancerous cells include:

1. the disregard for external and internal signals regulating cell proliferation;
2. the avoidance of suicide via apoptosis;
3. genetic instability;
4. the circumvention of programmed restrictions to proliferation, such as the evasion of replicative senescence and the avoidance of differentiation;

5. angiogenic propensity; and
6. the ability to metastasise (Nebert, 2002; Hanahan & Weinberg, 2000).

Consequently, current and prospective anticancer therapies are directed at these intrinsic properties of cancer cells. While surgery and radiotherapy may be sufficient to treat a primary or localised tumour, metastases generally require chemotherapy. According to Kelland (2005), ideal cancer targets are (a) specifically expressed in tumour cells and are not present in any normal cells or tissues and (b) critically involved in maintaining the malignant phenotype.

As explained by Dewick (2002), the term *anticancer drug* is emotive, and can build up false hopes for cancer sufferers. Researchers in the National Cancer Institute (NCI) programme have used the terms *cytotoxic*, *antitumour* and *anticancer* to differentiate between the activities of compounds according to the following definitions. A *cytotoxic* agent is toxic to tumour cells *in vitro*. If this toxicity transfers to tumour cells *in vivo*, the agent is considered to have *antitumour* activity. The term *anticancer* is reserved for compounds which are toxic to tumour cells in clinical trials (Dewick, 2002). The same definitions have been adopted to describe the activities of compounds throughout this thesis.

What follows is a brief overview of some of the more important different classes of anticancer drugs, including examples. A more comprehensive description of these drugs in relation to the specific behaviours of cancer cells they target is available in Appendix B.

1.2.3 Chemotherapeutic strategies to combat cancers

1.2.3.1 *Cytotoxic drugs*

Most current chemotherapeutic agents work by impairing cell division or interfering with DNA synthesis, which means that fast-dividing cells are preferentially targeted (Valeriote & van Putten, 1975). By definition, cancer cells proliferate more rapidly than their neighbours and so drugs that inhibit mitosis effectively kill neoplastic cells more than they kill normal cells. However, some normal cells of the body, including bone marrow cells and those responsible for hair growth and for replacing the intestinal epithelium, also rapidly divide and are affected by these cytotoxic drugs, leading to the well known side effects of popular chemotherapy, such as anaemia, hair loss, nausea, immunosuppression and vomiting as well as the long-term cardiac, renal, neurological and reproductive consequences (Kerbel & Kamen, 2004; Luqmani, 2005). This also means that some fast growing tumours (e.g. Hodgkin's lymphoma) are more sensitive to chemotherapy than other, more slow-growing tumours (e.g. prostate cancer), as a larger percentage are undergoing cell division at any given time (Furuya *et al.*, 1994; Hellawell & Brewster, 2002). Cytotoxic drugs include drugs that impair DNA synthesis like antimetabolites (e.g. 5-fluorouracil), camptothecins (e.g. topotecan), anthracyclines (e.g. doxorubicin), drugs that impair DNA integrity like the platinum compounds (e.g. cisplatin) and alkylating agents (e.g. melphalan) and drugs that impair mitosis, such as tubulin destabilising agents (e.g. vinblastine) and microtubule stabilising agents (e.g. paclitaxel).

1.2.3.2 *Drugs that target molecular abnormalities*

Because cancer arises as a result of a series of genetic changes in a cell, drugs targeting these molecular abnormalities (oncogenes and tumour repressors) have recently been developed.

Molecular antibodies (e.g. trastuzumab) and small molecule inhibitors (e.g. gefitinib) belong to this class of drugs.

1.2.3.3 *Drugs that target cell cycle control*

The frequent disruption of cell cycle regulation in cancer has flagged many molecules as potential therapeutic targets. Inhibitors of cyclin dependent kinases (see Appendix A2.1, e.g. flavopiridol) and activators of the retinoblastoma pathway (see Appendix A6.2.2, no clinically used drugs as yet) target cell cycle control.

1.2.3.4 *Drugs that target resistance to apoptosis*

Defective apoptosis is one of the characteristics of cancer cells and has been implicated in various stages of cancer development and progression. Furthermore, it is the ability to evade apoptosis that appears to provide tumour cells with their capacity to resist conventional chemotherapy and radiotherapy (Khosravi-Far & Esposti, 2004). However, while cancer cells inactivate elements of the apoptotic pathway, they never disable the complete signaling cascade. This implies that at least some molecules share function between cell proliferation and cell death (Maddika *et al.*, 2007). These authors go on to suggest that since cell survival, cell death and cell cycle progression pathways are interconnected, it should be possible to develop pharmacological agents that can selectively harness cell proliferation pathways and redirect them into the apoptotic process. They mention several viral molecules that can selectively kill cancer cells, but as yet, no drugs have been developed based on this theory. Other strategies exploiting cancer cells' resistance to apoptosis, however, have yielded promising new chemotherapeutics.

These include activators of the p53 pathway (see Appendix A6.2.1, e.g. Actinomycin D) and inhibitors of the ubiquitin-proteasome pathway (see Appendix A4, e.g. bortezomib) and other signalling pathways (no clinically used drugs as yet).

1.2.3.5 *Drugs that target tumour cell immortality*

A key property of cancer cells is their immortality. In tumours, cell replication is associated with the maintenance of telomere length and integrity, usually through the reactivation of a reverse transcriptase mechanism, whereby telomerase adds TTAGGG units to telomeres (see Appendix A5). Telomerase is constitutively overexpressed in the vast majority of human cancers and telomeres are critically shorter in most tumours compared to normal tissues. This makes targeting telomeres, or the telomerase machinery, an attractive, potentially broad-spectrum, approach to cancer therapy (Kelland, 2005) (no clinically used drugs as yet).

1.2.3.6 *Drugs that target angiogenesis*

The generation of a lethal tumour requires more than excessive tumour cell proliferation. A solid tumour must also have an adequate network of blood vessels (vasculature) from normal tissue to supply nutrients and oxygen and to remove waste products (Nishida *et al.*, 2006; Yano *et al.*, 2006). However, when a tumour becomes too large ($\sim 1\text{-}2\text{ mm}^3$) for its own blood supply to support further expansion, a stressful, hypoxic and acidic microenvironment develops within the tumour. The cells in these hypoxic regions are resistant to both chemotherapy and radiotherapy (Brown & Wilson, 2004). The harsh microenvironment provides a strong selection pressure for more aggressive cancer cells and the generation of signals necessary for the growth of new blood vessels (angiogenesis) (Thornton *et al.*, 2006; Boehm-Viswanathan, 2000; Adams, 2005). In fact, neovasculature is critical to the growth and spread of malignant tumours (Ferrara, 2004a), the generation of tumour mass having been shown to be impossible without endothelial cell proliferation (Folkman, 2006a). Hence, both tumour cell proliferation

and angiogenesis together are vital to turn a small solid tumour into a life-threatening neoplasm (Folkman, 2006a; Rahman & Toi, 2003; Zhong & Bowen, 2006).

In 1971 Folkman proposed his then provocative hypothesis that arresting the growth of a tumour may be achieved by attacking its blood supply (Folkman, 1971; Verhoef *et al.*, 2006). However, it was not until recently with the identification of molecular targets and cellular pathways of angiogenesis that antiangiogenic chemotherapy came of age as a viable approach to combating the growth of solid tumours (Thornton *et al.*, 2006). However, the consequences of antiangiogenic therapy seem to be short-lived, as withdrawal from the treatment induces rapid regrowth of tumour vessels and a subsequent relapse of tumour growth (Loges *et al.*, 2010). Nevertheless, angiogenesis inhibitors have now been approved for clinical use by the FDA in the USA and elsewhere in the world (Rosen, 2001; Rahman & Toi, 2003; Folkman, 2006a). These include vascular targeting agents, antiangiogenic factors (e.g. endostatin, in China) and inhibitors of proangiogenic factors (e.g. sorafenib).

1.2.3.7 *Drugs that target DNA repair mechanisms*

A major problem with cytotoxic drugs that target DNA synthesis (see Appendix B1.1) or integrity (see Appendix B1.2) (as well as ionising radiotherapy) is that they have been shown to induce secondary cancers several years after initial exposure. This is directly related to their potential to induce DNA damage in normal cells (Madhusudan & Hickson, 2005). Research into the possibility of using inhibitors of DNA repair mechanisms in combination with chemotherapy to selectively target tumours and enhance the efficacy of current therapies is consequently undermined by the enhanced risk of inducing secondary cancers. While convincing evidence exists supporting the validity of DNA repair proteins as viable drug

targets, as yet no anticancer drugs have been developed using this idea (Madhusudan & Hickson, 2005; Sánchez-Pérez, 2006).

1.2.3.8 *Drugs that target hormones*

Strictly speaking, this is not chemotherapy, but hormonal therapy. Cancers arising from certain tissues of the body (e.g. mammary or prostate glands) may be inhibited by alterations in hormonal balance. Tamoxifen is probably the best known example of a drug that inhibits cancer cell proliferation by inactivating a hormone that promotes cell growth. It inhibits the action of estrogen and so is particularly effective against breast cancer cells that express the estrogen and/or progesterone receptor, but has no effect on cancer cells that are estrogen receptor negative (Arpino *et al.*, 2005; Ravdin *et al.*, 2007).

1.2.4 Traditional, complementary and alternative medicines

Of course, there is another popular source of therapeutics used to treat cancer that is often overlooked by orthodox medicine. This might be considered understandable given the area of alternative medicine is obviously a controversial one. An online search of the keywords *traditional medicine* and *cancer* yields a host of alternative remedies claiming to be the long-sought-for cure for all cancers. *Quackwatch.com* is an interesting website detailing cancer therapies ranging from miracle diets, like macrobiotics and the grape diet to consuming shark cartilage to injecting hydrogen peroxide. The medical world is quite justifiably concerned that these so-called cancer cures not only give false hope to patients at their most vulnerable, but might also prevent them seeking proven, conventional medical treatment or interfere with medicines and therapies they are taking (Dufault *et al.*, 2001).

However, amongst these bizarre treatments, there are legitimate medicines that have been used by thousands of people worldwide with some convincing results. For example, green

tea, mistletoe, ginseng and turmeric have all been used by various peoples over the centuries for a range of ailments, including cancer. Their bioactive constituents have since been shown to inhibit cancer cell proliferation and survival in laboratory studies (Melnick, 2006b).

Traditional medicine is a broad term used to define any non-Western medical practice. Complementary and alternative medicine (CAM) has been defined as “those healthcare and medical practices that are not currently an integral part of conventional medicine” (Silenzio, 2002). CAM can include such treatments as acupuncture, massage, aromatherapy and prayer (Fennell *et al.*, 2009). Traditional, complementary and alternative medicine (T/CAM) is a major industry in Australia and other western nations, and there is an escalating push from the community and health-care professionals for more education and research into traditional medicines (Patwardhan & Patwardhan, 2006; Fennell *et al.*, 2009). Melnick (2006a) points out the irony of labelling these therapies “alternative” given their rich historical traditions that have formed the basis of many of modern therapies. It should be remembered that botany and medicine were closely allied until the 18th century when they were separated by advances in science (Dufault *et al.*, 2001). Nevertheless, orthodox medicine requires scientific validation of efficacy and until research endeavours provide this, individual T/CAMs will bear the tag and remain, in the eyes of some at least, second rate treatments.

There is a growing demand for herbals with therapeutic value that are available without prescription (McClatchey & Stevens, 2001). Already, nearly half the population in many industrialised nations regularly use T/CAM, and the proportion is as high as 80% in developing countries (Bodeker & Kronenberg, 2002; Melnick, 2006a). Various studies

have indicated that rates of T/CAM usage are generally higher among cancer patients than among the population as a whole (Lee *et al.*, 2002). Although some people are undoubtedly becoming dissatisfied with the limitations of orthodox medicine, this increasing demand for T/CAMs largely reflects the changing beliefs, needs and values of today's society (Parris & Smith, 2003). Indeed, Astin (1998) found that the majority of T/CAM users feel these alternatives more closely align with their own values, beliefs and philosophical orientations towards health and life. Many people have deep concerns about the toxicity of potent conventional medicines and have developed a respect for knowledge based on centuries of herbal use. However, critics say there is not enough science to justify the use of herbs and regulations guaranteeing that herbs can fulfil their claims are not strict enough (Dufault *et al.*, 2001).

So, while T/CAMs are often the only choice for patients in developing countries, conventional chemotherapy and radiotherapy remain the mainstays of oncological practice in the Western world. Therefore, it seems T/CAM has only a supporting role, perhaps as adjuvant therapy, to play in the treatment of wealthier cancer patients. Nevertheless, the placebo effect can be very strong in some people and herbs will continue to be sold based on the promise that they will work.

1.2.5 The need for new anticancer agents

Cancer is the most common cause of death in both men and women (Russo *et al.*, 2005) and, while great advances have been made in the treatment of various cancers, there remains an urgent need for more effective drugs with fewer side effects (McClatchey & Stevens, 2001; Tan *et al.*, 2006).

As already stated, most effective cancer chemotherapies have significant side effects, including emesis, anaemia, immunosuppression, bruising, bleeding and hair loss, the result of cytotoxic drugs preferentially targeting proliferating cells like cancer cells, but which also affect rapidly-growing normal cells of the intestine, bone marrow, skin and hair (Sikora *et al.*, 1999). Moreover, because cancer is a disease based on random genomic mutations in various cell types, chemotherapeutic agents necessarily have different mechanisms of action and not all existing anticancer drugs will work the same way for every patient (Kunick, 2004; Senzer *et al.*, 2005). In addition, resistance to drugs that may work initially can develop due to dose-limiting toxicities of chemotherapeutic agents on some normal cells (Keyomarsi & Pardee, 2003). Even worse, multidrug resistance (MDR), whereby exposure to a single cytotoxic agent results in cross-resistance to other, structurally unrelated classes of anticancer agents, is a common phenomenon in cancer. This is due to several membrane proteins, belonging to the ATP-binding cassette (ABC) family of proteins (e.g. P-gp and MRP1), which use the energy liberated from ATP hydrolysis to bind and efflux the drugs out of the cell (Boumendjel *et al.*, 2005).

Additionally, it is usually impossible to remove every single cancer cell through surgery and first-line chemotherapy or radiotherapy and these stray cells can be the cause of clinical relapse. When dormant cancer cells at the centre of a tumour are revived by vascularisation following destruction of the tumour periphery via chemotherapy or radiotherapy, they can have greater metastatic potential (Dubowchik & Walker, 1999). Perhaps even more alarming is that alkylating agents and topoisomerase II inhibitors, like ionising radiation, can induce secondary cancers several years after initial exposure. This is directly related to their modes of action whereby they can potentially cause mutagenic DNA damage in

normal tissues (Madhusudan & Hickson, 2005). Hence, it is the combination of these drugs which will most likely yield the greatest therapeutic benefit (Dent *et al.*, 2009).

Of the 92 anticancer drugs approved by the FDA to 1999, only 17 were seen by oncologists as having a high priority for widespread use, and 12 others as having some advantage in certain clinical settings (Sikora *et al.*, 1999). Furthermore, these drugs act on only a small number of molecular targets and most are cytotoxic in nature (Aherne *et al.*, 2002). Clearly then, more anticancer agents are desperately required.

Of course, developing new chemotherapeutics is an enormous undertaking. Following much time, money and effort invested in initial studies, many more years of clinical trials are required to ascertain a potential drug's effectiveness and safety in human patients. Discussing the issues involved in the latter processes of drug development is beyond the scope of this thesis. However, understanding that there are many strategies available to researchers for discovering new molecules that may be viable drug candidates is very relevant to this study. Thus, several of these will now be reviewed.

1.3 DRUG DISCOVERY

Firstly, it is necessary to define the terminology involved in drug discovery and development. Every drug discovery programme needs a starting point, known in the business as a *lead* (Lipinski *et al.*, 1997). A *lead series* is a set of compounds that has enough potential (based on various factors such as potency, selectivity and novelty) to be considered as a suitable chemical starting point for optimisation (Leach & Hann, 2000). *Optimisation* of a lead compound is simply the chemical modification of the molecule in order to enhance secondary properties such as absorption, metabolism and elimination and reduce toxicity so that the eventual drug has optimal function in a patient (Lee & Dordick,

2006; Hefti, 2008). A *hit* has been defined as “a compound of known structure that shows a dose-response in a primary screening assay” (Leach & Hann, 2000). In other words, a *hit* is what becomes a *lead* (Aherne *et al.*, 2002). A new chemical entity (NCE) is a compound not previously described in the literature (Wermuth *et al.*, 1998).

It should be noted here that drug development entails much more than drug discovery. In order to convert a hit into a commercial drug, many aspects must be considered. These include whether the potential drug will be clinically useful and safe to use, whether the chemical can be economically extracted, synthesised or produced on an industrial scale and whether the drug and its derivatives will be adequately protected by patents. Ultimately, drug companies must believe that the market is big enough to repay the typical US\$800 million development and marketing costs for the new drug (Firm, 2003; Verpoorte, 2005). However, these considerations are beyond the scope of this thesis which will focus on the first step in the drug discovery process, obtaining samples to screen for useful biological effects.

1.3.1 Structure-based (rational) drug design

Rational drug design, first popular in the 1970s, is the ability to make drugs to order based on structural information about the target (Petsko, 1996). Traditionally, new drugs were discovered by the trial and error testing of leads from a variety of sources in both *in vitro* and primary *in vivo* screening assays. Most of these lead sources had already undergone significant scientific investigation before being identified as a drug lead. Usually, the starting leads had physical properties consistent with previous knowledge of orally active compounds (Lipinski *et al.*, 1997). In contrast, rational drug design starts with knowledge

of specific biochemical mechanisms and targets these to fit a treatment profile (Mandal *et al.*, 2009).

In recent years, a better understanding of cancer cell biology has led to the rational design of several anticancer drugs. Imatinib (Gleevec[®]) was identified in the late 1990s by Novartis pharmaceuticals in high-throughput screens for tyrosine kinase inhibitors (Capdeville *et al.*, 2002). Mabs such as trastuzumab (Herceptin[®]) were similarly designed by scientists at Genentech using information about cancer cell biology (Goldenberg, 1999). Not only were these molecules designed from scratch, but these drugs are more selective than conventional anticancer chemotherapeutics that are generally non-specifically cytotoxic (Segota & Bukowski, 2004)

Another example of a rationally designed drug is caplostatin. Fumagillin, derived from the fungus *Aspergillus fumigatus fresnius*, exhibits broad-spectrum antiangiogenesis activity but, because it causes neurotoxicity, is not suitable for clinical use. Caplostatin, a nontoxic synthetic analogue of fumagillin conjugated to a water-soluble N-(2-hydroxypropyl methacrylamide (HPMA) copolymer, is a more suitable broad-spectrum angiogenesis inhibitor (Satchi-Fainaro *et al.*, 2005).

There are many other examples of novel anticancer drugs designed to specifically target biochemical abnormalities found in tumour cells. Not only have advances in molecular biology facilitated the identification of tumour markers to predict prognosis and therapeutic response, but various potential targets for anticancer therapies have been identified (Nahta & Esteva, 2003). Dubowchik and Walker (1999) extensively reviewed receptor-mediated and enzyme-

dependent targeting of anticancer agents, and since then many more targets for cancer therapy have emerged.

The sequencing of the human genome in 2001 (Venter *et al.*, 2001) means that new genes critical for cancer are regularly being identified. These oncogenes and tumour repressor genes represent potential targets for new anticancer drugs. In addition, there exists a wealth of structural data about biomolecules specifically expressed by cancer cells. The challenge now is for molecular and cell biologists, structural and medicinal chemists to work together to harness the wealth of information available to rationally design new anticancer therapies (Druker & Lydon, 2000; Huang & Oliff, 2001b).

1.3.2 Targeted libraries and fragments

Two other approaches that are used to meet the ever-increasing challenges of pharmaceutical objectives are targeted libraries and fragments. With targeted libraries, small banks of compounds representing scaffolds (molecules > 250 Da) known to inhibit a given class of target are tested, as these have a very high probability of delivering potent inhibitors of that target. Fragonomics uses the opposite approach in that it uses small (100 – 250 Da), simple molecules screened at high concentrations (typically > 1 mM) to generate leads. Fragments are also useful in that simpler molecules give more available chemical space for optimisation, particularly in view of the properties required for optimal oral bioavailability (Zartler & Shapiro, 2005, Carr *et al.*, 2005, Vieth *et al.*, 2004; Lipinski *et al.*, 1997).

1.3.3 High-throughput screening (HTS)

It has been estimated that, using current compound screening protocols, the chances of discovering one marketable drug is one in a million. This results in pressure to screen larger

and larger libraries, necessitating high-throughput screening (HTS), which is simply specific developments in laboratory automation designed to collect a large amount of experimental data as quickly as possible (Carnero, 2006).

In general, HTS does not actually identify a drug, but detects lead compounds and provides directions for their optimisation (Broach & Thorner, 1996). This is because many of the properties essential to the development of a successful drug, including bioavailability, toxicity, pharmacokinetics and absolute specificity, cannot be evaluated by HTS. Medicinal chemistry and pharmacological research are required to convert a lead from HTS into a useful drug; the eventual compound that becomes the drug is unlikely to have been the molecule initially screened (Broach & Thorner, 1996).

In the early 1990s HTS was still largely a manual process, but today HTS can test hundreds of thousands of compounds each day, due mainly to advances in robotics and miniaturization ("Robotic screening," 1996). Besides increasing the rate of screening, miniaturization reduces the cost, as running costs of assays depends considerably on sample volumes (Aherne *et al.*, 2002). However, as Archer (1999) states, to be truly effective, HTS should be based on more than merely improved automation. The entire system must be capable of operating at maximal capacity with no bottlenecks. Sufficient compounds to test are required, and these must be prepared in the correct layout, at the prescribed concentrations, with multiple targets in varying assay formats ready to go. In addition, all reagents must be readily available and instruments need to be capable of operating 24/7 for weeks at a time with minimal down-time for maintenance and repairs. Furthermore, flexibility and responsiveness should be built in to the HTS system to ensure it is quickly

adaptable to performing *in vitro* or functional, cell-based assays, and to allow for novel assay formats and new technologies (Archer, 1999).

HTS was developed in the late 1980s and combinatorial chemistry (CC) soon after to feed the process (Hogan, 1997; Aherne *et al.*, 2002). Other collections containing large numbers of compounds derived from various sources, including nature, exist in the pharmaceutical industry and academic institutions and these are also fodder for HTS (Aherne *et al.*, 2002). However, if fewer compounds could be tested without compromising the probability of success, the cost and time would be greatly reduced.

The advent of HTS meant the traditional method of solubilizing compounds in aqueous media under equilibrating conditions was no longer appropriate for the large numbers of compounds being screened. From then on it became normal practice for compounds to be dissolved in dimethyl sulfoxide (DMSO) and serially diluted in 96-well assay plates. This procedure results in a high discovery rate of bioactive compounds, but with poor solubility properties. In addition, while the efficiency of lead generation is high with HTS, the *in vitro* nature of the screening means that the leads generally have higher molecular weights and are more lipophilic than those obtained via rational drug design programmes. These are significant problems because most drugs are intended for oral therapy, for which high molecular weights and high lipophilicity (low water solubility) are undesirable, according to Lipinski's Rule of Five (Ro5) (Lipinski *et al.*, 1997). The Ro5 is a rule of thumb to evaluate a compound's "drug-likeness" in order to predict if it is likely to be orally active in humans. The Ro5 predicts that, in general, poor absorption or permeation is more likely when there is more than one violation of the following criteria:

1. Not more than 5 hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms);
2. Not more than 10 hydrogen bond acceptors (nitrogen or oxygen atoms);
3. A molecular weight under 500 Da; and
4. An octanol-water partition coefficient $\log P$ of less than 5.

It is so named because each cut-off value is a multiple of five (Lipinski *et al.*, 1997).

Medicinal chemists can quite easily increase the potency of a drug candidate *in vitro*, but introducing oral activity is not so simple. In fact, it is unpredictable, time and labour expensive and can easily become the rate-limiting step in drug development (Lipinski *et al.*, 1997).

1.3.4 Combinatorial chemistry (CC)

Combinatorial Chemistry (CC) was developed in the wake of HTS, but advances in CC have also driven improvements in HTS (Petsko, 1996; Newman & Cragg, 2007). CC was initially based on the simple idea that the probability of finding a molecule by random screening is proportional to the number of places one looks for it. It follows, then, that simultaneously generating numerous molecules provides numerous places to look for activity in a drug candidate (Hogan, 1996). However, CC has evolved from its early focus on generating large numbers of molecules to a potent tool for the production and optimisation of leads to drug candidates (Hogan, 1997).

The past decade or so has seen HTS of corporate compound libraries generated by CC become the main model used by major pharmaceutical companies to discover new drugs

(Keseru & Makara, 2006; Feher & Schmidt, 2003). However, Lahana (1999) questioned the reasoning behind the paradigm of CC/HTS, claiming that “After all....when trying to find a needle in a haystack, the best strategy might not be to increase the size of the haystack.”

CC is an umbrella term that covers a wide range of foci, such as the parallel synthesis of individual organic compounds or complex mixtures of organic compounds, vast phage display and nucleic acid libraries, and all the technologies associated with handling these libraries to obtain useful molecules from them (Szostak, 1997). The same author described CC as “a brute force alternative” to rational drug design.

According to Hogan (1996), the basic chemical requirements for producing combinatorial libraries are:

1. reactive complementarity of the building blocks;
2. the capacity to carry a wide variety of functional groups; and
3. non-equivocal reaction pathways.

CC has provided medicinal chemists with an unprecedented ability to synthesise very large numbers of molecules at a lower cost per unit than traditional synthetic methods (Leach & Hann, 2000). However, at least in the early days of CC, before sample quality and supporting computational techniques were more highly developed, the hit-rate was disappointingly low. Indeed, hopes for useful leads from unfocused combinatorial chemistry libraries of mixtures evaporated years ago (Li & Vederas, 2009).

The problem is that large numbers of molecules do not necessarily provide a drug candidate as the ability to generate molecules has inherent upper limits. This means that preselection of molecules for drug candidature is necessary (Hogan, 1996).

Large numbers of molecules also present a major challenge for managing the structural information obtained from biological screening assays. As the variety of molecules that must be produced is inversely proportional to the information the chemist starts with, when there are no data about a target, as many different molecular shapes and sizes as possible are required, and this is what drove the initial increase in CC (Hogan, 1996).

However, while CC allows for the synthesising of more and more compounds faster and cheaper than methods based on extracting molecules from natural products (NPs), there seems little point if those compounds do not show any biological activity. Moreover, most of our current drugs are derived from NPs (Newman & Cragg, 2007). Despite these statistics, during the 1980s and 90s, drug companies began to show an increasing reliance on the systematic testing of synthetic molecules, as opposed to NPs, as their favoured source of bioactive compounds. One reason is that CC gives researchers complete knowledge and control of what they are working with (Macilwain, 1998). Furthermore, as Hogan (1996) points out, screening a diverse array of compounds against a single target gives a huge amount of information on structure and activity, and even knowledge of what does not work can be very useful in later medicinal chemistry efforts. Another reason why some pharmaceutical companies continue to favour CC is that the methodology of CC has evolved such that libraries are now smaller and focused more towards specific biological targets or groups of related targets, or towards collections that contain much of the structural aspects of NPs, instead of towards ever-increasing diversity (Leach & Hann,

2000; Newman & Cragg, 2007). This is not to say that the power of CC should not be utilised to explore chemical space, but rather that the main aim should be to strike a balance between diversity and focus (Leach & Hann, 2000; Li & Vederas, 2009).

1.3.5 Bioprospecting for natural products (NPs)

The Australian Concise Oxford Dictionary (2009, p135) defines bioprospecting as “the search for animal and plant species from which medicinal drugs and other commercially valuable compounds can be obtained”. In the past, NPs were the prime source of new chemical entities (NCEs). NPs are typically secondary metabolites produced by living organisms in response to the specific requirements of their local environments (Verdine, 1996; Strohl, 2000).

Secondary metabolites are organic compounds not directly involved in the normal functioning of an organism. In fact, their biological function is not always obvious. However, they are not formed without reason. In plants, they are important factors in survival and propagation and are produced in response to the specific conditions of their local environment (Verdine, 1996; Strohl, 2000). For example, secondary metabolites may be involved in chemical defence by deterring herbivores from browsing or insect pathogens from attacking (Lord, 1987; Wink, 1999). In other cases, the compound may be important for the everyday existence of the organism, but have serendipitous activity in an unrelated biological system (e.g. egg-derived proteins) (Colegate & Molyneux, 2008).

Historically, NPs have been a principal source of new chemotherapeutic agents, and many successful drugs were initially synthesised to mimic the action of molecules found in nature (Feher & Schmidt, 2003). In fact, more than half of all drugs in current clinical use have a

NP origin (Cragg *et al.*, 1997). Furthermore, in the past century, drugs that trace their heritage to NPs have more than doubled the average lifespan of humans and ameliorated non-fatal, but still debilitating, conditions such as chronic pain and depression (Verdine, 1996). Of the over 1,000 NCEs approved as drugs between 1981 and 2002 by the FDA, 5% were secondary metabolites and a further 23% were derived from NPs (Clardy & Walsh, 2004). The proportion was even higher if compounds “inspired by” NPs are included (Newman & Cragg, 2007; Li & Vederas, 2009). While the expansion of synthetic medicinal chemistry during the 1990s caused the proportion of new drugs based on NPs to drop from 80% in 1990 to only about 50% by the end of the decade, 13 drugs derived from NPs were still approved by the FDA between 2005 and 2007, with five of those being the first members of new classes (Li & Vederas, 2009).

Just in the area of cancer over the past 65 years, 155 small molecules were discovered, 73% being from sources other than synthetic sources, with 47% actually being NPs or directly derived from them (Newman & Cragg, 2007). In contrast, only a single *de novo* NCE arising from CC, sorafenib mesylate (Nexavar[®], for renal cell cancer), has been approved as an anticancer drug in the past 25 years (Newman & Cragg, 2007).

The important properties of compound libraries, whether derived from CC or archiving, are quality and diversity (Aherne *et al.*, 2002). Obviously, nature has been performing CC for millennia and the amount of chemical diversity found in natural compounds is unrivalled (Petsko, 1996; Verdine, 1996). Natural compounds (and existing drugs) are substantially more diverse than combinatorial compounds, much of the chemical space covered by NPs and drugs containing no representative combinatorial compounds (Feher & Schmidt, 2003). Moreover, the diversity of combinatorial compounds is confined to an area where NPs

show little diversity, indicating perhaps the restricted region of chemical space explored by CC. This relative lack of diversity of synthetics could be why combinatorial libraries have failed to generate leads at the rates forecast and why the pharmaceutical industry is again turning to NPs instead of focussing on the production of synthetic drugs (Feher & Schmidt, 2003; Guevara-Aguirre & Chiriboga, 2005).

Another reason could be their lack of quality. The world of drug-like compounds is quite limited, with most compounds possessing drug-like properties existing in small tight clusters of chemical space (Lipinski, 2000). Desirable drug-like features (“drug-likeness”) include favourable absorption, distribution, metabolism, excretion and toxicity (ADMET) properties and their higher prevalence in NPs compared to synthetic compounds is partly explainable by evolutionary pressures (Lipinski, 2000).

Additionally, the chemical properties of NPs mean they are generally more suitable drug candidates than synthetic compounds. They often, but not always, adhere to Lipinski’s Rule of Five (Ro5) (Ganesan, 2008). Overall, the advantages of NPs include:

1. On average, natural compounds are more lipophilic than combinatorial compounds. This partly explains the low success rate of converting initial hits from HTS/CC to leads that can be further developed into drugs, in accordance with the Ro5 (Feher & Schmidt, 2003);
2. NPs usually have high binding affinities for specific receptor systems and their biological action is often highly selective (Feher & Schmidt, 2003). Synthetically modifying a molecule by introducing additional flexibility, decreasing its size or removing

chiral centres as often happens when synthetic analogues of NPs are made, generally leads to a loss in specificity and weaker activity (Feher & Schmidt, 2003);

3. NPs are less likely to interact in a detrimental way with common cellular components, such as membranes or DNA, because they have already been through the “evolutionary mill”. In contrast, the new types of organic compounds being examined as potential drugs might show high potency *in vitro*, but within the complex microenvironment of a cell, they may interact undesirably with other cellular components besides the target (Dobson, 2004); and

4. Many natural molecules are derived from a small group of core structures, including peptides, nucleosides, carbohydrates, steroids and alkaloids, each of which is generally associated with biological activity. The systematic structural variation of their substituents has been invaluable in guiding the development of new therapeutic agents (Hogan, 1996).

Thus, while the generation of combinatorial libraries has been restricted by the availability of reagents and suitable reactions, NP diversity has occurred within the constraints of available biosynthetic reactions and precursors and also in the context of biological utility. In other words, most NPs have a function, and the synthetic routes generating these compounds have coevolved with the requirements of ligand functionality. This means that for CC to be more effective, it must evolve beyond synthetic practicability to specifically address the construction of compounds with relevant physiological function (Feher & Schmidt, 2003). In other words, it must provide compounds that are more like NPs.

Of course, obtaining leads from NPs can also be problematic. The complexity of many NPs can restrict their optimisation for therapeutic use via chemical modifications. In addition, there are challenges involved in identifying the active components from NP extracts as they

typically contain several compounds. Furthermore, obtaining a renewable supply of active compounds from biological sources can be difficult (Clardy & Walsh, 2004). These limitations, coupled with the fact that each approach provides very different types of lead compounds, means that the production of unique bioactive libraries from NPs is complementary to the generation of synthetic libraries through CC and HTS (Feher & Schmidt, 2003). In fact, the total synthesis of the potent anticancer NP, discodermolide, in gram quantities (Smith *et al.*, 2000) highlights the increasing efficiency of synthetic organic chemistry in reducing the barrier posed by limited natural supply, even for molecules with very complex structures (Altmann, 2001), and suggests there will always be a place for skilled synthetic organic chemists (Petsko, 1996).

However, Nature is undoubtedly mankind's greatest combinatorial chemist, and many compounds that are still to be discovered in NPs are probably beyond the imaginations of even our best scientists (Lewis & Elvin-Lewis, 1977; Dufault *et al.*, 2001; Tulp & Bohlin, 2004). It makes sense that a multidisciplinary approach to drug discovery, involving the generation of novel molecules from NPs combined with CC may provide the best answer to the current drug productivity crisis (Newman & Cragg, 2007). This approach is best left to teams of medicinal chemists and large pharmaceutical companies. However, there is still an important role for the individual laboratory interested in particular NPs and their effects on specific diseases.

NPs can have a plant, animal or bacterial origin and be found anywhere in the world. As chemical diversity is a function of biodiversity, it is often assumed that tropical regions, where about two-thirds of biodiversity exists (Pimm & Raven, 2000), will provide us with a plethora of medicines (Tulp & Bohlin, 2002). However, it is important to note that the

world's oceans, which cover over 70% of the earth's surface, are also teeming with untapped biological diversity, each millilitre containing over 1,000 microbes (Cragg & Newman, 2002). In addition, soil contains over 1,000 species of microbial flora per cubic centimetre and should not be overlooked as a source of NPs. Moreover, it has been estimated that less than 1% of all microbes and less than 10% of fungi have been identified, so there exist further opportunities for drug discovery (Cragg & Newman, 2002; Ghisalberti, 2008).

Notwithstanding the future opportunities and, indeed, past leads and approved drugs obtained from animals (e.g. ES285 from a marine mollusc, Faircloth & Cuevas, 2006) and microbes (e.g. antibiotics), plants remain the principal focus of biologically driven drug discovery programmes. Indeed, the aim of the current project was to try to identify compounds in plants that may be of use in cancer therapy. Therefore, the next section of this review will focus on approaches used to discover drugs from plants. Particular emphasis is placed on anticancer agents derived from ethnomedical knowledge and issues related to using this approach.

1.4 DRUGS FROM PLANT SOURCES

Prominent pharmacognosists and ethnobotanists, such as Norman Farnsworth and James Duke, believe that “somewhere in the plant kingdom there is a remedy for everything” (Duke, 1990, p492). More than 7,000 compounds used in Western medicine are derived from plants (Clapp & Crook, 2002). It is well established that plants have been a prime source of highly effective conventional drugs, including many clinically relevant anticancer compounds (Cordell *et al.*, 1991; Ansah & Gooderham, 2002; Newman & Cragg, 2005). Many other currently used anticancer agents were derived and synthesised from lead compounds discovered in plants (Grever *et al.*, 1992; Newman & Cragg, 2005). In fact,

more than half of all pharmaceuticals, including cancer therapies, are derived wholly or partly from plants (Melnick, 2006a). Furthermore, the National Cancer Institute (NCI) in the USA screened samples from an estimated 12,000 species of plants between 1960 and 1982 for just one disease category, which yielded three major anticancer drugs. This represents a hit rate of 1 in 4,000 (Cragg *et al.*, 1998), much more economical than the one drug from a million molecules screened quoted for CC/HTS (Carnero, 2006).

Consequently, there are over 60 plant-derived compounds currently in clinical trials for cancer therapy alone (Saklani & Kutty, 2008). It is clear that, despite various challenges encountered in plant-based drug discovery, NPs isolated from plants will remain an essential component in the search for new medicines (Jachak & Saklani, 2007).

There are distinct advantages to using plants as the starting point in any drug development programme. In most cases plants are a renewable source of starting material. Plants provide a virtually unlimited source of novel and complex chemical structures that most likely would never be the subject of a beginning synthetic programme (Fabricant and Farnsworth, 2001).

Bioprospecting can result in whole new classes of materials that even our best chemists could probably never even imagine. And while the ability to synthesise new proteins is growing exponentially, our current understanding of structural biology is still too shallow to allow us to produce the kinds of molecules available in nature. While we cannot even dream of making all possible combinations, Nature has been experimenting for two billion years (Macilwain, 1998). In addition, any bioactive compounds obtained from plants that have been used long-term by people might be expected to have low human toxicity (Fabricant & Farnsworth, 2001).

Plants represent the second largest source of global biodiversity (15%) after insects ("New drug," 2002; Tan *et al.*, 2006). Estimates of the number of higher plant species on Earth range from 200,000 to 1,000,000 (Pimm, 1995) and only a small fraction have been thoroughly examined for medicinal value. It therefore seems logical that an abundance of drugs from plant sources are as yet undiscovered. Approaches to selecting plants to be tested for new bioactive compounds range from random selection to more guided selection strategies such as the chemotaxonomic approach and the ethnopharmacological approach (Cordell *et al.*, 1991; Soejarto, 1996; Shoemaker *et al.*, 2005) which are discussed in turn below. Usually a research group would choose just one of these strategies. However, they do not have to be mutually exclusive and several large databases have been set up that facilitate such a multi-pronged tactic. For example, to 2001 the NAPRALERT database (<http://www.napralert.org>) contained information covering the pharmacological/biological activity, taxonomic distribution and ethnomedical uses of nearly 44,000 species of higher plants, making it possible for researchers to correlate ethnomedical use with experimental biochemical or pharmacological activities to identify plants having both types of activity for a given effect (Fabricant & Farnsworth, 2001).

However, the search for anticancer agents from terrestrial plants is a complex process that demands the involvement of not only scientific expertise, but also expertise in a broad spectrum of human endeavours including diplomacy, international laws and legal understandings, social sciences, politics, anthropology, and basic common sense. Equally important is the fact that such endeavours must be governed by international bureaucratic and regulatory procedures (Tan *et al.*, 2006). Some of these issues will be discussed further in section 1.4.4.2.

1.4.1 Random approach

A widely adopted plant drug discovery approach is the screening of a large range of diverse plant samples for one or more phytochemicals or biological activities. The reasoning behind this approach is that, since each plant species produces a set of unique chemicals, a large array of taxonomic diversity reflects a great diversity of chemical compounds. This biodiversity-based screening method is often referred to as a “random” collection and screening approach. However, such a term can be misleading as botanists do not simply walk into the forest blindfolded. For example, certain plants, widely cultivated species and those that have been previously collected and tested for the same therapeutic category, are avoided in the collection process (Soejarto, 1996).

Following random selection, plants can be screened for phytochemicals. Screening for phytochemicals is simple to perform, but the proportion of false-positives and false-negatives is high, making results difficult to interpret. More significantly, it is usually impossible to relate one class of phytochemicals to specific biological targets. For example, the flavonoids produce a vast array of biological effects that are usually not predictable in advance (Fabricant & Farnsworth, 2001). Another option is to screen for biological activity. The most extensive programme that involved random selection of plants followed by one or more biological assays was sponsored by the NCI. Between 1960 and 1981, the NCI systematically screened 114,000 extracts of 40,000 plants for anticancer activity. As only three clinically active anticancer drugs came out of the programme, the NCI has since modified its strategy to prioritise known medicinal plants when relevant information is available (Cragg *et al.*, 1994; Dewick, 2002). Overall, in view of the large number of plants to be screened, and the host of therapeutic targets against which tests must be conducted, the biodiversity-based drug discovery approach is considered a very expensive process,

with little chance of delivering a hit (Soejarto, 1996). Dewick (2002) contended it is probably only justified in certain areas, where the current range of drugs is critically inadequate or inefficient. He explained that the NCI's initial adoption of the random mass-screening strategy was because cancer was considered to be in that category.

1.4.2 Chemotaxonomic relationships

Besides random selection, plants are often chosen based on their taxonomy. The rationale here is that, since related plants tend to produce similar chemical compounds, the closer the taxonomic relationship, the better are the chances that similar or related compounds may occur in these taxa (Verpoorte, 1998). Thus, when a compound is of medical or pharmaceutical importance, attempts are made to search for similar or related compounds in related taxa. These might be other varieties within the same species, other species within the same genus, or even other genera within the same family (Soejarto, 1996). The chemotaxonomic approach can also be used as a negative indicator. For example, if a cytotoxic compound has been found in several related species but experiments have shown it has no value as a lead, screening further related species could be stopped. Additionally knowledge of chemotaxonomy can be helpful in identifying active compounds (Verpoorte, 1998).

An example of the use of chemotaxonomic relationships to search for medically important compounds is provided by the field search of other *Taxus* species with higher paclitaxel content than *Taxus brevifolia* (see 1.4.4.1.3). In general, when searching for species that may contain similar or related compounds with the same biological activity, but in higher yields than the original species, chemotaxonomically-based field searching promises a productive return. However, when looking for compounds with different biological

activities, the chances of finding novel structures via this approach are too low to make it worthwhile (Soejarto, 1996).

1.4.3 Field observations

Alternatively, ecological information can be used to help select suitable plants for further screening. Given secondary metabolites are frequently produced by plants as a chemical defense against potential predators and pathogens (Wittstock & Gershenzon, 2002; Becerra *et al.*, 2009), field observations that point to such interactions between organisms might indicate the presence of useful NPs (Soejarto, 1996). Young leaves and seedlings might be expected to have more secondary metabolites than older parts of a plant so as to be more strongly protected from predators (Verpoorte, 2000).

Similar to the famous story of how Fleming discovered the *Penicillium* mould, careful observations of biological interactions could theoretically lead to the discovery of new drugs. For example, a novel antifungal flavonoid, kaempferol rhamnoside, was isolated following a bioassay-guided study of *Myrica gale* L. after field observations of this shrub, indicated an absence of predation by herbivorous insects and a lack of pathogenic fungal infestation (Soejarto, 1996). However, to date, no data could be found on actual NCEs discovered based on field observations of interactions between plants and other organisms.

1.4.4 Ethnopharmacology

Another undeniably valid approach to drug discovery is ethnopharmacology.

Ethnomedicine has been broadly defined as the use of plants by humans as medicines, but this use could be more accurately called ethnobotanic medicine (Fabricant and Farnsworth, 2001). Northridge (2002) defines ethnomedicine as the folk medicines of particular ethnic groups and asserts it is dependent on location. All over the world, indigenous cultures

traditionally used the plants available to them in their local environments to treat diseases and promote health and these folk remedies were generally passed down orally (Northridge, 2002). Fossil records indicate that people have used plants as medicines for at least 60,000 years (Kong *et al.*, 2003) and almost 65% of the world's population continues to use plants as their primary mode of healthcare (Fabricant & Farnsworth, 2001; Dufault, 2001).

1.4.4.1 *Advantages of ethnopharmacological approach*

Ethnopharmacology is a highly diversified approach to drug discovery involving the observation, description, and experimental investigation of indigenous drugs and their biological activities (Fabricant and Farnsworth, 2001). Intuitively, the rate of drug discovery through the use of ethnopharmacological data would be expected to be much greater than with random collection. Indeed, Chapuis *et al.* (1988) pre-selected plant species based on traditional medicinal use and demonstrated a relatively high proportion showed promising activity. Spjut and Perdue (1976) revealed in a retrospective (to that time) analysis of the early NCI programme in which many species of plants were tested for antitumour properties, that the percentage of active leads based on ethnomedical use was substantially above that based on either random screening or taxonomy.

Fabricant and Farnsworth (2001) point out that any of the 250,000 higher plant species on earth could conceivably produce a new drug. However, given the virtually limitless introduction of novel mechanism-based bioassays, there is a very low probability of collecting all 250,000 species and screening them for more than one biological activity. Thus, researchers should judiciously choose species most likely to produce useful activity and the biological targets must represent the activities best correlated with the rationale for plant selection. Hence, selection of plants based on long-term human use in conjunction

with appropriate biologic assays that correlate with the ethnomedical uses seems to be most appropriate (Fabricant & Farnsworth, 2001).

There are hundreds of examples of plants used by indigenous peoples to treat cancers and other ailments that have led Western medicine to anticancer agents (Graham *et al.*, 2000). While not necessarily discovered as a direct result of researchers utilizing ethnopharmacological knowledge as a guide, some of the most powerful anticancer drugs in current use, vinblastine, vincristine, etoposide and paclitaxel (Taxol[®]), were all originally developed from plant sources that have reported ethnomedical uses (Kong *et al.*, 2003; Cragg & Newman, 2005; Heinrich & Bremner, 2006).

There are over 120 distinct compounds currently used as major chemotherapeutic drugs, including several used in conventional cancer treatment, that were developed from botanical sources and 80% of these have had an ethnomedical use identical to or related to their current use (Fabricant & Farnsworth, 2001). That statistic should speak for itself.

This thesis is primarily concerned with finding compounds from plants traditionally used by Aboriginal people that may be of use in the treatment of cancer. However, it is important to understand that within local communities conditions referred to as “cancer” may not be what is clinically recognised as cancer. This is because cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicine (Newman & Cragg, 2005). Furthermore, generally speaking, indigenous cultures most commonly use plants to treat infectious diseases such as gastrointestinal problems, skin infections and respiratory illnesses rather than in the treatment of cancer. It is, therefore, frequently difficult to establish a direct link between traditional plant remedies and true neoplasms

(Heinrich and Bremner, 2006). The following examples are just a selection of the hundreds of plants used by indigenous people to treat “cancers” or other ailments that have led Western medicine to anticancer agents.

1.4.4.1.1 *Podophyllum peltatum*

One of the best known examples is etoposide, derived from the mayapple plant (*Podophyllum peltatum*). Native American Indian tribes used mayapple to treat various ailments, but scientific investigations led to the discovery of the bioactive constituent podophyllotoxin and ultimately to the semisynthetic compounds etoposide and teniposide (Cragg & Newman, 2005). Etoposide was approved by the FDA in 1983 for use in patients with various cancers (Hande, 1998).

1.4.4.1.2 *Catharantus roseus*

Long before modern researchers learned of the plant's valuable and varied properties, folk healers all over the world were using the Madagascar or rosy periwinkle (*Catharantus roseus*) for a host of medicinal purposes (Crellin & Philpott, 1989). Research teams from the University of Western Ontario, Canada, and the pharmaceutical company, Eli Lilly, independently became interested in this plant, on hearing of a "periwinkle tea" that was drunk in Jamaica as an antidiabetic. Subsequent testing found it to have no hypoglycaemic activity, but treated test animals became susceptible to bacterial infection and this observation resulted in extensive studies to determine if immunosuppressive principles caused these effects. Eventually, this research led to the development of two potent anticancer alkaloids, vinblastine and vincristine (Duffin, 2000; Dewick, 2002). In the 1960s, a diagnosis of Hodgkin's lymphoma was a virtual death sentence. Now, vinblastine achieves an 80% remission rate for sufferers of this disease (Mukherjee *et al.*, 2001; Kong *et al.*, 2003). Before the discovery of vincristine, only one in five childhood leukemia

victims lived. Today, 99% of them survive, thanks to the rosy periwinkle (Mukherjee *et al.*, 2001).

1.4.4.1.3 *Taxus brevifolia*

Paclitaxel, sold under the tradename Taxol®, is one of the most powerful and widely used anticancer drugs available today. Indeed, it is now the world's biggest selling anticancer drug (Walsh & Goodman, 2002). The natural source, the Pacific yew tree (*Taxus brevifolia*), is an environmentally protected species and one of the slowest growing trees in the world (Rowinsky & Donehower, 1995). Isolation of the active compound, which is contained in the bark, involves killing the tree, and the quantities available by this method are pitifully small (Blume, 1991). It would take three to six 100-year-old trees to provide enough paclitaxel to treat just one patient (Blume, 1991; Dewick, 2002). Luckily, a closely related analogue of paclitaxel, baccatin III, was discovered in the leaves of a related European species of ornamental shrub, *Taxus baccata* (Denis *et al.*, 1988). Although the extraction and subsequent chemical elaboration of baccatin III to paclitaxel was very laborious, the source was renewable, and sufficient quantities were obtained to carry out clinical trials. McGuire *et al.* (1989) showed that paclitaxel exhibited very promising activity against advanced ovarian cancer, and in 1992 the FDA approved paclitaxel for the treatment of this condition (Cragg *et al.*, 1993). Subsequently, paclitaxel was FDA-approved for advanced breast cancer in 1994 and for early stage breast cancer in 1999 (Feng & Huang, 2001). Since then, paclitaxel has also been approved for use in other countries too and for treating other types of cancers, including non-small cell lung cancer and Kaposi's sarcoma (Panchagnula, 1998; Singla *et al.*, 2002; Oberlies & Kroll, 2004). Despite numerous references in folk lore to the cancer healing properties of the yew tree, the "discovery" of paclitaxel came out of a random screen by the NCI, not from an ethnobiomedical lead (Lewis, 2000; Heinrich and Bremner, 2004). However, its bioactivity

has been known since ancient times. Native Americans have long used *T. brevifolia* for medicinal purposes and other *Taxus* species were important to medieval Europeans, Druids, Celts, ancient Greeks and Romans (Walsh & Goodman, 2002). Even Julius Caesar himself mentioned in his "Gallic Wars" that Catilvolcus committed suicide by consuming extracts from the yew tree (Caesar, 50-40 B.C.). Furthermore, the Himalayan yew (*T. baccata* ssp. *wallichiana* or *T. wallichiana*) was used in Ayurvedic and Tibetan traditional medicine specifically as a cancer treatment (Chattopadhyay *et al.*, 1996; Lewis, 2000). In retrospect, if researchers had been aware of these traditional uses of the yew, perhaps paclitaxel may have been developed as a drug much earlier.

1.4.4.1.4 *Curcuma longa*

Turmeric, a spice derived from the rhizomes of *Curcuma longa*, has been used in India for medicinal purposes for centuries and its use continues today. Its active constituent, curcumin, has been shown to exhibit a wide range of beneficial properties, including antiinflammatory, antioxidant, chemopreventive and chemotherapeutic activity (Hatcher *et al.*, 2008). For example, curcumin induces apoptosis in a wide variety of cell lines in culture (Roy *et al.*, 2002; Deeb *et al.*, 2007). Additionally, it down-regulates the expression of transcription factors (e.g. NF- κ B), enzymes (e.g. COX-2), cytokines (e.g. TNF), chemokines, cell surface adhesion molecules, cyclin D1 and growth factor receptors (e.g. EGFR and HER2) (Aggarwal *et al.*, 2003). These activities, combined with its low toxicity, have led to scientific interest in curcumin's potential for cancer therapy as well as cancer prevention and clinical trials are ongoing. Phase I clinical trials have been completed with patients affected by various types of cancer generating promising results and Phase III clinical trials are currently underway in patients with advanced pancreatic cancer (Karunagaran *et al.*, 2005; Hatcher *et al.*, 2008).

1.4.4.1.5 *Scutellaria baicalensis*

Scutellaria baicalensis (also known as Chinese skullcap or Huang Qin), a member of the mint family, is an important Chinese herbal medicine that has been used historically to treat many diseases, including cancer (Ye *et al.*, 2002). Several studies have verified this plant's effectiveness against various types of cancers. Baicalin, after conversion to baicalein in the gut, has been implicated as the active ingredient, possibly working by inducing cell cycle arrest and apoptosis and/or inhibiting cyclooxygenase (COX)-2 or lipooxygenase activity (Chan *et al.*, 2000; Ye *et al.*, 2002; Nelson & Montgomery, 2003; Zhang *et al.*, 2003). A more recent investigation suggested that another active constituent, wogonin, may also be responsible for its antitumour properties via induction of apoptosis and reduction of telomerase activity (Huang *et al.*, 2010). No clinical reports could be found in the published literature, but toxicological studies in rats and mice have established safe intravenous doses for wogonin (Qi *et al.*, 2009). Additionally, baicalin, as a major component of the commercially available herbal mixture, PC-SPES, has been safely used to treat prostate cancer patients (Ikezoe *et al.*, 2001; Nelson & Montgomery, 2003).

1.4.4.1.6 *Euphorbia peplus*

Closer to home is the case of *Euphorbia peplus*, known variously as radium weed, milkweed and petty spurge (Ogbourne *et al.*, 2007). Originally native to Europe and North Africa, the distribution of this prolific small annual herb is now almost cosmopolitan in disturbed locations (Jakupovic *et al.*, 1998). Historically, the milky sap of *E. peplus* has been widely used as a home remedy against many conditions, including carcinomas (Weedon & Chick, 1976). For example, Australian Aboriginal people and colonial physicians removed skin cancers by dabbing them with the sap of this weed (Low, 1990). Additionally, it was used in the Ukraine in the 1900s as a treatment for cancer of the stomach, liver and uterus while in Mauritius a decoction of the leaves was drunk to treat

dysentery and diarrhoea and in Saudi Arabia an infusion of the leaves was used to lower blood pressure (Schmelzer & Gurib-Fakim, 2008). However, herbalists have known of the medicinal properties of the corrosive latex since ancient times. In fact, Juba II, the king of Numidia (now Algeria/ Tunisia) and Mauretania (now Algeria/ Morocco) during the first century BC, named the genus after his personal physician, Euphorbus, who reportedly used the sap as an ingredient in his medicines (Scott & Karp, 1996; Jassbi, 2006). Galen, working in the second century, also used sap from *Euphorbia* species as part of his remedies (Toledo-Pereyra, 1973; Scarborough, 1978). Guided by such ethnopharmacological knowledge imparted by his mother, Dr Jim Aylward and a team led by Professor Peter Parsons from the Queensland Institute of Medical Research (QIMR) successfully identified a potent anticancer agent from the sap of *E. peplus* (Ogbourne *et al.*, 2004; Hurst, 2009). The active principle, ingenol 3-angelate (I3A), formerly known as PEP005 but now called ingenol mebutate, is being developed as a safe topical treatment of non-melanoma skin cancers and precancerous actinic keratoses (Siller *et al.*, 2009). Subsequently, ingenol mebutate has shown potential in the treatment of bladder cancer and leukemia (Hampson *et al.*, 2005; Ogbourne *et al.*, 2007).

1.4.4.1.7 *Scaevola spinescens*

Scaevola spinescens, otherwise known as Maroon bush, Currant bush or Prickly fanflower, is another example of a plant used by Aboriginal people to treat ailments, including cancer (Ghisalberti, 2004). Anecdotal evidence led to the 1957 instigation of a programme whereby the Chemistry Centre of Western Australia (CCWA) prepared and supplied water extracts of *S. spinescens* free of charge to terminally ill cancer patients (Ghisalberti, 2004). No new patients were added after 1991, but eight patients diagnosed with terminal cancer prior to this, having received approval from their doctors, were still being treated up until at least 2000 (Kerr, 1999; CCWA, 2001). In 1989, a sample of a methanol extract of *S.*

spinescens was sent to the NCI, and shown to be active in all 60 cell lines tested. However, although the results indicated significant activity in this material, its lack of selective toxicity for different cell types precluded it from having great interest. Kerr *et al.* (1996) identified the presence of several taraxerenes in various extracts of *S. spinescens* and isolated the most abundant, myricadiol. These authors suggested that these compounds may be of potential use as lead compounds for synthetic anticancer agents. However, subsequent studies showed that some taraxerenes themselves have antitumour properties (Takasaki *et al.*, 1999). For example, while myricadiol failed to show any antiproliferative effects against the leukemic and lung cancer cell lines tested, the same study revealed its moderate inhibitory activity against COX-1 and COX-2 (Lee *et al.*, 2006). Given that specific COX-1 and COX-2 inhibitors have demonstrated antiproliferative effects against bladder, prostate and breast cancer *in vitro* (Mohseni *et al.*, 2004; Farivar-Mohseni *et al.*, 2004; McFadden *et al.*, 2006), myricadiol may be useful against these types of cancer. Alternatively, it may have a potential role in antiangiogenesis therapy (Rahman & Toi, 2003; Zhong & Bowen, 2006).

1.4.4.2 *Challenges of the ethnopharmacological approach to bioprospecting*

It seems plenty of evidence exists to support the ethnopharmacological approach to bioprospecting. Indeed, in the early 1990s this appeared to be the way pharmaceutical companies were heading. Developing nations of the tropics, where the bulk of terrestrial biodiversity exists, began to prepare for a veritable gold rush of Western scientists seeking new drugs (Mendelsohn & Balick, 1995; Macilwain, 1998). Treaties endorsing the concept of nations holding property rights to their indigenous species were signed and impoverished governments rubbed their hands together as environmental ministers of Third World

countries publicly declared their readiness to strike hard bargains. However, the anticipated rush did not materialise (Macilwain, 1998).

As already stressed, since plant-derived drug discovery efforts began, the ethnopharmacological approach to screening has been the most successful (Chapuis *et al.*, 1988; Fabricant & Farnsworth, 2001). However, the random collection of plants provides the highest biodiversity and is surging ahead as the method preferred by Big Pharma. This approach, followed by automated, robotised, *in vitro* screening requires significantly more financial resources than the ethnopharmacological approach, which is more suited to academic institutions who have less capital, but (arguably) more time to invest (Fabricant & Farnsworth, 2001) and are inherently more liable to spend resources on altruistic pursuit.

So, if the ethnopharmacological approach to drug discovery is so successful, why aren't all the big drug companies climbing over themselves to be the first to engage indigenous cultures and tap into their traditional knowledge (TK) of medicinal plants? Several reasons are postulated below.

1.4.4.2.1 *Loss of habitat*

One reason might be that terrestrial (and marine) ecosystems are diminishing rapidly. It is no secret that Man's activities have had, and continue to have, a devastating effect on the natural environment. Continued deforestation due to clearing for farming and housing to support the world's population of over 6 billion people is an obvious factor. Other agricultural practices as simple as slash-and-burn methods to more modern techniques involving fertilising and pesticides are primarily responsible. However, besides farming, industry and urban living also produce air and soil pollution with far-reaching consequences. For example, energy consumption, waste disposal and poisonous products of

modern lifestyles all combine to destroy natural habitats on a global scale. Exacerbating the problem is the impact of Man's activities on water pollution and the level of the water table. Then, of course, there is global warming. While there is still heated debate on whether climate change has been induced by man's activities or not, there is much scientific evidence in support of global warming itself and this undeniable phenomenon is having a very real impact on natural ecosystems (Dessler & Parson, 2010). Add to all this the destructive effects of invasive exotic species with no natural predators introduced into local habitats by Man and it is no wonder that the Earth is a very different place to what it was even a century ago (Wilson, 2002). A typical estimate of the number of recent plant and animal extinctions is 27,000 species per year. Even more conservative estimate put the figure at about 13,500 species lost, or at least committed to extinction, each year. This is in stark contrast to 785 species, the total number of documented extinctions worldwide in the past 500 years. However, the total number of extinctions is unknown as many species have likely become extinct before they were even known to exist (Sax & Gaines, 2008).

Habitat destruction is the leading cause of species extinction (Pimm & Raven, 2000). In the biodiversity-rich tropical rain forests alone, clearing now eliminates about 1 million sq. km every 5-10 years, and burning and selective logging severely damages several times the area that is cleared (Pimm & Raven, 2000). Looking ahead, it is calculated that by 2050, human land-use practices will have reduced the habitat available to Amazonian plant species by approximately 12–24%, resulting in 5–9% of species becoming committed to extinction (Feeley & Silman, 2009). While this is significantly fewer than previous estimates (Hubbell *et al.*, 2008), it still represents over 2,000 vascular plant species, and is obviously of real concern to conservationists. Big Pharma are undoubtedly aware of the statistics too. Perhaps their preference for the random approach to selecting plants for their

drug discovery programmes is simply a reflection of their anxiety to screen as many as they can before it is too late and they disappear forever. Intuitively, it would seem likely that plants with a known ethnomedical history would have a higher chance of being conserved than those with no known use and so the urgency is focused on testing random samples.

1.4.4.2.2 *Environmental laws*

Quite apart from the alarming rate of species extinction through habitat destruction, new regulations in environmental protection protocols have compounded the difficulty of finding and identifying new biological materials (Gollin, 1999). While environmental legal milestones such as the well-known Rio de Janeiro Biodiversity Treaty and the Kyoto Protocol were designed to protect the world's ecological health while still allowing economic development, their regulations may be seen as somewhat of an inconvenience or even a barrier to generating profit by the more short-sighted drug companies. However, as Gollin (1999) points out, if the new rules for bioprospecting succeed in reducing biodiversity loss (the greatest risk to NP research), while still allowing research to continue, any inconvenience they create will be justified.

The Convention on Biological Diversity (CBD), a groundbreaking initiative adopted by most of the world's governments, is dedicated to promoting sustainable development. The CBD was established at the Rio De Janeiro Earth Summit in 1992 and has three main goals: (i) the conservation of biological diversity, (ii) the sustainable use of its components, and (iii) the fair and equitable sharing of the benefits from the use of genetic resources. It is recognised as a landmark document, reminding decision makers that natural resources are not infinite. While past conservation efforts aspired to protect particular species and habitats, the CBD recognises that whole ecosystems, as well as individual species and even

genes must be sustainably used for the benefit of all mankind. However, this must be done so as not to cause the long-term decline of biological diversity (CBD, 2000).

Guevera-Aguiree and Chiriboga (2005) also stressed the importance of bioprospectors placing the interests of the collective ahead of their own and protecting (as their own) the very source that generates any potential earnings: a healthy and rationally used environment. They believe that environmental law enforcement, locally and internationally, is the cornerstone to guarantee the proper use, and not abuse, of natural resources. Indeed, this principle makes excellent economic sense because, after all, as Wilson (1992, p282) asserted, “useful products cannot be harvested from extinct species”.

This new approach to biodiscovery, which has been effective in identifying new bioactive leads, is an important step towards understanding the medicinal value of biodiversity and is also a practical way to link drug discovery with conservation (Coley *et al.*, 2003).

1.4.4.2.3 Access and benefit sharing

Another reason inhibiting the wide-spread adoption of the ethnopharmacological approach to drug discovery might be the regulations set in place to protect indigenous cultures from exploitation (Gollin, 1999). There are two issues surrounding the ethics of biodiscovery processes, access to genetic resources and traditional ecological knowledge and the equitable sharing of any benefits that may arise from them. As described below, neither has a simple solution. Consequently, perhaps some drug companies have found that conforming to all the new regulations and ethical requirements might just be all too much trouble for them to deal with.

In the past, there have been many instances of companies becoming very rich by using ethnomedical knowledge at the expense of the indigenous people who provided it. For

example, in defiance of local legislation, cinchona (Peruvian bark) seeds were smuggled out of South America in the 1850s and plantations set up in Asia for the purpose of supplying the world with the precious antimalarial compound, quinine (Gollin, 1999; Philip, 2001). This effectively pushed South American countries out of the world market (Bravo, 1998). Additional pharmaceutical uses of quinine, quinidine and their derivatives were discovered which expanded their worth, but not one cent of profit reverted to the countries where cinchona originally grew, or to the indigenous people who provided the ethnomedical knowledge (Wallace, 1996; Alter, 2000). Such plunder of natural resources is now commonly referred to as *biopiracy* (Krishnamurthy, 2003).

Traditionally, genetic resources were seen to be “the common heritage of mankind” (Krishnamurthy, 2003; Roa-Rodríguez & van Dooren, 2008). In practice, that meant that industrialised nations took freely of those resources and sold them back to developing countries as commercial products (Mukerjee, 1994). However, new rules have been introduced that have shifted the imbalance. As a result, some researchers may be discouraged from NP research by expensive or extremely time-consuming bureaucratic requirements put in place out of fears of commercial exploitation (Blaustein, 2006).

The new regulations for bioprospecting and NP research derive from three sources: international treaties, national laws and professional self-regulation (Gollin, 1999). The most important international, bilateral treaty protecting nations and indigenous cultures from biopiracy is the CBD, endorsed by the United Nations (UN) as the authority to set the standards for the cross-boundary access of genetic resources (Heinrich & Bremner, 2006; Blaustein, 2006). The CBD established sovereign national rights over biological resources and committed member countries to conserve them, develop them sustainably, and share

the benefits resulting from their use (Gollin, 1999). This protection of rights is legally binding, but countries voluntarily agree to commit to the ideals of the Convention and must make their own laws to enforce them (Blaustein, 2006; Roa-Rodríguez & van Dooren, 2008). Furthermore, a historically prominent hotbed of biopirates, the USA, has still not voted to ratify the CBD, despite President Clinton signing in 1993. With Andorra recently announcing it will soon endorse the treaty, the USA will become the sole non-signatory of the CBD (Holding, 2010). The powerful pharmaceutical industry lobbying Congress is a major reason why the US has not yet ratified the accord. This has prompted nations such as Brazil and India to exercise their sovereign rights, implementing stricter controls on medicinal plants. Unfortunately, as a consequence, much genuine scientific collaboration has been thwarted, tied up in bureaucratic red tape (Verma, 2002; Blaustein, 2006).

On the other hand, many scientists point out that researchers were already developing more equitable relationships and implementing specific mechanisms for accessing genetic resources before the advent of the CBD (Cox & Balick, 1994). They argue that all the CBD did was enhance awareness and expectations on the part of the host country (Blaustein, 2006).

As well as seeking direct access to plant resources, bioprospectors often want access to the TK¹ of the indigenous peoples who have been the guardians and repositories of those resources. In many cases they also seek intellectual property (IP) rights, and the exclusive legal entitlement to exploit the resources and TK for commercial purposes. There is no doubt that researchers add immense scientific value during product development. Creating

¹ see Dutfield (2001) for discussion of what exactly constitutes TK.

this scientific value requires enormous amounts of money, time and human resources, and the failure rate in drug development is very high. Obviously, the results deserve to be legally protected (Chacko & Sambuc, 2003). However, it has been recognised for some time that the TK practitioners who provided the original leads have also contributed to develop that TK into innovation, thus they must rely on more industrialised countries (Stone, 2008). Consequently, disputed claims are common and can hamper fantastic potential for medical breakthroughs as well as the development of sustainable industries for the indigenous people, in which case everybody loses (Chacko & Sambuc, 2003).

It is no longer a question of whether indigenous peoples should benefit from products that have been developed from their TK. The CBD, the World Intellectual Property Organization (WIPO), individual ethnobiologists and organizations, such as the Society of Economic Botany, the International Society of Ethnobiology and the American Anthropological Association, have emphasised the importance of ethical reciprocal conduct by all parties who perform research with indigenous peoples. The challenge is to ascertain what the indigenous peoples themselves see as being benefits and how these can be provided by the organizations collaborating with them (Bierer *et al.*, 1997). For example, it might seem logical to compensate indigenous cultures for their TK by simply according them a percentage of the profits from a drug once it is commercialised, but this may require a five to ten year waiting period, if it ever occurs at all, in which case the local people would not see any benefits (King *et al.*, 1996).

In 2002, member states of the CBD adopted the voluntary *Bonn Guidelines on Access to Genetic Resources and Fair and Equitable Sharing of the Benefits Arising out of their Utilization*, which offer guidance in the roles and responsibilities of the various parties

involved in access and benefit sharing. Similarly, the *Aboriginal Knowledge and Intellectual Property Protocol*, developed after extensive dialogue with Aboriginal organisations and researchers, has been recognised by the UN as breaking new ground in respectful relations between researchers and Indigenous people in world terms (DKCRC, 2008; Ferguson, J., personal communication, 31st May, 2010). Ultimately, however, the value of voluntary initiatives depends on public pressure and they are most effective when corporations fear consumer reaction. This means that, when it is time to commercialise an innovation arising from the use of TK, it will be completely up to corporate goodwill whether a company decides to recognise or compensate an indigenous community (Roehrs, 2007).

A base requirement of the CBD is that permission must be obtained before biological samples can be collected. To conform to the CBD, an Access and Benefit Sharing Agreement (ABA) must be agreed upon by the researcher and the source country providing the NP. A complicating factor for drug screening programmes is that the ABA must stipulate what the NP will be used for. In this way source countries know up-front how the genetic resources will be exploited and the benefits that will be shared. Benefits may include support for research and conservation, contributions of equipment and materials, assistance to indigenous and local communities, up-front fees, milestone payments and royalties. If a collector does not agree in advance to provide an equitable share of benefits to the source country, they will likely be denied access to the samples. Additionally, if a biological sample is obtained without informed prior consent its value is substantially reduced and the collector will not be able to pass it on to collaborators, partners or third partners, the usual procedure for researchers (Gollin, 1999).

There are strict penalties for failing to comply with an ABA. If the CBD is not observed, the NP can be treated as being poached, resulting in extremely serious consequences for the company that failed to uphold the deal. For example, unless all public knowledge about the genetic resource and its use are fully disclosed, any patent on that NP can be revoked. Additionally, if a researcher illegally obtains a biological sample and then profits from the material, they may be sued. The risk is that the conditions a court may impose for a successful product may be far greater than what would have been negotiated as part of an ABA at the outset, when success is still a highly unlikely outcome. Thirdly, any company accused of biopiracy will suffer commercially from a bad reputation. Given the existence of an alternative, “greener” competitor, consumers will likely boycott the products of branded “biopirates”. Lastly, criminal charges, including jail terms, may apply to biodiversity thieves in the same way that hunters are often jailed for poaching or trespassing (Gollin, 1999).

Given the potential risks, many organizations have seen the wisdom in entering into ABAs for every collection. Other companies have taken the initiative to adhere to voluntary requirements such as the Bonn Guidelines. For example, Novozymes, a Danish biotechnology company voluntarily follows disclosure requirements in many developing countries. Generally, though, the pharmaceutical industry favours contracts and dogmatically contends that disclosure of origin should not have to be included in patent applications (Roehrs, 2007).

In 2004, Mt Romance, a West Australian company producing products from the sandalwood tree, provided the world’s first case of indigenous intellectual accreditation through their formal partnership with Aveda (a US-based multinational cosmetics

corporation) and the Kutkabubba community (Marinova & Raven, 2006). Since then, other companies have also signed agreements with indigenous groups. Notably, in a partnership initiated by the Jarlmadangah Burru Aboriginal Community (JBAC) of the Kimberley region of Western Australia, Griffith University and JBAC were joint applicants on several patents, the most recent filed in October 2008, for exclusive rights in relation to the bark of the marjarla tree, traditionally used by Aboriginal people for healing and pain relief. They have since been joined by a third party, a start-up biotechnological company, Avexis, who have signed a formal ABA, agreeing to share income from sales and to provide the opportunity to cultivate and supply the plant in sufficient quantities to make the product marketable in return for the entitlement to commercialise the product (Janke *et al.*, 2009).

Additionally, this PhD project was partly funded by the Desert Knowledge Cooperative Research Centre (DKCRC), who, in conjunction with the University of Western Australia (UWA) and the Aboriginal communities represented by the company Ninti One Ltd are all stakeholders in the event that any commercialised product arises from these studies. However, some less ethical pharmaceutical companies who are more interested in their bottom lines than in the idea of equitable sharing of profits, may be deterred altogether from engaging indigenous cultures to aid in their drug screening programmes.

However, while new legislation concerning benefit sharing has been introduced in many countries, there is widespread concern that the laws are not stringent enough (Roehrs, 2007). Despite the new regulations, often only an extremely small percentage of a company's huge profits are given to those who have nurtured the TK from which the medicine is derived (Chacko & Sambuc, 2003).

The WTO's trade-related aspects of intellectual property rights (TRIPS) agreement came into force in 1995 after nearly ten years of negotiations, but it remains controversial. The agreement recognises that IP rights are, in essence, private rights and was adopted to protect and enforce IP rights, thus promoting technical innovation, transfer, and dissemination of technology to the mutual advantage of both the producers and users of the information (Goel, 2008). The TRIPS agreement recognises that most of the value of new medicines and other high technology products lies in the amount of invention, innovation, research, design and testing involved and aims to help protect those rights through the patent system as well as settle disputes between members of the World Trade Organization (WTO) ("Intellectual property," n.d.). It is often argued that the current IP system and its underlying assumptions are inadequate regarding TK (Dutfield, 2001). There are even suggestions that patents may not be the most appropriate tool to guarantee equitable distribution of benefits and recognition of total value of knowledge (Marinova & Raven, 2006). There have been recent instances whereby companies have misappropriated TK by taking advantage of loopholes in the existing legal framework. For example, in an extreme case, under the TRIPS agreement, it suddenly became illegal for Indians living in the US to use the ancient Ayurvedic herbal turmeric in traditional remedies, although the patent was revoked after a long and costly legal challenge mounted by the Indian government (Agrawal & Saibaba, 2001; Kalua *et al.*, 2009). Many believe the TRIPS agreement is tipped too that the new biodiversity laws have actually facilitated biopiracy by universalizing the US legal system of IP rights (Agrawal & Saibaba, 2001; Tedlock, 2006). However, until a better system is proposed, ABAs and TRIPS are the only devices available that might protect indigenous knowledge from those who would exploit it for commercial gain.

1.4.4.2.4 Cost

Yet another factor alienating Big Pharma from the ethnopharmacological approach is cost. Pharmaceutical companies generally prefer synthetic compounds for proprietary economic reasons. This is understandable given it costs millions of dollars to develop a new drug. When fees and royalties have to be paid to host countries, the expense is even higher. Moreover, the company has only 10 years to recoup its investment before a patent expires and generic manufacturers can copy the design (Dufault *et al.*, 2001).

Thus, companies that do have NP drug discovery programmes are generally seeking bioactive compounds from plants that will serve as lead compounds for synthetic or semisynthetic development, thus assuring patent protection. This reduces the need to isolate novel bioactive structures from plants, since the ultimate goal is to use the active constituents to produce synthetic derivatives with higher efficacy and lower toxicity (Fabricant & Farnsworth, 2001). However, there is no incentive for pharmaceutical companies to determine which alternative phytochemical is best because plants are not patentable. Indeed, even if a NP works better, they often prefer the semi-synthetic compound as it guarantees higher profits (Dufault *et al.*, 2001).

1.4.4.2.5 Variation

Another problem associated with any drug discovery programme based on plants, either chosen on the basis of ethnomedical data or randomly selected, is that, as biological systems, plants display inherent individual variability in their chemistry and can therefore display varying degrees of bioactivity (Fabricant and Farnsworth, 2001). In these authors' experience, possibly 25% of all plants showing promising biological activity in preliminary assays, failed to have the bioactivity confirmed on subsequent recollections. They suggested that this was possibly because of variability in the chemistry of plants, although

they acknowledged it could also be due to variable bioassay systems, incorrect taxonomic identifications or simply mix-ups in labelling of samples. Assuming that discrepancies are due to the inherent variability between plant samples, then factors such as environmental conditions, season of harvesting and age of the plants must all be taken into account when sampling plants for assay.

However, in some cases variable activities between plant collections are very likely due to incorrect species identification. This can be especially significant when the ethnopharmacological strategy is employed as locals often only know plants by their vernacular names and these can change from region to region (Guarrera & Lucia, 2007). In Australia, Aboriginal there can be many names for the same species between different Aboriginal languages and even within one language. For example, *Euphorbia drummondii* has 8 different names within a radius of about 200 km of Alice Springs. Within an area less than 600,000 sq. km, the same plant is known variously by 17 different Aboriginal names. To complicate matters further, different species may have the same Aboriginal name. For example, the Alyawarr plant name, “amikwel” can refer to either *Euphorbia tannensis* or *Sarcostemma australe*. In the Pitjantjatjara language “ipi-ipi” refers to *E. drummondii*, *E. tannensis*, *E. eremophila* and any plant with milky sap in general (Latz, 1995). Therefore, to ensure plants are correctly identified each time they are collected, it is vital that collections are always documented with representative voucher specimens and that these are preserved for independent verification (Farnsworth & Bingel, 1977; Soejarto, 1996; Hildreth *et al.*, 2007).

1.4.4.2.6 Time constraints

While collecting plant samples randomly in a specific geographic area can be done simply and quickly, collecting plants on the basis of ethnomedical knowledge is a much more time

consuming process. Not only does it require considerable preliminary planning to determine where each plant grows and how plentiful it is, particular arrangements must also be made to collect the plants, such as acquiring permits and finding local botanists familiar with the flora of the region (Fabricant and Farnsworth, 2001). Moreover, many indigenous cultures, including Australian Aboriginal communities, operate on a slower timeline compared to the faster pace of modern society. Indeed, researchers are often taken aback by the almost complete lack of awareness of time as a concept by Aboriginal people (Zur, 2007). This type of seemingly timeless culture actually operates on what has been coined *polychronic* time (Hall & Reed Hall, 2001). In extreme polychronic cultures, events take place as solitary entities, separate from each other, and in no way connected by an ongoing timeline as many Westerners understand it. In such cultures, even when it appears that everything is at a standstill, a great deal is probably going on behind the scenes. Scheduling cannot happen until meetings have taken place to permit essential discussions. Hence, the amount of time, especially the amount of lead time, required in forging collaborative relationships is high. Therefore, it is vital that researchers are always aware of the local time system so they can make the necessary allowances (Hall & Reed Hall, 2001).

The fact that traditional Aboriginal society is classified as polychronic is of particular relevance to this project. In fact, *traditional* and *contemporary* are only words that nonindigenous people have used to describe indigenous periods in time, and have no traditional meaning to Indigenous Australians. Indeed, Aboriginals do not even have a word for *time* as an abstract concept. This is largely due to the way time is perceived according to the *Dreaming*, which is the concept of time as an “Everywhen,” containing both future and past (Zur, 2007). To further highlight this significant difference in time perception, a local

Aboriginal woman explained to Zur (2007) that NT also stands for “Not Today; Not Tomorrow...”.

Compounding the research obstacles associated with the polychronicity of Aboriginal society is their notion of equality in knowledge sharing. Relationships must be established before knowledge is shared. The key word is *shared*, so there is an expectation of a two-way exchange of information. This is facilitated by face-to-face communication, which is also desirable for cultural reasons of trust and respect. The amount of time necessary to build this trust and respect is naturally not up to the researcher. Therefore, multiple trips to remote communities are required and this obviously consumes yet more time (Zur, 2007). All this means that Aboriginal related research takes much longer than a Western person might expect. The obligatory abandonment of usual scheduling procedures can be extremely frustrating, especially for drug companies which are generally driven by profit and therefore pathologically attached to rigid timetables.

1.4.4.2.7 *Cultural differences*

Other cross-cultural differences can also be a barrier to collaborative ethnopharmacological research efforts. When people unwittingly apply their own rules to another culture, critical steps can be omitted and effective research rendered unachievable. If two people from different cultures meet, they can have difficulty relating because they are not “in-sync”. This is significant because synchrony, the subtle ability to move together, is imperative for all collaborations. Therefore, respecting cross-cultural differences by paying attention to aspects of “the silent language” is paramount (Hall & Reed Hall, 2001). For example, the processes of observation, recording, and other typical Western means of generating data are often in direct opposition to the way knowledge is traditionally shared in Aboriginal society. Whereas physical possessions have been traditionally valueless to Indigenous

Australians, knowledge has always been treated as a significant possession. This means researchers cannot simply sit down and begin asking questions or they risk exhibiting rudeness severe enough to ruin their chances of ever getting close enough to learn. The concept of equality in knowledge sharing means that, in return for TK, researchers must be able to offer something back to the community that is seen as valuable. However, it is important to avoid neocolonialism, which could occur if the researcher attempts to impose West-centric structures and ideas in a completely inappropriate context which in no way meets the needs of the people sharing their knowledge. Therefore, an essential first step is to communicate effectively and respectfully how the results of one's research may help the people involved (Zur, 2007).

1.4.4.2.8 *Communication difficulties*

Besides disparities in time perception and the concept of sharing, other cross-cultural differences may hinder information exchange. For example, where there is a language barrier, a skilful interpreter might be required. However, it is vital that the interpreter chosen must have an appropriate awareness of cultural nuances and obey the unwritten laws of custom so as not to offend the local people (Hall & Reed Hall, 2001).

While we have gleaned some of what our ancestors knew from current and ancient texts, valuable data have not always been recorded (Lewis & Elvin-Lewis, 1977). This is especially true of traditional indigenous cultures, including Australian Aboriginals, who did not have a written language. Additionally, some of the knowledge is highly sacred and is often kept secret, even from members of the particular culture (Low, 1990; Turton, 1997; Fabricant & Farnsworth, 2001). It therefore makes sense to study the practices of indigenous peoples in case they are lost forever, either through neglect or because the vegetation has been irrevocably changed. Today's researchers, with a broader insight and

scientific expertise, have much greater scope to utilise this information than any previous generation (Lewis & Elvin-Lewis, 1977). Some might consider it tantamount to gross negligence if we did not seize the opportunity before it is too late.

1.4.4.2.9 *Previous failure*

Despite the complications of ethnopharmacological approach to drug discovery, one high-profile company did appreciate the potential benefits. Shaman Pharmaceuticals, based in San Francisco, aspired to become a model of ethnobotanical bioprospecting, discovering new medicines while maintaining a commitment of reciprocity to the indigenous cultures. However, while Shaman Pharmaceuticals did develop several medicinal products by integrating traditional plant chemistry, ethnobotany, medicine and medicinal chemistry, the money-losing firm could not afford to comply with FDA requirements that it do more tests on a proposed drug to treat diarrhoea in AIDS patients. It reinvented itself as Shaman Botanicals in a bid to operate in the more loosely regulated field of herbal remedies (Abate, 1999), but this company, too, eventually declared bankruptcy in 2001. This company's failure led *Economist* ("Shaman loses its magic," 1999) to decry ethnobotanical bioprospecting as a concept, branding it out of date and advocating its abandonment. However, Clapp and Crook (2002) contend that Shaman's experience was simply one of bad luck and bad timing. The company's demise was due more to mismanagement and its inability to maintain the triple bottom line than to the failure of its vision.

1.4.4.2.10 *Misconceptions*

Despite the fact that TK has been tried, practised and preserved over many generations in many parts of the world, the value of this research has been largely dismissed by Western science. While the quality of data and format of data collection may be very different from modern evidence-based research, TK is still very valuable. Furthermore, while clinical studies for modern medicines typically take just 5-10 years, ethnomedical data have been

accumulated over centuries (Chacko & Sambuc, 2003). However, the prevailing misconception seems to be that something old is worth less than something new. Someone once said that there are but two types of fools: one professes “this is old and therefore is good,” and the other says, “this is new and therefore better.” However, when judging the medicinal value of ethnobotanical data, neither view is scientifically valid (Lewis & Elvin-Lewis, 1977).

The virtually exclusive use of the research-oriented approach to biodiscovery with little regard for data acquired through the empirical method, has meant that many potential benefits have been delayed (or even missed altogether). For instance, it is unfortunate that Western medicine’s first tranquilizer, reserpine, derived from the roots of Indian snakeroot (*Rauvolfia serpentina*), was not used generally until 1952, despite its long history of use in Ayurvedic medicine (Monachino, 1954; Lewis & Elvin-Lewis, 1977). Similarly, cromolyn sodium (Intal®), the preventative drug for asthma, was only “discovered” in 1965, although it was used in the form of seeds from bishop’s weed (*Ammi visnaga*) for centuries as part of Bedouin folk medicine (Lewis & Elvin-Lewis, 1977; Edwards, 2005). Similarly, there are numerous examples in the area of anticancer therapy, including the few already discussed (1.4.4.1). It is worth re-emphasising here that, at least until 2001, over half of all major chemotherapeutic drugs were developed from plants and 80% of those had an ethnomedical use identical to or related to their conventional use (Fabricant & Farnsworth, 2001; Melnick, 2006a).

According to Heinrich and Bremner (2006), the contribution of ethnobotanical research to drug discovery is often simply ignored at best, or regarded as irrelevant at worst. Sometimes, ethnobotanical data is frequently taken at face value and the role of the

ethnobotanist is perceived to be that of a simple scribe, recording information for the bioscientists to utilise. These authors explain how ethnobotanists investigate the complex relationship between humans and plants and how their research is generally based on a detailed observation and study of the use a society makes of plants, including all the traditional beliefs and cultural practices associated with this use. Using several examples of ethnobotany-driven anticancer research, they highlight the potential and complexity of the multidisciplinary approach to ethnopharmacology and describe what challenges today's ethnobotanists face.

Heinrich and Bremner (2006) emphasise that “ethnobotanists have a responsibility to both the scientific community as well as to the indigenous cultures”, a belief that is the foundation upon which the research described in this thesis was based. I would now like to summarise the objectives of the project, the methodology involved and reveal what was discovered.

1.5 SCOPE OF THE CURRENT PROJECT

1.5.1 Approach

Many native plants have been traditionally used as medicines by the indigenous Aboriginal people of Australia (ACNTA, 1988; Isaacs, 1987; Low, 1990; Palombo & Semple, 2001; Mijajlovic *et al.*, 2006; Locher & Currie, 2010). However, Western medicine requires scientific evidence before pharmaceuticals can be developed, endorsed and supplied to the public. This means rigorous basic and clinical research is absolutely necessary. The effects of potential treatments need to be thoroughly investigated through placebo-controlled, randomised, scientifically valid experiments, and the subsequent data published in peer-reviewed journals (Parris & Smith, 2003; Trachtenberg, 2002). Additionally, it is important

to realise that the continued knowledge of and use of these resources requires not only their recognition as local knowledge, but also their multidisciplinary study. Obviously, this is only possible if the traditional keepers of this knowledge have a say in its future use and benefit somehow from such research and development (Heinrich and Bremner, 2006).

As outlined previously, recently there has been an increased appreciation of the value of ethnomedical knowledge as a guide to identifying new therapeutic agents, and a heightened interest in their modes of action (Kong *et al.*, 2003; Heinrich and Bremner, 2006; Melnick, 2006a). This project aimed to evaluate the anticancer potential of plants identified by Aboriginal people as having medicinal properties. The goal was to discover or identify a compound or compounds that may be of use in the treatment of cancer.

As discussed further in section 1.4.4.2.6, it can be a delicate and time-consuming process to earn the trust of Aboriginal community leaders so as to enlist their cooperation in identifying plants with traditional medicinal value. However, as a consequence of various Desert Knowledge Cooperative Research Centre projects, such cooperation was obtained and therefore access to hitherto restricted knowledge and plant resources was available. Although previous workers had used human cell lines to investigate the anticancer potential of a range of plant species, including those used as medicines by other indigenous cultures of the world (Chinkwo, 2005; Shoemaker *et al.*, 2005; Lampronti *et al.*, 2003; Lee & Houghton, 2005; Tai *et al.*, 2004) and Aboriginal people (Mijajlovic *et al.*, 2006), before now nobody had tested many of the particular Australian plants I evaluated. This was the original contribution made through my PhD studies.

1.5.2 Overview of experiments

After the harvesting and processing of appropriate plant species, the antineoplastic activity of methanolic extracts was quantitated using the MTT assay as a measure of inhibition of cell proliferation *in vitro* (Kicic *et al.*, 2002; Budman *et al.*, 2002). The selectivity of the most bioactive extracts for various human cancer cell lines was then assessed. Initial studies focused on producing concentration curves to determine IC₅₀ values of extracts and comparing those to published values.

It was then necessary to determine the specificity of active extracts for cancer cells compared with nontumorigenic proliferating cells. Ideally, promising extracts display no bioactivity against normal cells. However, even if an extract just showed relatively less effect on normal cells it was still considered of interest, as most drugs already in clinical use affect normal cells as well as cancer cells; they just preferentially inhibit cancer cell growth. This is often due to the greater rate of proliferation of neoplastic cells compared to normal cells (Lord, 1987; Blagosklonny, 2006; Senthilnathan *et al.*, 2006).

The initial screen revealed that some methanolic extracts were more bioactive than others. Therefore, several plant species were chosen for further evaluation. More samples were collected and extracted to give aqueous, methanol or ethyl acetate fractions. These were subjected to various chemical analyses and assessed for their specificity and selectivity against cancer cells. Time course experiments were then conducted to establish whether the effects were reversible when the extracts were removed. These kinetics experiments suggested that the bioactivity of extracts was due to cytotoxic effects. In addition, morphological changes that occurred to cells upon exposure to the extracts revealed more

about their modes of action. General nonspecific cytotoxicity was evaluated by studying the lethal effects of various extracts on brine shrimp.

From these data, the most promising bioactive extract was chosen for further characterization. Active constituents were identified by LC-MS and shown to be mostly flavonoids. Therefore, the cytotoxic activities of pure compounds of these flavonoids were compared to that of the whole extract. It was shown that the observed cytotoxic effects of the extract tested were not due to the flavonoids tested, but some other, undetermined compound. Finally, cells were exposed to the selected extract and Ca^{2+} entry into the cells was measured to obtain more clues as to its mode of action. These studies suggested that the inhibitory effect of the selected extract on cancer cells was not due to a nonspecific mechanism, but rather to a physiological response such as apoptosis. However, more experiments are required to confirm this.

1.5.3 Specific aims

So, in summary, the specific aims of this PhD project are:

1. to use ethnomedical knowledge as a guide to identifying plant compounds that may be of use in the treatment of cancer;
2. to scientifically verify that plants used as Aboriginal medicines are bioactive, with the ultimate goal being that Indigenous communities benefit from their traditional knowledge.

Chapter 2: Materials and Methods

2.1 MATERIALS

Unless otherwise specified, reagents were all of analytical grade or better.

Table 2.1 outlines the sources of the various reagents used in this study.

Common salts not listed were from BDH (Kilsyth, Vic, Australia) or Ajax chemicals (Auburn, NSW, Australia).

Table 2.1 Sources of Reagents

Reagent	Supplier	Catalogue No.	Australian distributor
Camptothecin (CPT)	Sigma-Aldrich	C9911	Sigma-Aldrich; Castle Hill, NSW
Deferoxamine mesylate (DFO)	Sigma-Aldrich	D9533	Sigma-Aldrich; Castle Hill, NSW
Deferriprone (L1, CP20, 1,2-dimethyl-3-hydroxypyrid-4-one)	Professor D.R. Richardson and Prof. P. Ponka		Gifts
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D5879	Sigma-Aldrich; Castle Hill, NSW
DMEM solution (phenol red free)	Sigma-Aldrich	D1145	Sigma-Aldrich; Castle Hill, NSW
DNA (salmon sperm)	Sigma-Aldrich	D1626	Sigma-Aldrich; Castle Hill, NSW
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	12800-017	Invitrogen; Mt Waverley, Vic
Etoposide (ETO)	Alexis Chemicals	ALX-270-209-M100	Sapphire Bioscience; Redfern, NSW
F12 Nutrient Mixture (Ham's F12)	Gibco	21700-075	Invitrogen; Mt Waverley, Vic
F12 Nutrient Mixture Kaighn's Modification (Ham's F12K)	Gibco	21127-022	Invitrogen; Mt Waverley, Vic
Fluorouracil-5 (5FU)	Sigma-Aldrich	F6627	Sigma-Aldrich; Castle Hill, NSW
Foetal Calf Serum (FCS)	Gibco	10099-141	Invitrogen; Mt Waverley, Vic
Fura-2-AM ester	Biotium Inc.	50033-1	Jomar Diagnostics; P/L, Stepney, SA
Histamine	Sigma-Aldrich	H7125	Sigma-Aldrich; Castle Hill, NSW
Hoechst 33258 dye	Boehringer Mannheim	663409	Roche Diagnostics; Castle Hill, NSW

Ionomycin	Sigma-Aldrich	I9657	Sigma-Aldrich; Castle Hill, NSW
Isopropanol, anhydrous (2-methyl-1-propanol)	Sigma-Aldrich	278475	Sigma-Aldrich; Castle Hill, NSW
L-Glutamine	Gibco	21051-016	Invitrogen; Mt Waverley, Vic
Luteolin	Sigma-Aldrich	L9283	Sigma-Aldrich; Castle Hill, NSW
MEM non-essential (NE) amino acids solution	Gibco	11140-050	Invitrogen; Mt Waverley, Vic
Minimum Essential Medium Eagle (MEM)	Sigma-Aldrich	M2279	Sigma-Aldrich; Castle Hill, NSW
Paclitaxel (PAC)	Alexis Chemicals	ALX-351-001-M025	Sapphire Bioscience; Redfern, NSW
Penicillin-G	Sigma-Aldrich	P3032	Sigma-Aldrich; Castle Hill, NSW
Phenol Red	BDH	20090 4U	Lab Supply; Mascot, NSW
Pluronic F127	Biotium Inc.	59000-F	Jomar Diagnostics; P/L , Stepney, SA
Quercetin dihydrate	Sigma-Aldrich	Q0125	Sigma-Aldrich; Castle Hill, NSW
RPMI Medium 1640	Gibco	31800-022	Invitrogen; Mt Waverley, Vic
Rutin hydrate	Sigma-Aldrich	R5143	Sigma-Aldrich; Castle Hill, NSW
Sodium dodecyl sulfate (SDS)	Bio-Rad Laboratories	161-0301	Bio-Rad; Gladesville, NSW
Sodium pyruvate	Gibco	11360-070	Invitrogen Mt Waverley, Vic
Streptomycin sulfate	Sigma-Aldrich	S9137	Sigma-Aldrich; Castle Hill, NSW
Synthetic Sea Salt	Aquasonic Ocean Nature		Pet City; Perth, WA
Tamoxifen citrate (TAM)	Alexis Chemicals	ALX-550-095-G001	Sapphire Bioscience; Redfern, NSW
Thiazoyl blue tetrazolium bromide (MTT)	Sigma-Aldrich	M2128	Sigma-Aldrich; Castle Hill, NSW
Trypan blue solution	Sigma-Aldrich	T8154	Sigma-Aldrich; Castle Hill, NSW
Trypsin-EDTA (10X)	Gibco	15400-054	Invitrogen; Mt Waverley, Vic
Vinblastine sulfate salt (VIN)	Sigma-Aldrich	V1377	Sigma-Aldrich; Castle Hill, NSW

2.2 EQUIPMENT

2.2.1 Cell culture

2.2.1.1 *Sterilisation*

All glass pipettes and plastic pipette tips were sterilised at 120°C under 125kPa pressure for 20 min in a Siltex 250D autoclave (B Scientific; Perth, WA, Australia). Other glassware was sterilised at 200°C for 2 h in an Axyos oven (Brendale, Qld, Australia). Media made up from powder was filter-sterilised using the Millipore system under pressure. All other liquids, including glutamine, penicillin-streptomycin and dissolved plant extracts and controls, were sterilised through an Acrodisc[®] 25 mm PF syringe filter, 0.8/0.2 µm Supor[®] membrane (Pall Corporation; Ann Arbor, MI, USA).

2.2.1.2 *Aseptic conditions*

Cell culture was performed aseptically in a class II biological safety cabinet (Email Westinghouse; NSW, Australia). This cabinet also served to protect the user from biohazards, such as chemicals and potential diseases within human cell lines.

2.2.1.3 *Incubation*

Cell lines were maintained at 37°C in a humidified atmosphere (85% relative humidity) consisting of 5% CO₂/ 95% air in a Thermo Forma Steri-cult CO₂ HEPA Class 100 incubator (Biolab; Scoresby, Vic, Australia). This appliance was also used for incubation steps during the MTT assay.

2.2.2 General procedures

2.2.2.1 *Centrifugation*

Sedimentation of cells for subculturing was carried out in a Centurion bench top centrifuge (Thermoline Scientific; Smithfield, NSW, Australia). Small samples in Eppendorf tubes were spun in an Eppendorf 5415D centrifuge (Crown Scientific; Minto, NSW, Australia).

Other washing procedures utilised a refrigerated IEC GP8R centrifuge with a 210 rotor (Selby Biolab; Malaga, WA, Australia).

2.2.2.2 *Weighing*

Small quantities of reagents and samples were weighed out using an A&D HA 120-M balance with an accuracy of 0.0001 g (Lab Supply; Mascot, NSW, Australia). Larger quantities were measured on a Mettler PE 360 DeltaRange[®] (accuracy 0.001 g) balance (Greifensee, Zurich, Switzerland).

2.2.2.3 *pH measurement*

pH was measured using an Activon model 210 pH/mV/ATC/temp meter (North Ryde, NSW, Australia) with an Ionode IJ44 electrode (Tennyson; Qld, Australia), or an Aqua pH cube from TPS (Brisbane, Qld, Australia).

2.2.2.4 *Osmometry*

Osmolality was measured via freezing point depression using a Fiske F1-10 osmometer (Needham Heights, MA, USA).

2.2.2.5 *Refractometry*

A Uricon-NE hand-held refractometer (Cat No. 2722) from Atago Co. Ltd. (Tokyo, Japan) was used to measure the refractive index of samples.

2.2.2.6 *Liquid dispension*

Graduated glass pipettes and a Drummond Pipet Aid XP were used for millilitre quantities. Gilson micropipettes were used for smaller volumes that were only required one or two times. When several aliquots of the same sample were required, an Eppendorf Combitip multipipette system (Crown Scientific; Minto, NSW, Australia) was used. When many aliquots of the same solution were required (e.g. DMSO in the MTT assay), a Titertek Plus multichannel, variable volume pipette (ICN; Seven Hill, NSW, Australia) was employed.

2.2.2.7 *Spectrophotometry*

At first, absorbance of 96-well microtitre plates was measured at a wavelength of 595 nm on a Model 3550 microplate reader (Bio-Rad Laboratories; Gladesville, NSW, Australia). Later, an ELx808 Ultra microplate reader (BioTek, Millennium Science; Surrey Hills, Vic, Australia) linked to Gen5 software was used to measure absorbance at $\lambda = 570$ nm. A Cary 50 Bio UV/VIS spectrophotometer from Varian (Mulgrave, Vic, Australia) was used for other spectrophotometric measurements, including scans, of larger sample.

2.2.2.8 *Microscopy*

Cultured cells were generally viewed under 400X magnification using a Nikon Diaphot TMD1 phase contrast microscope (FSE Scientific; Burwood, NSW, Australia) to observe cell morphology, viability and proliferation. Dead brine shrimp (see section 2.6.3) were scored by viewing under 20X magnification with a VMT stereo microscope (Olympus; Mount Waverley, Vic, Australia).

2.2.2.9 *Fluorometry*

Fluorescence intensity measurements were performed on 96-well plates using a FLUOstar Optima Multidetector Microplate Reader from BMG Labtech (Mount Eliza, Vic, Australia).

2.2.2.10 *Cellscreen (CS) system*

The Cellscreen (CS) system purchased from Innovatis AG (Bielefeld, Germany) was used to analyse the percentage confluence of cells grown in 96-well microtitre plates (cat. No. 167008, Nunc; Scoresby, Vic, Australia). This system is comprised of an IX50 inverted phase microscope (Olympus), a motorised X-Y stage and a KPF100 CDD camera (Hitachi) with a resolution of 1024 x 1024 pixels. Pattern recognition software analysed captured images of 4 areas of each well viewed under the 4X objective. Experimental settings for

acquisition of images and results were stored in a database linked to a personal computer and were accessed via the graphical user interface.

2.2.2.11 *Ca²⁺ measurement apparatus*

The apparatus required for measuring intracellular Ca²⁺ (described briefly in section 2.6.4 and in more detail in section 6.2.4) consisted of a high-intensity xenon arc lamp, a photomultiplier tube and an OptoScan monochromator all from Cairn Research Ltd. (Kent, UK) and a TE2000 inverted microscope equipped for epifluorescence (Nikon; Tokyo, Japan).

2.2.3 Solutions

Unless stated otherwise, all solutions were prepared in double deionised water (DDW) and adjusted to pH 7.4. DDW was purified by passing distilled water through a Milli-Q system (Millipore; North Ryde, NSW, Australia). All were stored at 4°C unless specified.

2.2.3.1 *Isotonic saline*

Isotonic (300 mOsm/kg) saline was made by dissolving 91.4 g of NaCl in 10L of DDW to give a final concentration of 155 mM. It was stored at room temperature (RT).

2.2.3.2 *Isotonic phosphate*

Approximately 70 mL of 155 mM NaH₂PO₄ was added to 400 mL of 103 mM Na₂HPO₄ until the pH reached 7.4. The final concentrations were therefore approximately 23.1 mM NaH₂PO₄ and 87.7 mM Na₂HPO₄.

2.2.3.3 *Phosphate buffered saline (PBS)*

Phosphate buffered saline (PBS), pH 7.4, was prepared by adding approximately 470 mL of isotonic phosphate (2.2.3.2) to 10 L of isotonic saline (2.2.3.1).

2.2.3.4 *Phenol red stock solution*

A 0.5% (w/v) solution of phenol red was prepared by dissolving 0.5 g of phenol red powder in 100 mL DDW with the aid of a few drops of 10M NaOH. This solution was passed through filter paper and stored at RT, where it was stable for many years.

2.2.3.5 *Buffered salt solution (BSS)*

Balanced salts solution (BSS) comprised 0.15 mM NaH_2PO_4 , 1.3 mM Na_2HPO_4 , 6.0 mM NaHCO_3 , 5.6 mM D-glucose, 137 mM NaCl, 2.7 mM KCl, 1.0 mM CaCl_2 , 1.0 mM MgCl_2 and 0.0005% (w/v) phenol red adjusted to pH 7.2 and 300 mosm/kg.

2.2.3.6 *Calcium and magnesium free salts (CMFS)*

As for BSS (2.2.3.5), but without CaCl_2 , MgCl_2 and phenol red.

2.2.3.7 *Basal media*

Media used for cancer cell line maintenance was generally made up from powdered sachets according to the manufacturer's directions. Appropriate amounts of NaHCO_3 were added and the pH adjusted to 7.2 before filter-sterilisation (see section 2.2.1.1) to allow for the 0.1-0.3 unit rise in pH upon filtration so that the final pH was 7.4. Ham's F12K was obtained in liquid form (see Table 2.1), as was media containing no phenol red, which was used during assays.

2.2.3.8 *Complete media*

Complete medium varied for individual cell lines, but in this text, refers to the appropriate basal medium including all supplements, as indicated in section 2.3.1. Complete medium is what the cells were actually cultured in.

2.2.3.9 *Trypsin-EDTA solution*

10X trypsin-EDTA stock was diluted 1 in 10 in CMFS. The concentrations of this working solution were 0.05% trypsin (w/v) and 0.4 mM EDTA.

2.2.3.10 *L-Glutamine*

A 100X stock solution of glutamine was made by dissolving 2.92 g of L-glutamine in 100 mL of DDW. This was filter-sterilised and stored in 10 mL aliquots at -20°C until required. 1 mL of this solution was added to 100 mL of basal medium to give a final concentration of 2 mM. Glutamine was replenished fortnightly as it is unstable at 4°C.

2.2.3.11 *Penicillin-streptomycin*

A 100X stock solution of penicillin-streptomycin was prepared by dissolving 0.633 g of penicillin-G (1575 U/mg) and 1.0 g of streptomycin in 100 mL DDW. After filter-sterilisation, this was divided into 10 mL aliquots and stored at -20°C until required. A 1 mL aliquot of this solution was added to 100 mL of medium to give final concentrations of 100 U/mL penicillin and 100 µg/mL streptomycin.

2.2.3.12 *Freeze medium*

A cryoprotective medium for freezing cells was prepared by mixing one part DMSO with 9 parts FCS. This was protected from light with aluminium foil around the tube.

2.2.3.13 *MTT solution*

Thiazoyl blue tetrazolium bromide (MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was originally dissolved at 5 mg/mL in PBS on the day of use before filter-sterilisation. However, an adjustment to the protocol halfway through the project resulted in MTT being dissolved at 1 mg/mL in the DMEM containing no phenol red.

2.2.3.14 *Extraction buffer*

The original buffer used to solubilise formazan crystals consisted of 10% SDS in 50% isobutanol in 0.1 M HCl, stored at RT for up to several months. Later in the project, 100% DMSO was used for extraction instead (see section 3.2.2).

2.2.3.15 *Artificial sea water (ASW)*

“Synthetic sea salt” was dissolved at 33.34 g per L of deionised water and the pH checked to be between 8 and 8.5 to make artificial sea water (ASW), which was stored at RT.

2.2.3.16 *Physiological rodent saline (PRS)*

The base buffer used in the measurement of Ca^{2+} was physiological rodent saline (PRS). This consisted of 138 mM NaCl, 2.7 mM KCl, 1.06 mM MgCl_2 and 12.4 mM HEPES, pH 7.3 which was supplemented with 1.8 mM CaCl_2 and 5.6 mM glucose on the day of experimentation.

2.2.3.17 *Fura-2-AM*

The Ca^{2+} -sensitive fluorescent dye, Fura-2 bound to an acetoxymethyl ester group (Fura-2-AM), was dissolved in DMSO to give a stock solution of $1\mu\text{g}/\mu\text{L}$ (= 1 mM) which was stored at -20°C .

2.2.3.18 *Pluronic F-127*

The non-ionic detergent and dispersing agent, Pluronic F-127, was dissolved at 20% (w/v) in DMSO and stored at -20°C .

2.2.3.19 *TNE buffer*

A 2X stock solution of TNE buffer consisted of 20 mM Tris base, 2 mM EDTA and 4M NaCl, pH 7.4 and was stored at RT.

2.3 CELL CULTURE

2.3.1 Cell lines

All cell lines used were of human origin in order to more closely mimic how plant extracts would affect human cancer cells. Cells were generally cultured in 10 mL of appropriate medium in 75 cm^2 tissue culture (T-75) flasks at 37°C in a humidified atmosphere of 5% CO_2 / 95% air (see 2.2.1.3). Cells were passaged weekly and medium replaced fortnightly.

2.3.1.1 *Breast cancer*

The human breast adenocarcinoma epithelial cell lines MDA-MB-468 and MCF7, are estrogen receptor negative and estrogen receptor positive, respectively. MDA-MB-468 cells were a generous gift from Professor Peter Leedman's laboratory (West Australian Institute of Medical Research, Royal Perth Hospital, WA, Australia) and MCF7 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The MDA-MB-468 cancer cell line was routinely cultured in a 1:1 mixture of DMEM and Ham's F12 media, supplemented with 10% FCS, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, while MCF7 cells were maintained in DMEM with the same composition of supplements.

2.3.1.2 *Skin cancer*

MM253 (skin melanoma) cells were kindly provided by Professor Peter Parsons (QIMR, Qld, Australia) and cultured as above, except the basal medium was RPMI-1640.

2.3.1.3 *Colon cancer*

Caco-2 (colorectal carcinoma) cells from ATCC via Professor Trevor Redgrave's laboratory (Physiology, University of Western Australia (UWA), Crawley, WA, Australia) were maintained in DMEM, supplemented with 15% FCS, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/mL penicillin and 100 g/mL streptomycin.

2.3.1.4 *Lung cancer*

A549 (lung carcinoma) cells were kindly provided by Professor Geoffrey Stewart's laboratory (Microbiology and Immunology, UWA). These cells were cultured in Kaighn's

modification of Ham's F12 medium supplemented with 10% FCS, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

2.3.1.5 *Prostate cancer*

DU145 (prostate carcinoma) cells were also kindly donated by Professor Peter Parsons (QIMR). A second prostate adenocarcinoma cell line, PC3, was another kind gift from Professor Geoffrey Stewart (UWA). Both these cell lines were maintained in RPMI-1640 medium containing 10% FCS, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

2.3.1.6 *Non-tumourigenic*

Normal lung fibroblasts, MRC5, were kindly provided by Professor Des Richardson's laboratory (Bosch Institute, University of Sydney, Sydney, NSW, Australia). The MRC5 cell line was derived from normal lung tissue of a 14-week-old male foetus and was originally purchased from ATCC. MRC5 cells, initially obtained at passage 6, were maintained in RPMI 1640 medium supplemented with 10% FCS, non-essential (NE) amino acids, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

A summary of the various cell lines used in this study is provided in Table 2.2.

Table 2.2 Human Cell Lines Used

Name	Organ	Morphology	Disease	Derived from primary tumour	Growth medium*
MDA-MB-468	Breast	Epithelial	Adenocarcinoma	No; (pleural effusion)	DMEM/Ham's F12/FCS ₁₀
MCF7	Breast	Epithelial	Adenocarcinoma	No; (pleural effusion)	DMEM/FCS ₁₀
Caco-2	Colon	Epithelial	Adenocarcinoma	Yes	DMEM/FCS ₁₅
MM253	Skin	Melanomic	Melanoma	No; (secondary melanoma)	RPMI/FCS ₁₀
A549	Lung	Epithelial	Carcinoma	Yes	Ham's F12K/FCS ₁₀
PC3	Prostate	Epithelial	Adenocarcinoma	No; metastatic site (bone)	RPMI/FCS ₁₀
DU145	Prostate	Epithelial	Carcinoma	No; metastatic site (brain)	RPMI/FCS ₁₀
MRC5	Lung	Fibroblast	Non-tumourigenic	Not applicable	RPMI/FCS ₁₀ /NE amino acids

* Also contained 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine

2.3.2 Subculture

Medium was removed by aspiration and adherent cells were washed with 5 mL of Calcium and Magnesium Free solution (CMFS) to remove residual medium, which contained FCS, an inhibitor of trypsin. In order to digest the extracellular matrix and suspend the cells, approximately 1 mL of a 1X solution of trypsin-EDTA in CMFS (see 2.3.8) was added to each flask. These were incubated at 37°C for no more than 10 min, the cells being monitored microscopically for signs of detachment over this period. Trypsin was inactivated by the addition of 9 mL of appropriate medium, containing FCS. Subcultivation ratios of between 1/5 and 1/50 were used, depending on the cell line and when the cells were required.

2.3.3 Experimental set-up

Usually, cells were studied in exponential growth phase after seeding 100 μ L at 100,000 cells /mL in a Cellstar[®] 96-well flat bottom microtitre plate (Greiner bio-one, Interpath; Heidelberg West, Vic, Australia). These cells were generally from cultures of between 70 and 90% confluency.

2.3.4 Cell line storage

Cells were removed from flasks with trypsin as outlined in section 2.4.2. However, after addition of 9 mL of appropriate medium containing FCS to inactivate trypsin, cells were transferred to a 15 mL centrifuge tube and spun at 400 x *g* for 5 min. The cell pellet from a single confluent T-75 flask was resuspended in about 1 mL of ice-cold freeze medium (2.3.12) and transferred to a 1 mL Nunc CryoTube[®] vial (Biolab; Scoresby, Vic, Australia). This was placed in an insulated rack to ensure slow cooling and stored at -80°C overnight (O/N). The vial was then transferred to a tank of liquid nitrogen for long term storage.

2.3.5 Cell line thawing

Every two to three months new stocks of frozen cells were thawed quickly in a 37°C water bath and transferred to a 15 mL centrifuge tube. About 9 mL of appropriate medium was added and the cells centrifuged at 1200rpm for 5 min. The supernatant containing the cryopreservative (DMSO) was aspirated and the cell pellet was resuspended in 10 mL of appropriate medium, before transfer to a T-75 flask. The following day, cells were microscopically assessed for viability and adherence. Usually, the medium containing dead cells was removed and replaced with fresh medium.

2.4 PLANT EXTRACTS

2.4.1 Collection and handling of plant material

Samples of plants pointed out by local Aboriginal people as having any medicinal properties were collected on two occasions from Titjikala in the Northern Territory (NT) (Batch 1 and Batch 2) and on one occasion from Scotdesco (Ceduna) in South Australia (SA). Batch 1 was collected in July 2004, Batch 2 in October 2004 and Batch 3 in April 2005. The samples were freighted by air or road to the School of Pharmacy at Curtin University of Technology in Perth (WA) in foam boxes as soon as possible after collection. In most cases they arrived at the School of Pharmacy within two days. The first batch of samples was stored in a quarantine-approved facility at 7°C and extraction of the volatiles was commenced as soon as possible, in most instances within one or two days. Samples collected later (Batch 2 and Scotdesco samples) were dried at 37 °C in a quarantine-approved facility for subsequent Soxhlet extraction. Voucher specimens of all plant species collected were sent to either the Western Australian Herbarium or the Alice Springs Herbarium for confirmation of correct identification.

When completely dry, samples were broken into small pieces using a mortar and pestle or powdered using a hammer mill. These samples were then stored in an air-tight container and protected from light. Plant material was extracted with methanol using a Soxhlet apparatus for several days until the extract appeared colourless. The solvent was evaporated under reduced pressure using a roto-evaporator and dried to constant weight in a vacuum oven. In total, 25 plant samples were collected and their methanolic extracts stored in the dark for subsequent bioactivity screening.

For convenience and confidentiality, the extracts were labelled as shown in Table 2.3. As mentioned previously, sometimes there is more than one vernacular name for a particular species. The common names presented were provided by the collectors.

2.4.2 Methanol extraction

Plant material was stored in paper bags in a quarantine approved area at 37°C until completely dry. It was then separated into small pieces using a mortar and pestle or powdered using a hammer mill, before being stored in an air-tight container protected from light. Samples were subsequently extracted with methanol (see section 4.1.2) in a suitably sized Soxhlet apparatus for several days until the extract of the new cycle appeared colourless. The solvent was then evaporated under reduced pressure using a roto-evaporator and dried to constant weight in a vacuum oven. This methanol extract was then stored in a suitable container protected from light. Approximately one quarter was posted to this laboratory for cytotoxic screening. Material was also sent to other associated research groups for pharmacological testing. One portion was tested for general toxicity using the brine shrimp and daphnia acute assays at the Centre for Sustainable Mine Lakes, Curtin University of Technology. Another quarter was screened for antibacterial and antiviral activity at the University of South Australia. The remaining quarter of the material was used for a range of basic chemical screening tests undertaken at the School of Pharmacy, Curtin University of Technology.

Table 2.3 Source and Designation of Plant Extracts

ID	Code	Scientific name	Common name	Plant part
Titjikala: Batch 1				
1 (i)	1	<i>Eremophila longifolia</i>	Emu Bush	Leaves, stems
2 (i)	2	<i>Eremophila latrobei</i>	Native Fuchsia	Leaves, stems, flowers
3 (i)	3	<i>Thysanotus exiliflorus</i>	Walda-Walda (Pitjantjatjara)	Roots
5 (i)*	4	<i>Eremophila sturtii</i>	Turpentine/Kerosene Bush	Leaves, stems, flowers
5 (i)	5	<i>Eremophila sturtii</i>	Turpentine/Kerosene Bush	Leaves, stems, flowers
8 (i)	8	<i>Eremophila freelingii</i>	Rock Fuchsia	Leaves, stems, flowers
10 (i)	10	<i>Sarcostemma australe</i>	Ipi Ipi (Pitjantjatjara)	Stems
11A (i)	9	<i>Acacia tetragonophylla</i>	Dead Finish	Leaves, stems
11B (i)	11	<i>Acacia tetragonophylla</i>	Dead Finish	Leaves, stems, flowers
12 (i)	12	<i>Hakea divaricata</i>	Fork-leafed Corkwood	Bark
15 (i)	6	<i>Euphorbia drummondii</i>	Caustic/Milk Weed, Mat Spurge	Leaves, stems, flowers, fruit
Titjikala: Batch 2				
T19	19	<i>Eremophila duttonii</i>		Leaves, stems
T20	20	<i>Euphorbia tannensis</i>		Stem (leaves)
T21	21	<i>Eremophila duttonii</i>		Leaves, stems
T22	22	<i>Hakea sp.</i>		Bark
T24A	23	<i>Hakea divaricata</i>	Fork-leafed Corkwood	Inner bark
T24B	24	<i>Hakea divaricata</i>	Fork-leafed Corkwood	Outer bark
T25	25	<i>Codonocarpus cotinifolius</i>		Stems, leaves
T26	26	<i>Euphorbia tannensis</i>		Stem (leaves)
T27	27	<i>Eremophila freelingii</i>	Rock Fuchsia	Leaves, stems
T28	28	<i>Acacia tetragonophylla</i>	Dead Finish	Root bark
Scotdesco: Batch 3				
S1.1	A	<i>Eremophila alternifolia</i>	Narrow-leaf fuchsia bush	Leaves, stems
S1.2	B	<i>Eremophila alternifolia</i>	Narrow-leaf fuchsia bush	Leaves, stems
S3	C	<i>Scaevola spinescens</i>	Fan flower, maroon bush	Leaves, stems
S7	D	<i>Eremophila alternifolia</i>	Narrow-leaf fuchsia bush	Leaves, stems

* filter paper residue

2.5 CONTROLS

2.5.1 Positive controls

Two positive controls used were the Fe-chelating molecules, deferoxamine mesylate (DFO) and deferriprone (CP20 or L1), shown previously to inhibit cell proliferation *in vitro* and to be active clinically (Estrov *et al.*, 1987; Donfrancesco *et al.*, 1992). 10 mM solutions of these were prepared in 10% DMSO in complete medium and sterilised through a 0.22 μm filter. 10 μL of one of these chelators was added to cells at a final concentration of 1 mM. Another positive control, sometimes included, was 1 mM HgCl_2 , which would be expected to kill all cells (Cantoni *et al.*, 1984; Guo *et al.*, 1998; Schurz *et al.*, 2000). This was prepared by dissolving 10 mg HgCl_2 per mL of complete medium containing 10% DMSO.

Other positive controls used were tamoxifen (TAM), camptothecin (CPT), fluorouracil-5 (5FU), etoposide (ETO), vinblastine (VIN) and paclitaxel (PAC). Milligram quantities of these were dissolved in DMSO to give stock concentrations of 10 mM, which were stored at -20°C . These stock solutions were diluted 1/10 in complete medium and filter-sterilised to give 1 mM working solutions. However, these concentrations were still above optimal for some reagents and required further dilutions. The working solutions were serially diluted in complete medium in 1.5 mL Eppendorf tubes under aseptic conditions and 10 μL added to 100 μL of adherent cells in microtitre plates. The range of final concentrations for each control is summarised in Table 2.4.

Table 2.4 Concentrations of Positive Controls Used

Control	Code	Final Concentration Range (μM)
Deferoxamine mesylate	DFO	1,000-0.5
Deferriprone	L1	1,000-0.5
Camptothecin	CPT	100-0.05
5-Fluorouracil	5FU	100-0.05
Tamoxifen citrate	TAM	100-0.05
Etoposide	ETO	10-0.005
Paclitaxel	PAC	10-0.005
Vinblastine sulfate	VIN	10-0.005

2.5.2 Negative controls

The negative (vehicle) control was incubation medium containing DMSO at a concentration corresponding to the amount in the test solution.

Spectrophotometric scans of extracts between 200 nm and 800 nm (Appendix D) showed a considerable variation in absorption, therefore other controls, consisting of extracts added to wells containing no cells, were also used to account for background absorbance.

2.6 ASSAYS

2.6.1 MTT assay

Cell proliferation was assessed after the addition of plant extracts using a modification of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay routinely used in this laboratory (Kicic *et al.*, 2002) and in many others (e.g. Amirghofran *et al.*,

2007; Sun *et al.*, 2007b; Zhang *et al.*, 2007). This colourimetric assay is found to be accurate and reproducible and is based on the measurement of mitochondrial dehydrogenase activity of living cells.

Briefly, cells were seeded into 96-well microtitre plates at an initial concentration of 1×10^4 cells/well and incubated at 37°C for approximately 18-24 h before adding 10 µL of plant extracts or controls. Cells were incubated at 37°C for 48 h, and then 10 µL of MTT at a concentration of 5 mg/mL in PBS (2.2.3.13) was added. After 2 h at 37°C, 100 µL of extraction buffer (2.3.14) was added and the mixture was incubated for another 90 min at 37°C before reading the absorbance at 570 nm or 595 nm on a microplate reader (2.2.2.7).

However, an alternative method, described by Fox *et al.* (2005) was encountered halfway through the project which provided potential advantages over this protocol. In this alternative method (discussed further in section 3.2.2), culture medium was removed prior to addition of MTT, which was added at 1 mg/mL in medium (2.2.3.13). Cells were incubated with MTT at 37°C for 1 h, instead of the 2 h incubation period required for the original protocol of Kicic *et al.* (2002). The MTT solution was then removed prior to the addition of 100 µL DMSO, which was effectively the new extraction buffer used to solubilise the dye. This step did not require incubation and the absorbance at 570 nm was read within about 10 min. Hence, not only was the new method “cleaner” in that there was little colour interference from interactions with various components of the medium, plant extracts and MTT, but it was also much quicker. Therefore, this new protocol was implemented for later experiments.

2.6.2 DNA assay

DNA content of cells was measured according to the method of Rago *et al.* (1990). Briefly, cells in 96-well microtitre plates were first lysed by inverting the plates to drain them of medium and placing them in the -80°C freezer O/N. After warming to RT, 100 µL of sterile DDW was added to each well and the plates returned to the freezer for at least 3 h. During this time, salmon sperm DNA was dissolved in TNE buffer at 10 mg/mL and heated at 50°C until dissolved. This was diluted to 250 µg/mL in TNE, from which standards ranging from 10 ng to 5,000 ng per well were prepared. Hoechst 33258 dye was dissolved at 200 µg/mL in DDW and diluted 1/10 in either 1X (for standards) or 2X (for test samples) TNE buffer to give a working solution of 20 µg/mL. This was added to lysed cell samples or DNA standards at 100 µL per well and mixed briefly, giving final concentrations of 10 µg/mL. Fluorescence of 6 or 8 replicate wells was measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm using the fluorometer described in section 2.2.2.9.

2.6.3 Brine shrimp lethality assay (BSLA)

The brine shrimp (*Artemia salina*) lethality assay (BSLA) proposed by Michael *et al.* (1956) and later developed by Vanhaeke *et al.* (1981) and Sleet and Brendel (1983) is a useful tool for the assessment of the general cytotoxicity of various types of compounds (Carballo *et al.*, 2002; Turker & Camper, 2002). A positive correlation between brine shrimp toxicity and some cancer cell toxicity has been reported (Turker & Camper, 2002). Pertinently, it has been widely employed to evaluate the bioactivity of plant extracts, including those used traditionally as medicines (e.g. Desmarchelier *et al.*, 1996; Krishnaraju *et al.*, 2005; Turker & Camper, 2002). This bioassay is a simple, inexpensive, reliable and convenient method of determining general cytotoxicity. Brine shrimp eggs are

readily available and the nauplii (larvae) hatch quickly (Krishnaraju *et al.*, 2005).

Furthermore, only a very small amount of test compound is required (Amaro *et al.*, 2009).

In this study, brine shrimp eggs (stored at 4°C) were hatched into nauplii O/N in a 1 L separating funnel. Approximately half a teaspoon of brine shrimp eggs were added to 500 mL of artificial sea water (ASW) maintained at between 28 and 32°C using heat lamps and subjected to constant aeration and illumination. After 24 h, unhatched eggs which had settled to the base of the funnel were removed and live nauplii were collected in a small beaker. As the brine shrimp are phototropic (Carballo *et al.*, 2002; Turker & Camper, 2002), a torch was used to illuminate one side of the beaker in order to concentrate shrimp in a smaller area. Using a Pasteur pipette, swimming shrimp were collected and transferred to a petri dish which was placed on a dissecting (Olympus VMT stereo) microscope. Light was then directed to one part of the petri dish to attract shrimp to a smaller area and, under 20X magnification, an adjustable micro pipette was used to draw up 20 µL of ASW containing as many live shrimp as possible. At least 8 shrimp were delivered to wells of a 96-well micro plate prepared with 180 µL aliquots of varying concentrations of the extracts or compounds being tested. Dead shrimp were then scored by viewing wells under 20X magnification at intervals over a 48 h period. Nauplii were only considered dead if they remained completely immobile, including antennae and mouth parts, for several seconds of observation. Total shrimp in each well were counted after sacrificing surviving nauplii with 40 µL of methanol per well. The percentage of dead shrimp for each time point was calculated and compared to a control which contained DMSO only.

2.6.4 Intracellular Ca^{2+} measurement

Changes in intracellular Ca^{2+} were measured in cells loaded with the fluorescent Ca^{2+} indicator, Fura-2, as described in more detail in Section 7.2.4.2. Briefly, Fura-2-loaded cells were placed on the stage of an inverted epifluorescence microscope attached to the Ca^{2+} measurement apparatus (see Section 2.2.2.11) and illuminated at alternating excitation wavelengths of 340 and 380 nm. Fluorescence emission at 510 nm was captured after being filtered by a 510 bandpass filter. Acquisition and analyses of data were performed with an Optiscan control system and software supplied by Cairn Research Ltd. (Kent, UK). The concentration of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) was taken as the emission ratio of the fluorescence intensities at the two excitation wavelengths (F_{340}/F_{380}) (Grynkiewicz *et al.*, 1985; Jackisch *et al.*, 2000).

2.6.5 Protein assay

Protein content was assessed using the Pierce[®] BCA[™] Protein Assay Kit (Prod # 23227) from Thermo Scientific (Rockford, IL, USA) according to the manufacturer's directions. This assay is based on the combination of the reduction of Cu^{2+} to Cu^+ by protein under alkaline conditions (the Biuret reaction) with the highly sensitive and selective colourimetric detection of Cu^+ using a reagent containing bicinchoninic acid (BCA) (Smith *et al.*, 1985). Briefly, 25 μL samples were aliquotted in quadruplicate into wells of a 96-well microtitre plate and 200 μL of working reagent added. After thorough shaking for 30 s, the plate was incubated at 37°C for 30 min. Upon cooling at RT, OD₅₇₀ readings were compared to those of known BSA standards prepared in parallel.

2.7 STATISTICS

2.7.1 Means and standard errors

Only positive values were included when calculating means and their standard errors, which were done using Excel. Student's unpaired, two-tailed t-tests were performed to compare two means using the InStat programme (GraphPad Software, La Jolla, CA, USA). The Welch correction was employed when the variances were unequal.

2.7.2 Analysis of variance

One-way analyses of variance (ANOVAs) were performed on mean values to compare differences between samples, particularly the effects of individual extracts versus controls. This was done in InStat using Dunnet's Multiple Comparisons Test. Student Newman-Keuls (SNK) tests were used to compare differences between mean values of other samples.

2.8 CURVE FITTING

2.8.1 Linear regression

Linear regression curves were fitted to standard curves using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The squared correlation coefficient (r^2 value) was reported to indicate goodness of fit.

2.8.2 Nonlinear regression

Percent of control values were plotted against the logarithm of the concentration of samples and these data were analysed using GraphPad Prism to fit non linear regression curves based on a sigmoidal dose-response, allowing for a variable slope. In this way, the extract concentration which inhibited cell proliferation by 50%, the IC₅₀ value, was calculated for each sample. It is important to note that for each sample, the mean data were analysed in

four separate ways: (i) by the default 4-Parameter (4P) curve-fitting model in which neither the top nor the bottom values were fixed, (ii) by fixing the top value at 100, (iii) by fixing the bottom value at 0, (iv) or by fixing both the top and bottom values at 100 and 0, respectively. The optimum model for each combination of extract and cell line was chosen based on the narrowest range of 95% confidence intervals of the standard error (SE) of the calculated IC_{50} value and the r^2 value.

Chapter 3: Optimisation of Experimental Techniques

INTRODUCTION

The validity of the ethnopharmacological approach to identifying potential anticancer agents has already been discussed in Chapter 1, where it was argued that using this multidisciplinary strategy is a very worthwhile pursuit. Nevertheless, few studies have used Australian Aboriginal traditional medical knowledge as a guide to identifying plants that may have anticancer properties. Following chapters will describe results obtained by using this uncommon approach. However, before screening studies could commence, it was necessary to validate the proposed methodology. Therefore, this chapter seeks to justify the specifics of the experimental design. It will focus on why the particular cell lines, controls and assays were chosen and how conditions were optimised in order to study the effects of the various provided plant extracts on cancer cells.

3.1.1 Cell lines

The research described within this thesis utilised human cancer cell lines as models for studying the effects of plant extracts on cancer cells. Well characterised cell lines are invaluable tools for initial drug screening procedures, allowing researchers to study drug effects *in vitro* before unnecessarily expending precious, and more expensive, live animals. Continuous cell lines are usually of monoclonal origin and are in an arrested state of differentiation characteristic of cancer cells. Thus, they provide more reproducible data as the cell population responds as one. However, their main advantage is their virtually unlimited proliferation, resulting in an almost limitless supply of cells that can be stored and recovered as required. The philosophy behind using cell lines derived from cancerous human tissue was simply to provide data appropriate for comparison to human patients (Korting & Schafer-Korting, 1999). In other words, the effects of plant extracts on cells of

human origin would be expected to more closely reflect how those extracts would affect human cancer cells *in vivo* than if animal models were utilised. Indeed, it was for these very reasons that the National Cancer Institute (NCI) switched from screening potential anticancer drugs on leukemic mice to a battery of 60 human cancer cell lines representative of various tumour types (Alley *et al.*, 1988; Shoemaker, 2006). However, the NCI quickly ascertained an unexpected, additional advantage of using several types of cancer lines in that patterns of relative drug sensitivity and resistance generated can provide insights into mechanisms of drug action (Shoemaker, 2006).

The cell lines used in these studies were chosen based on their availability, ease of maintenance and clinical significance. Excluding non-melanocytic skin cancer (NMSC), which is at least four times more common than all other cancers combined, the five most commonly diagnosed cancers in the Australian population in order of incidence are prostate, colorectal, breast, skin and lung. In 2005 these five cancers accounted for >60% of all new cases (AIHW & AACR, 2008). The cell lines employed in this study represent these five most common cancers and have been used by others, including the NCI, to study the cytotoxic effects of potential anticancer agents *in vitro* (see Table 3.1).

Table 3.1 The Five Most Commonly Occurring Cancers in Australia

Type of Cancer	Incidence*	Representative Cell Line/s	References
Prostate	16,349	PC3 and DU145	Budman <i>et al.</i> , 2002; Ogbourne <i>et al.</i> , 2004; Russo <i>et al.</i> , 2005; Alley <i>et al.</i> , 1988
Colorectal	13,076	Caco-2	Russo <i>et al.</i> , 2005; Chen <i>et al.</i> , 1998; Visanji <i>et al.</i> , 2004
Breast	12,265	MCF7 and MDA-MB-468	Lampronti <i>et al.</i> , 2003; Tai <i>et al.</i> , 2004; Lee & Houghton, 2005; Alley <i>et al.</i> , 1988
Skin	10,684	MM253	Parsons & Morrison, 1978; Parsons <i>et al.</i> , 1982
Lung	9,182	A549	Yin <i>et al.</i> , 2004; Cheng <i>et al.</i> , 2005; Amirghofran <i>et al.</i> , 2007; Sun <i>et al.</i> , 2007b; Alley <i>et al.</i> , 1988

*No. of new cases diagnosed in Australia in 2005 (AIHW & AACR, 2008).

3.1.2 MTT assay

The principal method used to determine cell proliferation in this study was the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The principle of the MTT assay is that mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of MTT, yielding MTT formazan crystals (Figure 3.1). Solutions of MTT solubilised in tissue culture media (without phenol red) are yellowish in colour; MTT formazan crystals are purple. Crystals are dissolved in an extraction buffer and the resultant purple solution can be measured spectrophotometrically at a wavelength of around 570 nm. Since tetrazolium salts are cleaved only in metabolically active cells, the number of viable cells is proportional to the amount of MTT formazan crystals and, hence, absorbance (or optical density, OD) (Mosmann, 1983). By measuring cell numbers over time, the MTT assay can therefore give an indication of changes in the rate of cell proliferation.

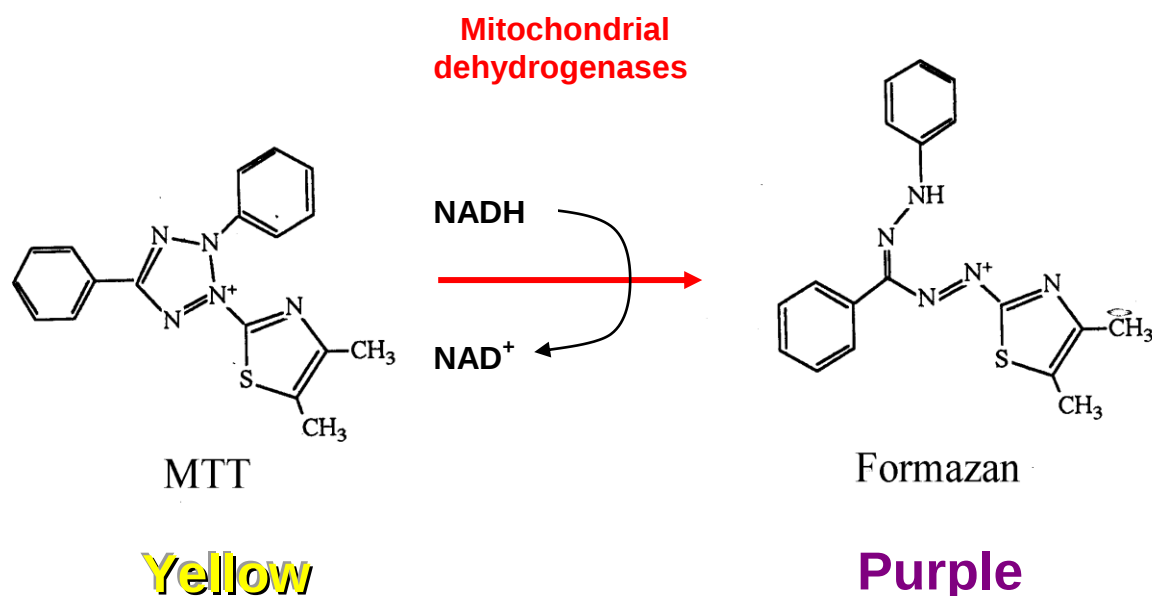


Figure 3.1 Principle of the MTT assay

First developed by Mosmann (1983), the MTT assay is among the most widely used *in vitro* methods to evaluate cell proliferation and viability (Ahmad *et al.*, 2006; Wang *et al.*, 2006; Abe & Matsuki, 2000) as it generates data that are both accurate and reproducible (Edwards *et al.*, 2008; Sargent, 2003). Additionally, the MTT assay lends itself to high through-put screening because it is rapid and sensitive (Kawada *et al.*, 2002; Mosmann, 1983; Wu *et al.*, 2008b) and has been used by the NCI for the large scale screening of potential anticancer drugs (Alley *et al.*, 1988). Moreover, it is simple and quantitative and has been routinely used in this laboratory for many years. For all these reasons, the MTT assay was selected for these studies above alternative methods of assessing changes in viable cell numbers.

However, although the MTT assay has been extensively used on other cell lines, it was important to confirm its validity in determining changes in cell numbers using the particular culture conditions of these studies. Hence, MTT assay-derived data were compared to other measures of cell viability and proliferation. Additionally, experiments were conducted which aimed to optimise its efficiency in assessing the cytotoxic effects of plant extracts against the panel of cell lines employed in these studies.

Basically, the MTT assay consists of three steps:

1. Incubation of cells with MTT;
2. Solubilisation of formazan crystals; and
3. Spectrophotometric measurement of resultant solution.

Each of these steps was adjusted in order to optimise the efficacy of the overall procedure.

3.1.3 Vehicle

Ideally, plant extracts would have been dissolved in water, more closely mimicking the bush medicine remedies of Aboriginal tradition. Unfortunately, however, complete aqueous dissolution was not possible for most of the crude methanolic extracts, even at concentrations below 100 mg/mL. As this was a quantitative study, this situation was obviously not tolerable. Therefore, unless otherwise specified, the vehicle used to dissolve all controls and plant extracts was DMSO. This solvent was chosen above others as it is routinely used in this laboratory and, indeed, is in more common use generally than methanol, acetone or ethanol in cell culture studies (Stanislav *et al.*, 1999).

3.1.4 Controls

Besides a control to account for effects of the vehicle (DMSO), positive controls were required to show that the assay system was working. Potential positive controls were tested for their ability to inhibit cell proliferation, as measured by the MTT assay. The compounds chosen for testing have been used clinically to treat various cancers. They included the ferric chelators, desferrioxamine mesylate (DFO) and deferiprone (L1), the selective estrogen receptor modulator tamoxifen (TAM), the topoisomerase I inhibitor camptothecin (CPT), the topoisomerase II inhibitor etoposide (ETO), the mitotic inhibitors vinblastine (VIN) and paclitaxel (PAC), and the pyrimidine antimetabolite 5-fluorouracil (5FU). Table 3.2 lists some relevant references together with the IC_{50} values (50% maximal inhibitory concentrations) those authors obtained using similar culture conditions and cell lines.

$HgCl_2$ and Triton X-100 (t-octylphenoxy polyethoxy ethanol) were also used to ensure 100% cell death was obtained. Triton X-100 (TX-100) is a non-ionic surfactant which, via permeabilisation of the cell membrane, leads to fast cell death by necrosis (Weyermann *et al.*, 2005), while $HgCl_2$ rapidly induces single strand breaks in DNA (Cantoni *et al.*, 1984).

3.2 METHODOLOGY

3.2.1 Cell lines

Cell lines were cultured in appropriate media under conditions recommended by previous workers as outlined in section 2.4. To establish the growth characteristics of each cell line, the commonly used trypan blue exclusion method was used to identify viable cells present over five consecutive days. Each culture was initially seeded at 10,000 cells per well in 96-well tissue culture plates and incubated in appropriate medium under standard conditions.

Each day, cells in each well were washed with 100 μ L CMFS and harvested by adding 50 μ L trypsin-EDTA and incubating at 37°C for 10-15 min. Cells from quadruplicate wells were harvested and an additional 100 μ L CMFS used to rinse out residual cells, which were pooled. Aliquots of 80 μ L of pooled cells were mixed with 20 μ L of trypan blue and direct cell counts performed using a haemocytometer. Parallel experiments were also performed in which cell proliferation was assessed via the MTT assay.

Additionally, in the last few months of this project it became possible to employ a sophisticated instrument to directly measure the confluence of cells grown in microtitre culture plates. The Cellscreen (CS) system (Innovatis AG, Germany), described by Viebahn *et al.* (2006) summarised in section 2.2.2.10, analysed captured images of 4 views of a single well and automatically averaged the data when given the plate layout. In this way, it was possible to quickly and accurately monitor growth of cells without destroying them. The great advantages afforded by the non-invasive nature of this technique meant that the same population of cells could be easily studied over a period of time. Hence, latter experiments were performed in 96 well microtitre plates utilising the CS system.

3.2.2 MTT assay

Initially, the MTT assay was performed as outlined in section (2.6.1). Briefly, 10 μ L of a 5 mg/mL solution of MTT in PBS was added directly to 100 μ L of cells per well and incubated at 37°C for 2 h, after which time 200 μ L of extraction buffer (consisting of 10% SDS, 50% isobutanol, 0.1 M HCl) was added to solubilise the formazan crystals. After a further 90 min at 37°C, the absorbance was read at 595 nm.

However, high background absorbance levels resulting from interactions with MTT, ingredients of the culture medium and test agents were a frequent occurrence. This was particularly concerning after treatment with cytotoxic plant extracts or positive controls when few viable cells were present as the background could be very close to (or even less than) recorded absorbances. Hence, further refinement of the technique was desirable.

Therefore, an alternative method (Fox *et al.*, 2005) was adopted midway through the project which provided potential advantages over the original protocol. In this alternative procedure, culture medium was removed prior to addition of MTT. Cells were incubated with 1 mg/mL MTT in medium at 37°C for 1 h, instead of the 2 h incubation period required for the original protocol. The MTT solution was then removed prior to the addition of 100 µL DMSO, which was added instead of the original extraction buffer to solubilise the dye. This step did not require incubation and the absorbance at 570 nm was read within about 15 min. Appropriately stored spectrophotometric-grade DMSO was used as this provides stable absorbance levels for up to 2 h, compared to non-spectrophotometric DMSO preparations or DMSO pre-exposed to air, which both result in ever-increasing levels of “background” absorbance within 15 min of solvent addition (Alley *et al.*, 1988).

One final modification was that, while Fox *et al.* (2005) removed the culture medium and MTT solution by aspiration, no significant difference was obtained when these solutions were removed by vigorous inversion and subsequent blotting over paper towels (data not shown). Therefore, this inversion system was adopted as it was much more efficient.

Around the same time, a new microplate reader (An ELx808 absorbance microplate reader (BioTek) linked to Gen5 software) was purchased. This new instrument was fitted with a more appropriate filter for the MTT assay, allowing the absorbance at the optimum wavelength (570 nm) to be read. The closest filter the old reader had was 595 nm, which was adequate but not optimal. This meant that after the new reader was employed, absorbance readings were slightly higher than with the old reader, and hence more accurate (see 3.3.2).

Vehicle

Plant extracts were dissolved at 100 mg/mL (w/v) in 100% DMSO. After appropriate dilutions in PBS or medium, and because 10 μ L aliquots were added to 100 μ L of cells in 96 well plates, the maximum concentration of DMSO that cells were exposed to was only 1%.

3.2.4 Controls

After O/N incubation, 10,000 cells in 100 μ L medium were exposed to 10 μ L of the relevant control for a further 48 h. To determine the optimum concentration of each potential positive control, compounds were dissolved at various concentrations in medium containing 10% DMSO and diluted in PBS or medium to give a range of final concentrations spanning published IC₅₀ values as outlined in Table 3.2.

To achieve total cytotoxicity, final concentrations of 0.1% TX-100 or 1 mg/mL HgCl₂ were added to other control wells. At between 3- and 10-fold higher than what others have used, these concentrations were in excess of that required to adequately kill all cells (Dias *et al.*, 2003; Borner *et al.*, 1994; Duncan-Achanzar *et al.*, 1996).

The negative control was simply cells incubated with the vehicle (DMSO) alone at a final concentration corresponding to the amount in the appropriate wells (less than 1%). All other readings were expressed as a percentage of the absorption obtained in wells containing DMSO only.

Table 3.2 Range of Concentrations of Positive Controls Used

Compound	Abbreviation	Concentration Range (μM)	Published IC₅₀ (μM)
Desferrioxamine	DFO	0.5 - 1,000	110-210 (Kicic <i>et al.</i> , 2001); 22 (Richardson <i>et al.</i> , 1995); 20 (Le & Richardson, 2004); 11 (Bernhardt <i>et al.</i> , 2005)
Deferiprone	L1	0.5 - 1,000	180 (Le & Richardson, 2004); 70-110 (Kicic <i>et al.</i> , 2001)
Camptothecin	CPT	0.05 - 100	0.15 (Adams <i>et al.</i> , 2006); 0.12 (Fujimori <i>et al.</i> , 1996); 0.007-0.25 (Jones <i>et al.</i> , 1997); 0.01-0.02 (Kouniavsky <i>et al.</i> , 2002)
5- Fluorouracil	5FU	0.05 - 100	38-45 (Kouniavsky <i>et al.</i> , 2002); 10 (Hernandez-Vargas <i>et al.</i> , 2006); 2.2-15.3 (Mans <i>et al.</i> , 1999); 3 (Evrard <i>et al.</i> , 1999);
Tamoxifen	TAM	0.05 - 100	242 (MDA-MB-435) (Guthrie <i>et al.</i> , 1997); 0.108 (MCF7) (Guthrie <i>et al.</i> , 1997); 17.5 (MCF7) (Panasci <i>et al.</i> , 1996); 8 (MCF7) 5, 20 (Cai & Lee, 1996);
Paclitaxel	PAC	0.001 - 10	0.0037- >32 (Georgiadis <i>et al.</i> , 1997); 0.0027 (He <i>et al.</i> , 2000); 0.0024-0.0038 (Perez & Buckwalter, 1998) 0.0004-0.0034 (Engblom <i>et al.</i> , 1996)
Vinblastine	VIN	0.001 - 10	0.0025-0.0072 (Budman <i>et al.</i> , 2002); 0.0031 (Yang <i>et al.</i> , 2005); 0.001 (Verdier-Pinard <i>et al.</i> , 2000); 0.00068 (Sobottka & Berger, 1992)
Etoposide	ETO	0.001 - 10	5.4 (Kouniavsky <i>et al.</i> , 2002); 2.5 (Ohsaki <i>et al.</i> , 1992); 0.59-0.77 (Perez & Buckwalter, 1998); 0.122-2.42 (Budman <i>et al.</i> , 2002)

Additionally, since a photometric assay was employed and many of the test extracts had colour of their own, it was necessary to include controls to account for background absorption. Absorbance readings from wells containing no cells but 100 μL of incubation medium and 10 μL of test or control compounds only were subtracted from corresponding readings before other calculations were made.

3.3 RESULTS

3.3.1 Cell lines

As discussed above, the cell lines chosen were representative of the five most common types of cancer. Six of the cancerous cell lines had epithelial morphology, while MM253 were melanomic, and the non-cancerous cells, MRC5, were fibroblasts (Volpi *et al.*, 2000).

All cell lines used were adherent but, while all could be removed from the tissue culture surface with trypsin-EDTA, some lines attached more stringently than others, and required longer incubation periods. DU145 cells were the most adherent, requiring at least 10 min at 37°C and vigorous pipetting to be completely removed from the surface of the flask. MM253 and MRC5 cells were the least adherent, easily detaching within minutes.

It was desirable that cells were in logarithmic phase before exposure to the plant extracts because cancer cells are steadily dividing. This meant it was necessary to understand the growth characteristics of the individual cell lines. For each cell line, direct cell counts were performed using trypan blue exclusion and phase contrast microscopy over consecutive days to generate growth curves such as those depicted in Figure 3.2.

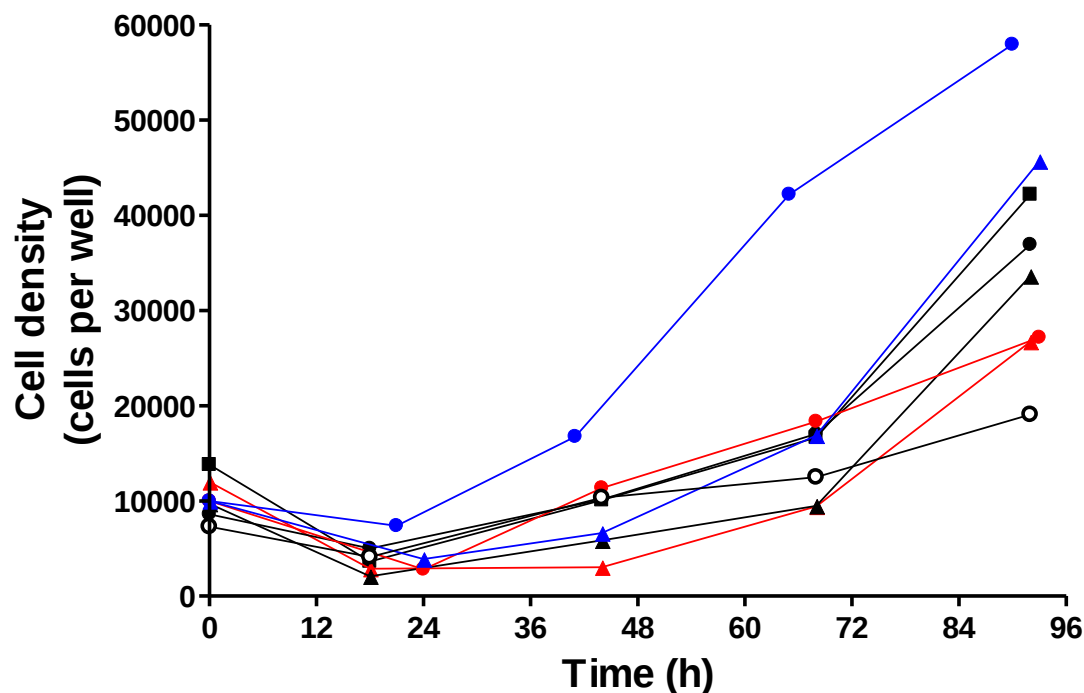


Figure 3.2 Proliferation of cell lines over time (direct cell counts)

MDA-MB-468 (■), MCF7 (▲), Caco-2 (●), MM253 (▲), A549 (●), PC3 (●) and DU145 (▲) and MRC5 (○) cells were seeded at 10,000 cells per well and incubated under standard conditions for 4 days. Approximately every 24 h, cells from quadruplicate wells were harvested and pooled and, after mixing well, a sample aliquot directly counted using a haemocytometer and a phase-contrast microscope. Except for MRC5 cells, at least 200 cells were counted per sample in up to 18 chambers in order to directly determine the number of cells per well for each cell line. Viability was greater than 90% as assessed by trypan blue dye exclusion. Although data points in this figure are from a single experiment, similar results were obtained on two other occasions.

Parallel experiments were also performed in which proliferation of cells incubated under identical conditions in the same plates was measured spectrophotometrically via the MTT assay, a representative experiment of which is shown in Fig. 3.3.

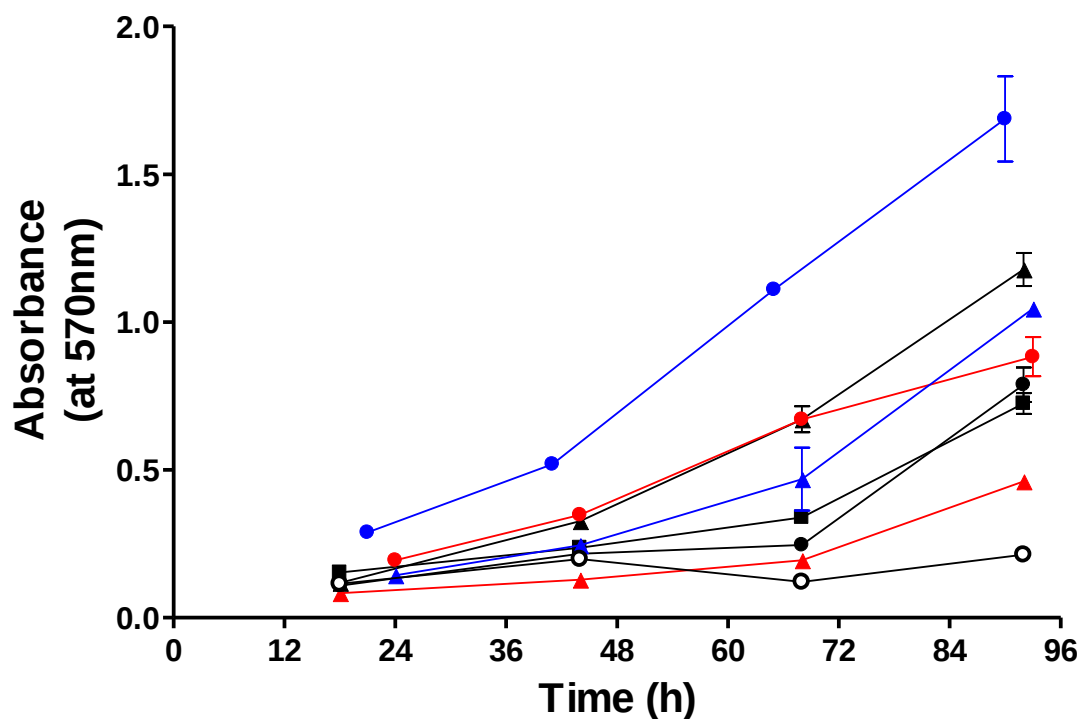


Figure 3.3 Proliferation of cell lines over time (MTT assay)

MDA-MB-468 (■), MCF7 (▲), Caco-2 (●), MM253 (▲), A549 (●), PC3 (●), DU145 (▲) and MRC5 (○) cells were seeded at 10,000 cells per well and incubated under standard conditions for 4 days. Approximately every 24 h, wells were assessed for cell proliferation using the MTT assay. Data are the mean OD_{570} values $\pm$ SE of quadruplicate wells from a single experiment, although similar results were obtained from two other independent experiments.

From both Figures 3.2 and 3.3 it can be seen that after an initial lag phase, cells continued to proliferate even until the last time point measured (94 h). Growth was shown to be exponential over the duration of the experiment in all cell lines, with very high r^2 values obtained when exponential nonlinear regression lines were fit to the data (Table 3.3). This was confirmed in experiments performed much later when the Cellscreen (CS) apparatus became available. As this equipment made monitoring cell growth a much simpler matter, growth curves were obtained which included more time points over a longer period (Fig. 3.4).

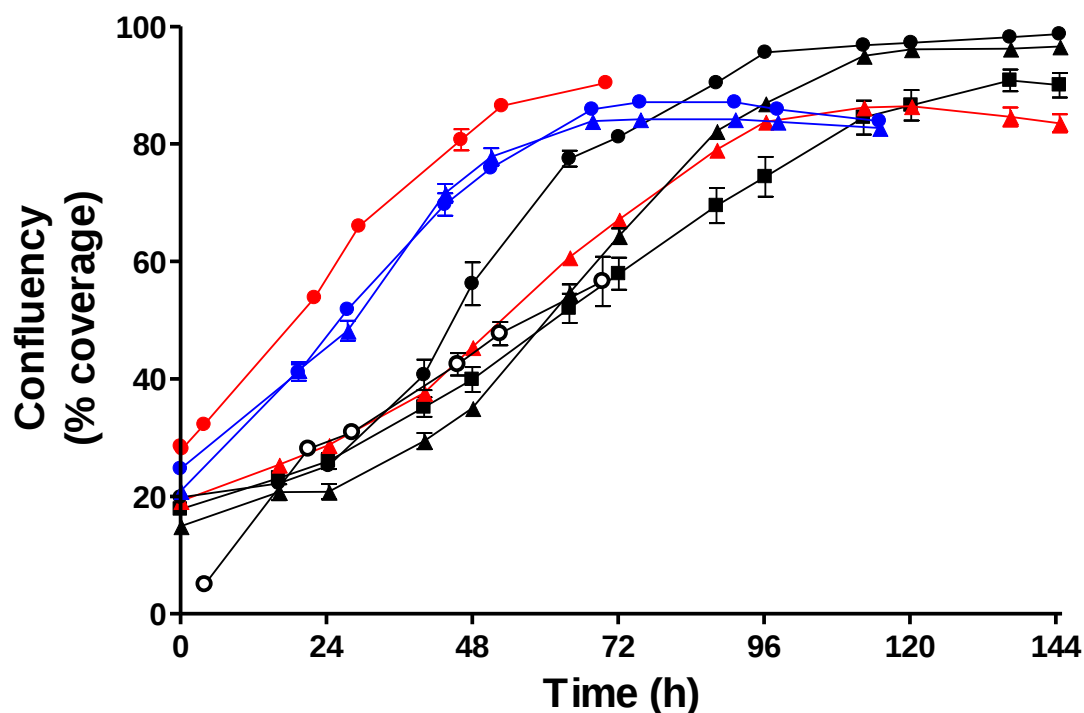


Figure 3.4 Proliferation of cell lines over time (Cellscren)

MDA-MB-468 (■), MCF7 (▲), Caco-2 (●), MM253 (▲), A549 (●) and MRC5 (○) cells were seeded at 10,000 cells per well, PC3 (●) and DU145 (▲) at 5,000 cells per well, and incubated under standard conditions for up to 1 week. Twice per day, wells were assessed for cell proliferation using the CS apparatus. Data are the mean % confluency values $\pm$ SE of quadruplicate wells from a single experiment, although similar results were obtained from two other independent experiments.

Clearly, when cells were seeded at densities translating to about 20% confluency, conditions were optimal for logarithmic growth between 24 and 72 h, the period chosen for incubation with test agents. When fewer cells or more cells than this were seeded, the exponential growth phase was correspondingly delayed or occurred earlier.

Table 3.3 Goodness of Fit of Exponential Growth Curves

Cell Line	r² Cell counts	r² MTT assay	r² Cellscreen
MDA-MB-468	0.988	0.977	1.000
MCF7	1.000	0.980	0.995
Caco-2	0.993	0.938	0.980
MM253	0.984	0.996	0.985
A549	0.996	0.940	0.950
PC3	0.918	0.973	0.980
DU145	1.000	0.999	0.978
MRC5	0.964	-	0.981

Growth curves were used to determine the mean doubling times of each cell line (Table 3.4). Cell numbers, OD₅₇₀ readings or confluency levels were transformed to log values and plotted against their corresponding time points. For each cell line, the doubling time was taken as the log of 2 divided by the slope of the regression line as fitted by GraphPad Prism.

This analysis was based on the formula:

$$T_d = \frac{t \cdot \log_{10} 2}{\log_{10} N_2 - \log_{10} N_1}$$

Where T_d = the population doubling time
 N_1 = the initial population number, OD₅₇₀ or % confluency
 N_2 = the population number, OD₅₇₀ or % confluency at final time point
 t = time difference between measurements.

Only the exponential phase of the growth curves was used, such that N_2 corresponded to the final time point and N_1 was taken to be the number of cells (or OD₅₇₀ or % confluency) at the beginning of the log phase. The doubling times presented are mean values derived from data from 2-4 independent experiments, including those depicted in Figures 3.2 to 3.4.

Table 3.4 Mean Doubling Times of Cell Lines Established via Different Methods

Cell Line	Cell counts (h)	MTT assay (h)	Cell-screen (h)	Published values* (h)
MDA-MB-468	25.2	29.6	42.9	60-72 (Brinkley <i>et al.</i> , 1980); 29.9 (Watanabe <i>et al.</i> , 2001); 24 (Busse <i>et al.</i> , 2000)
MCF7	17.7	29.1	37.7	36.9 (Smith <i>et al.</i> , 1999); 30.2 (Watanabe <i>et al.</i> , 2001); 24 (Lacroix & Lippman, 1980); 22 (Fujimori <i>et al.</i> , 1996)
Caco-2	19.9	28.3	22.9	32 (Visanji <i>et al.</i> , 2004); 26.5 (Basson <i>et al.</i> , 1995); 23.5 (Scaglione-Sewell <i>et al.</i> , 2000)
MM253	19.8	23.0	35.5	28 (Goss & Parsons, 1977); 24 (Parsons & Morrison, 1978); 23 (Parsons <i>et al.</i> , 1982)
A549	26.7	25.9	46.7	28.4 (Jones <i>et al.</i> , 1978); 19.8 (Uchiyama <i>et al.</i> , 1997); 18.7 (Newman, 1990);
PC3	21.0	25.2	38.6	28 (Nucciarelli <i>et al.</i> , 2003); 24 (Parker <i>et al.</i> , 1998); 23.2 (Yasumoto <i>et al.</i> , 2004)
DU145	20.2	27.3	42.0	29 (Sramkoski <i>et al.</i> , 1999); 25 (Gao <i>et al.</i> , 2001); 16-20 (Quinones <i>et al.</i> , 2002)
MRC5	54.4	89.9	74.8	96 (Carballo <i>et al.</i> , 2002); 44.0 (Uchiyama <i>et al.</i> , 1997); 22 (Lovejoy & Richardson, 2002)

* obtained via a variety of different methods.

As can be seen, the doubling times derived from the direct cell count curves were generally less than those calculated from the OD₅₇₀ readings after incubation with MTT, which were more in agreement with published values. Cellscreen-derived values were usually higher

than those calculated from direct cell counts or the MTT assay, but still mainly within the range of published values.

It should be noted here that, due to its nature, the non-tumourigenic cell line, MRC5, was much slower growing than the cancerous cell lines. This made it comparatively more difficult to grow and meant that sometimes not enough cells were available to complete all the experiments performed on the other cell lines.

Additionally, the data, in agreement with others, indicate that the 8 different cell lines proliferated at different rates. It was, therefore, necessary to subculture them at differing split ratios to synchronise their readiness for experiments, i.e. when the various cell lines were in mid log phase, when most cells were dividing. This was determined to be between 48 and 72 h after subculture. Ideally, cells were required at about 70-90% confluency once per week. Typically, this meant splitting them at the ratios outlined in Table 3.5, dependent upon their state of confluency from the previous passage.

Table 3.5 Subculture Conditions of Cell Lines

Cell Line	Split Ratio
MDA-MB-468	1/15 - 1/20
MCF7	1/15 - 1/20
Caco-2	1/15 - 1/20
MM253	1/20 - 1/40
A549	1/20 - 1/40
PC3	1/30 - 1/50
DU145	1/20 - 1/40
MRC5	1/5 - 1/10

3.3.2 MTT assay

Extensive studies were carried out to optimise the efficiency of the MTT assay routinely performed in this laboratory (Kicic *et al.*, 2002). This involved many experiments consuming several months. However, only the initial screening studies were performed utilising this method before a superior protocol was encountered. Therefore, the results of some of these preliminary studies are presented as an appendix only (Appendix C).

Despite the original method producing good results for the earliest experiments in this study, an alternative method based on the protocol of Fox *et al.*, (2005) was adopted for later experiments. Because of reduced incubation times, this new method was much quicker than the original and, therefore, enabled more experiments to be performed. More importantly, the new method gave “cleaner” results in that background absorbance values were very low. This was due to the inclusion of a step in which culture medium was discarded before incubation with MTT, resulting in an absence of colour interference from interactions with various components of the medium, plant extracts and MTT and/or extraction buffer. Figure 3.5 depicts absorbance readings from an experiment comparing the old method with the new one.

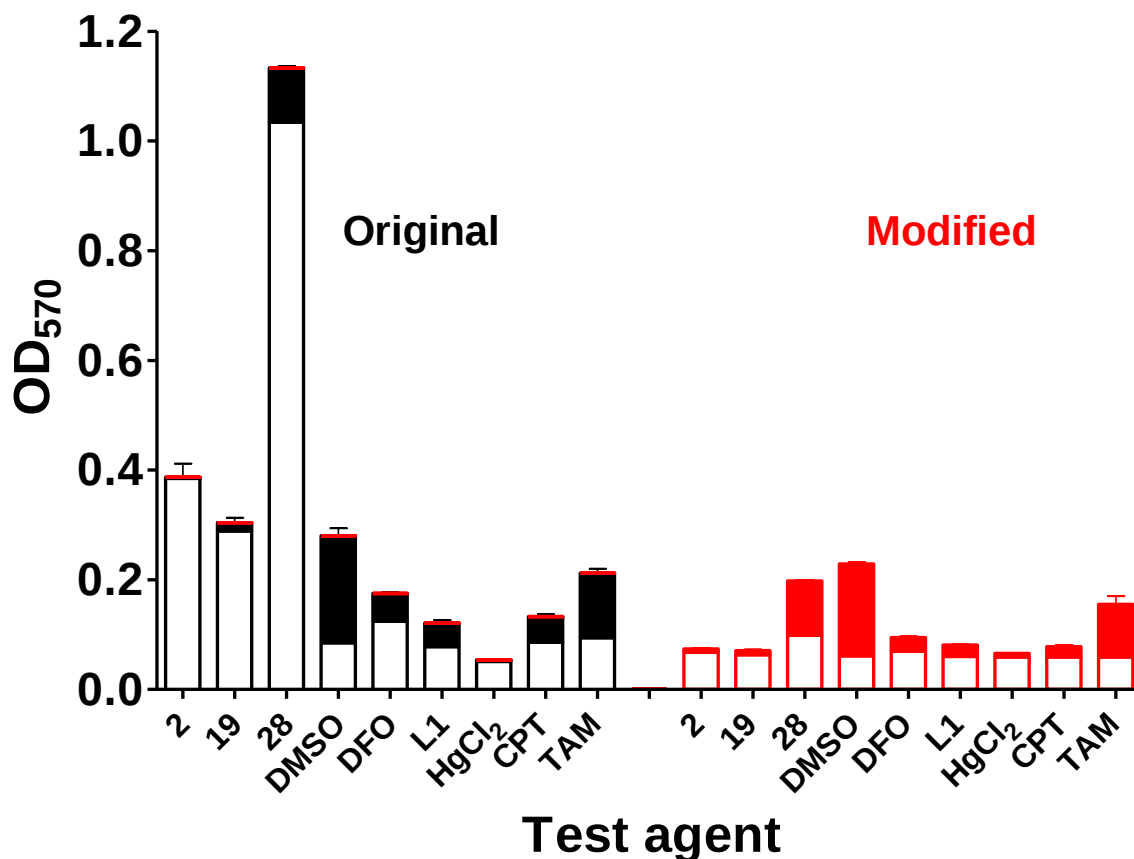


Figure 3.5 Absorbance values from original and new MTT assay protocols.

100 μ L of MDA-MB-468 cells were seeded at a density of 10,000 cells per well in parallel microplates and, after O/N incubation at 37°C, exposed to various test agents for a further 48 h. MTT assays were then performed on the cells, according to either the original protocol (■: 10 μ L of 5 mg/mL MTT in PBS for 2 h, 100 μ L extraction buffer for 90 min, both added directly to cultures) or the modified method (■: culture medium discarded, 100 μ L of 1 mg/mL MTT in medium, solution discarded, 100 μ L DMSO), and the absorbance read at 570 nm. The details of the test agents are not important to the interpretation of this experiment. Results are mean OD₅₇₀ values $\pm$ SE of quadruplicate wells. The white section of each bar represents the component of total absorbance due to background.

One of the most vital details to establish was that data obtained from the MTT assay directly related to the number of cells in each well. To this end, the number of cells counted manually was plotted against the absorbance values in wells corresponding to cells incubated under exactly the same conditions for the same period of time (Fig. 3.6).

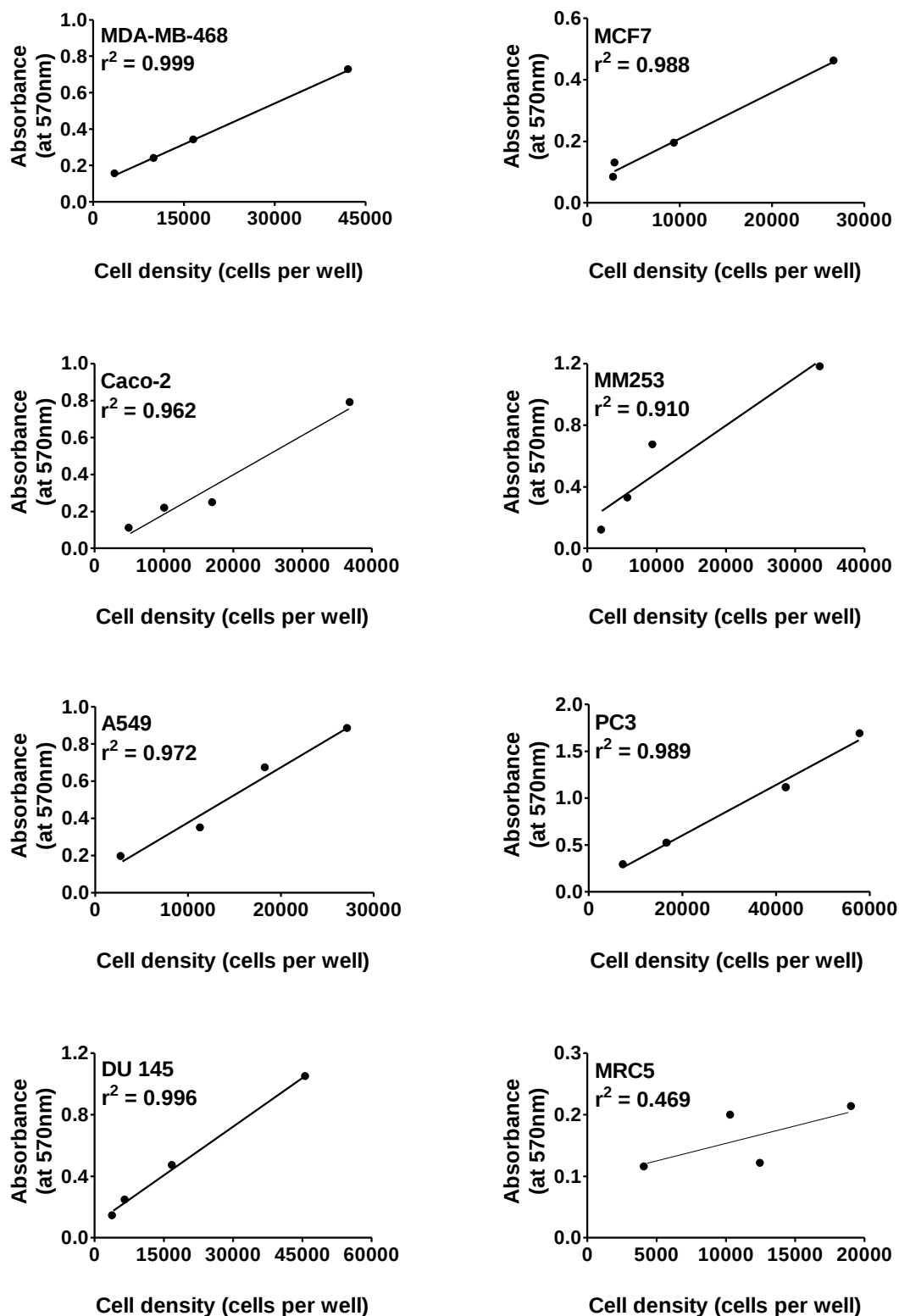


Figure 3.6 Relationship between absorbance values and cell numbers.

For each cell line, MTT assays were performed on cells in wells every day for 4 days and the OD_{570} readings obtained were plotted against direct cell counts. This experiment was carried out on quadruplicate wells on three separate occasions and these curves are typical of the results obtained.

As can be seen from Figure 3.6, for every cancer cell line tested, the correlation between direct cell counts and OD₅₇₀ values from the MTT assay was very high. Squared correlation coefficient (r^2) values were above 0.9 in all cell lines over a 4-day period, except for the non-tumourigenic cell line, MRC5.

Additionally, the relationship between OD₅₇₀ values obtained via the MTT assay and data obtained using an assay to measure DNA content (see section 2.7.2) was also assessed (Fig. 3.7).

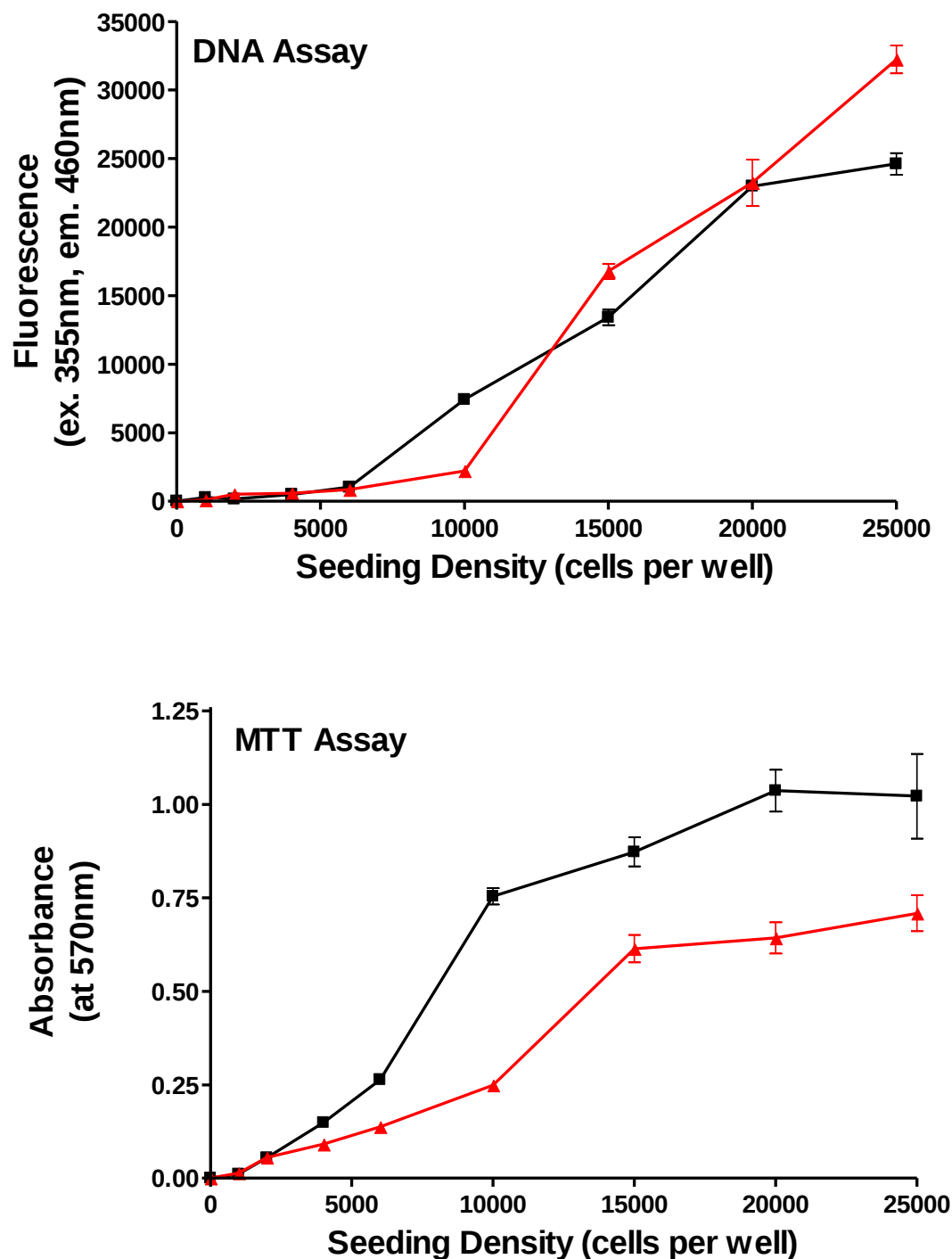


Figure 3.7 DNA assay vs MTT assay.

MDA-MB-468 (■) and MCF7 (▲) cells were seeded at varying densities and incubated under standard culture conditions. After 72 h, MTT and DNA assays were performed on identical plates in parallel. Results are the mean $\pm$ SE absorbance (570 nm) or fluorescence (ex. 355 nm, em. 460 nm) values after the blanks had been subtracted of 4 and 6 wells, respectively.

From Figure 3.7 it is evident that there are differences between the shapes of the curves generated from data acquired from the DNA assay compared to those from the MTT assay. The shapes of both DNA assay curves are steeper than those of the MTT assay, which start to flatten out at a seeding density of around 15,000 cells per well. Nevertheless, when these data were subjected to linear regression analysis, it was shown that the relationship between the MTT and DNA assay results was very close, with r^2 values of 0.936 and 0.953 for MDA-MB-468 and MCF7 cells, respectively. This correlation was even better if only the low range points (0-6,000 cells/well) were used (MDA-MB-468 $r^2=0.959$, MCF7 $r^2=0.979$). For MDA-MB-468 cells, an r^2 value of 0.990 for the high range points (10,000 to 25,000 cells/well) was obtained, while for MCF7 cells an r^2 value of 0.945 was recorded.

Based on other experiments in which absorbance values from the MTT assay were compared to cell numbers obtained via direct cell counting, a seeding density of 10,000 cells per well corresponded to a final cell density of about 16,665 MDA-MB-468 and 9,428 MCF7 cells after 72 h growth. From the DNA standard curve ($r^2=0.998$), a seeding density of 10,000 cells per well corresponded to 912 and 293 ng of DNA 72 h later for MDA-MB-468 and MCF7 cells, respectively. Therefore, it might be extrapolated that the DNA content of MDA-MB-468 cells was about 55 pg/cell and of MCF7 cells was 32 pg/cell.

MTT assay-derived OD_{570} values were also compared with the protein content of cells in parallel wells which had been lysed with 1% TX-100 (Fig. 3.8). Standard curves generated from the BCA protein assay kit employed (see section 2.7.5) had r^2 values greater than 0.999.

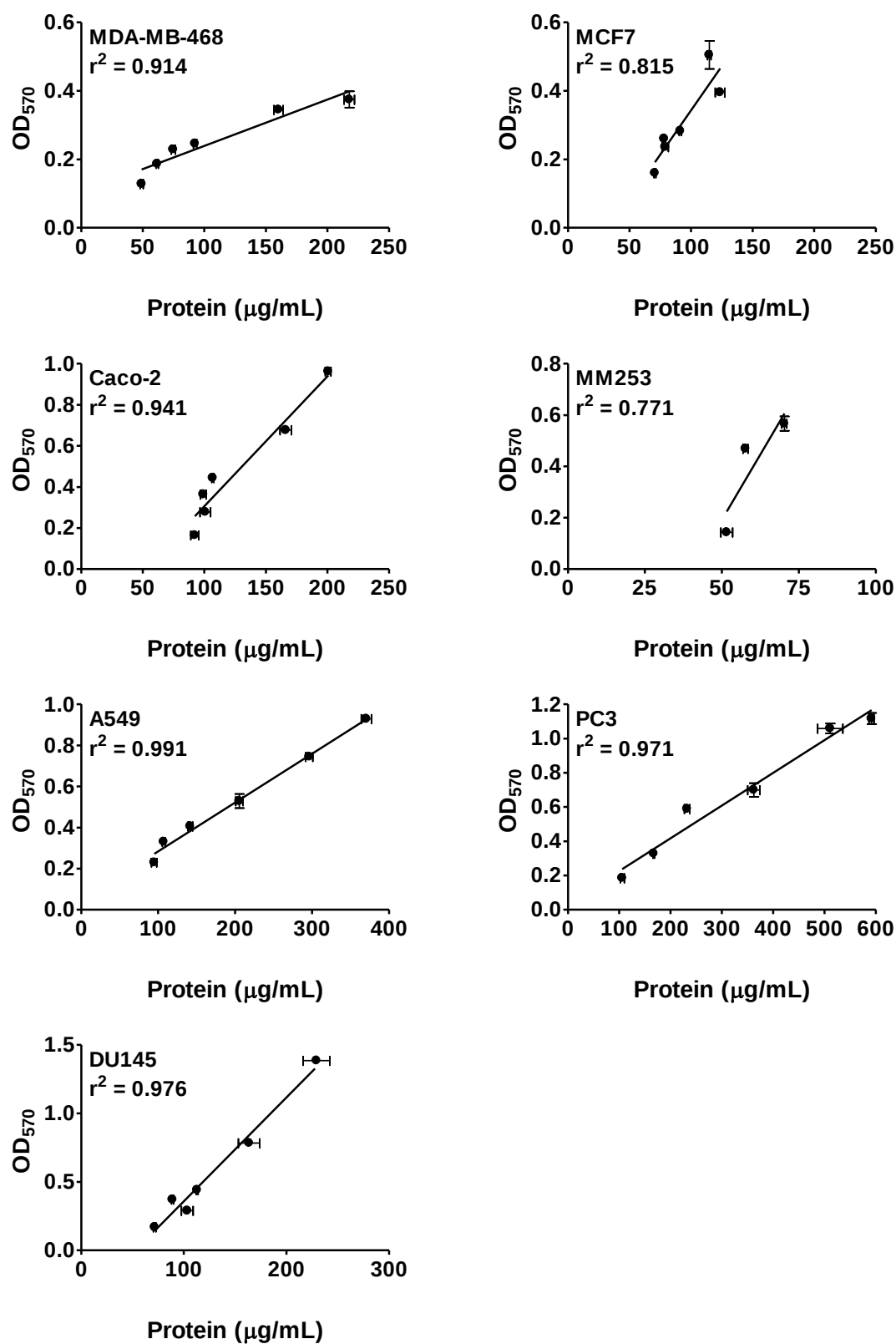


Figure 3.8 Relationship between protein content and MTT assay-derived data.

For each cell line, % of control data obtained from MTT assays were plotted against protein content of cells in parallel wells. This experiment was carried out in quadruplicate wells and data are the means $\pm$ SE for both measurements. Similar results were obtained in another experiment.

As can be seen, OD₅₇₀ values obtained from the MTT assay correlated very well with the amount of protein in cells cultured under the same conditions.

Later, the Cellscreen (CS) system was used to reinforce the validity of the MTT assay. For every cell line, very good correlations were found between % control values obtained via the MTT assay and the % confluency of wells as measured by the CS (Fig. 3.9).

Additionally, results obtained via the CS system itself were compared to those obtained in an assay for protein performed on the same cells directly after the last CS measurement was recorded. Figure 3.10 illustrates the very good correlation between these two methods of assessing cell numbers.

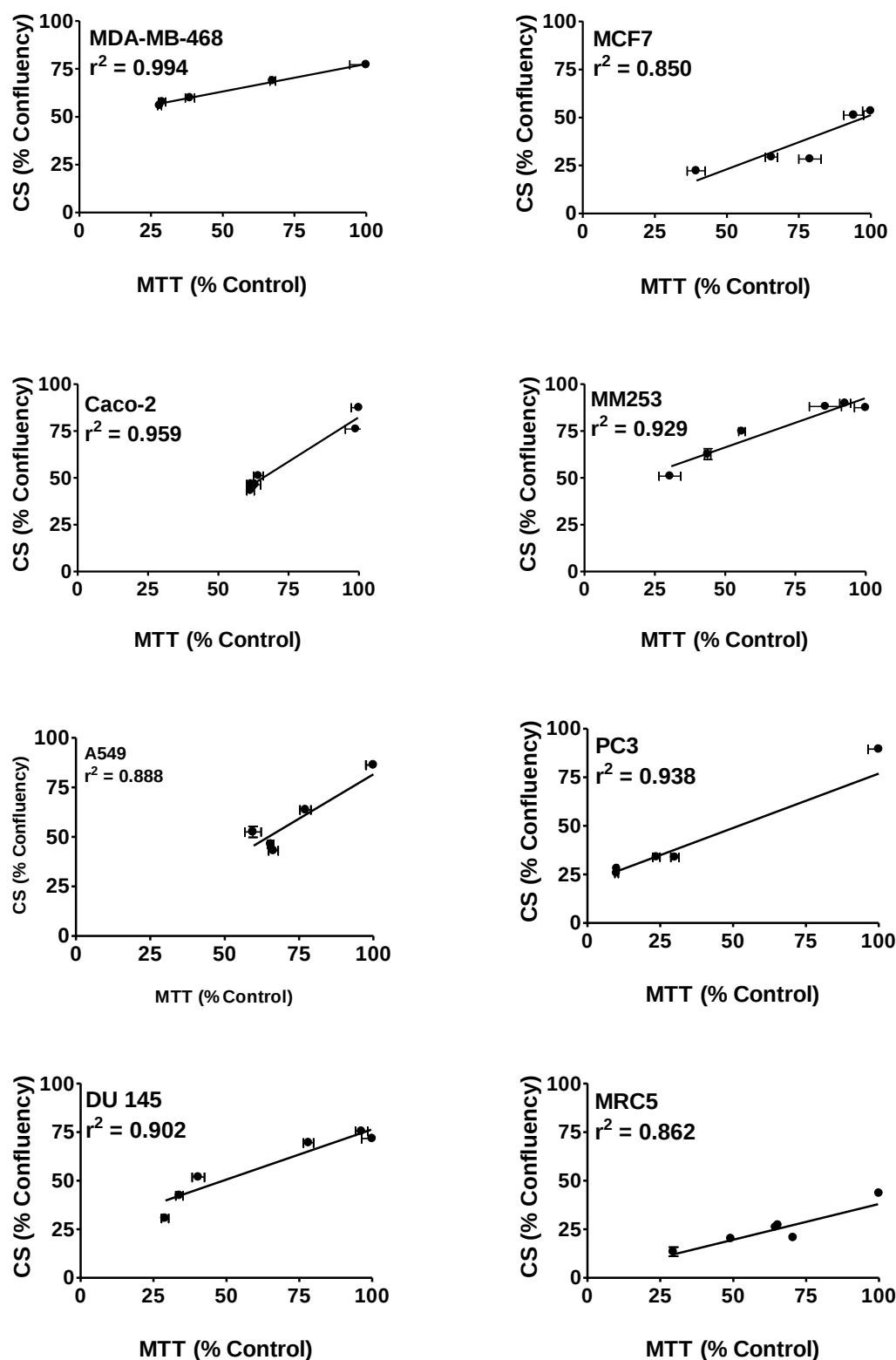


Figure 3.9 Relationship between MTT assay-derived data and CS-derived data.

For each cell line, the percentage of the wells covered by cells (% confluency) was plotted against % of control data obtained from MTT assays performed directly afterwards on the same cells. This experiment was carried out in quadruplicate wells and data are the means $\pm$ SE for both measurements. Similar results were obtained in another experiment.

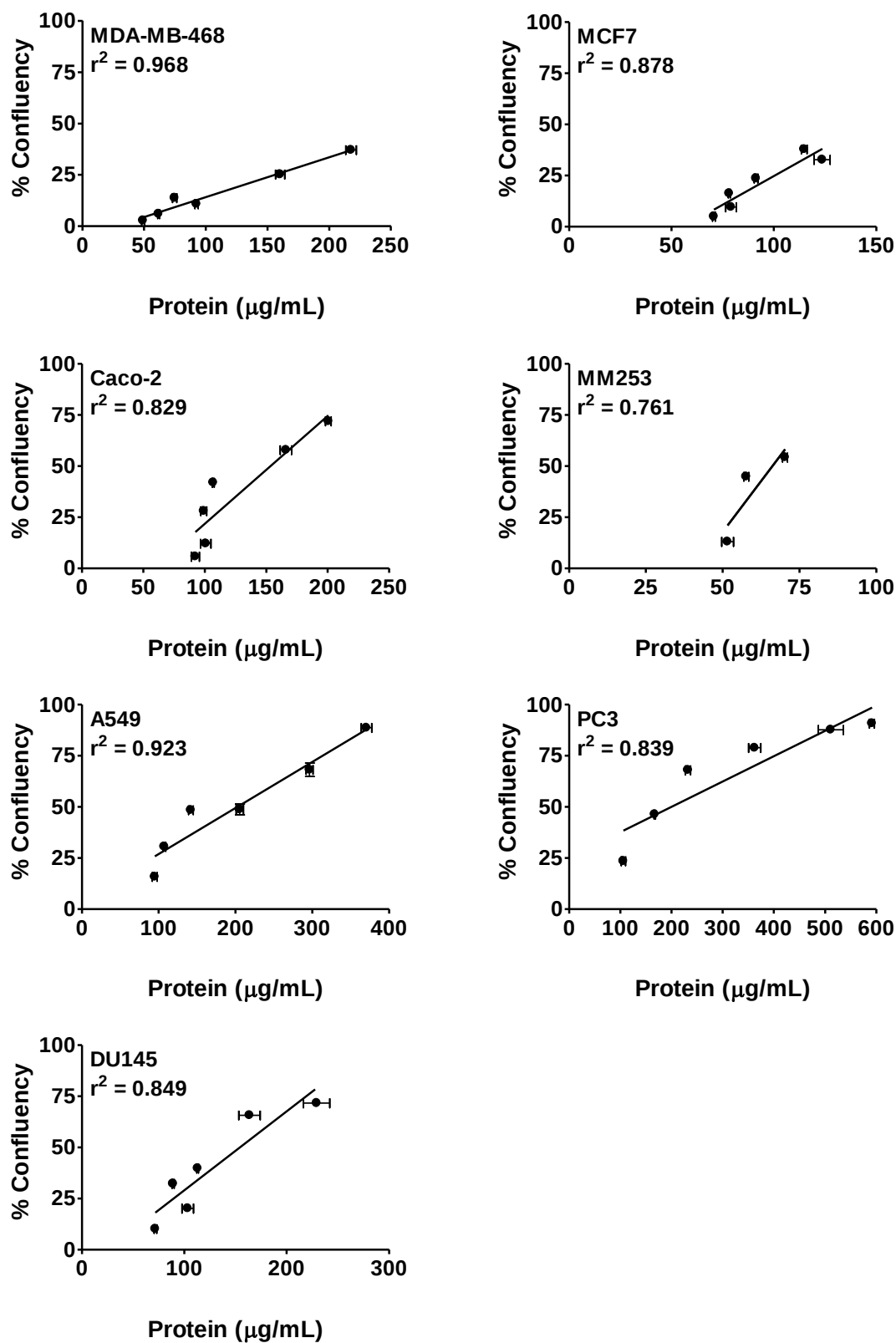


Figure 3.10 Relationship between protein content and CS-derived data.

For each cell line, the percentage of the wells covered by cells (% confluency) was plotted against protein content of cells in parallel wells. This experiment was carried out in quadruplicate wells and data are the means $\pm$ SE for both measurements. Similar results were obtained in another experiment.

3.3.3 Vehicle

Not all plant extracts tested were water-soluble, but all were effectively dissolved in DMSO. Cells were exposed to a maximal concentration of DMSO of 1%, a concentration which had little effect on cell morphology (Fig. 3.11).

However, DMSO did inhibit cell proliferation in a dose dependent manner over the high concentration range examined (Fig. 3.12). Clearly, some cell lines were more susceptible to DMSO than others. While 1% DMSO had a minimal effect on most cell lines in the panel, the faster growing lines, PC3, A549 and MM253 (Table 3.4, MTT-derived doubling times) were sensitive to this concentration.

Cells exposed to DMSO only were considered the negative control and all other values expressed as a percentage of these data to give the proportion of living cells after treatment with positive controls or test extracts.

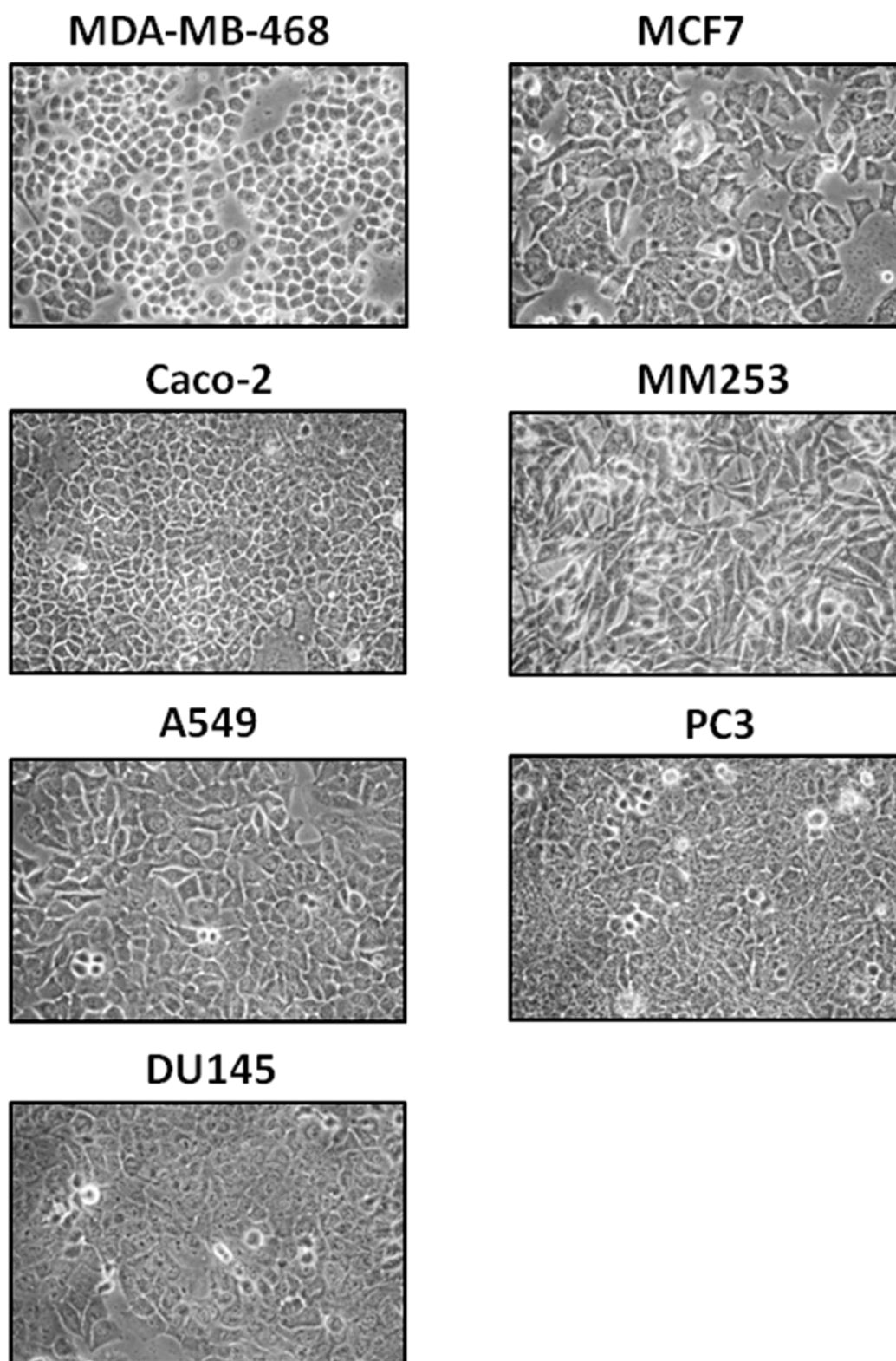


Figure 3.11 Micrographs of cancer cells exposed to DMSO.

Cancer cells were incubated at 37°C with 1% DMSO for 48 h and examined under a phase-contrast light microscope (200X).

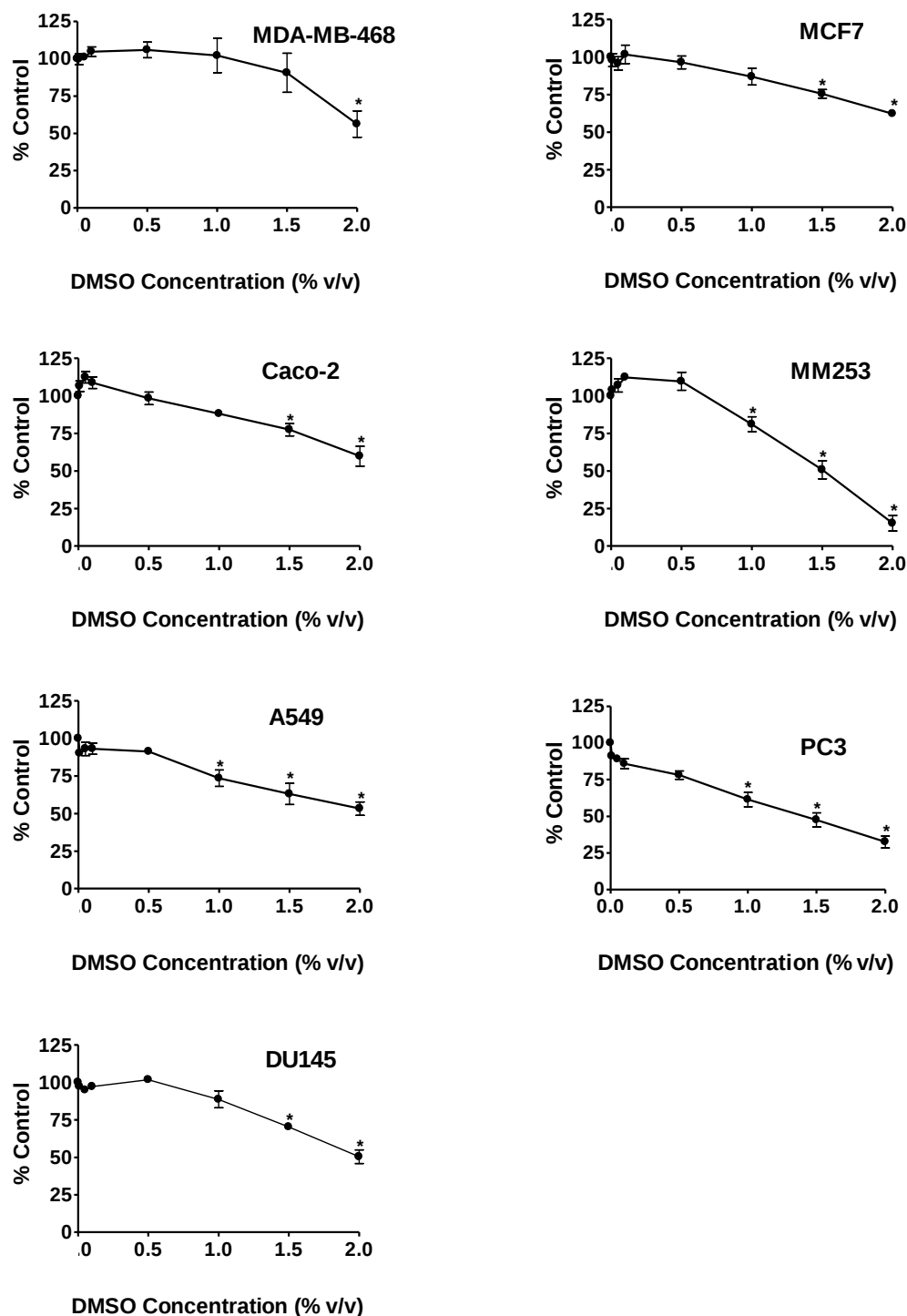


Figure 3.12 Effect of DMSO on proliferation of different cell lines.

Cancer cells were incubated at 37°C with a range of concentrations of DMSO for 48 h and the OD₅₇₀ expressed as a percentage of the negative control (cells not exposed to DMSO). These data represent the means $\pm$ SE of 3 separate experiments, each containing quadruplicate wells. For each cell line, repeated measures ANOVAs were performed comparing raw absorbance values (minus background) to those containing no DMSO (Dunnett's Multiple Comparisons Test). * denotes significantly different from control (P < 0.01).

3.3.4 Controls

Figure 3.13 illustrates the effect various compounds tested as potential positive controls had on the two breast cancer cell lines, MDA-MB-468 and MCF7. Where possible, these curves were used to calculate the IC_{50} value for each compound and these are presented in Table 3.6.

Table 3.6 IC_{50} Values of Compounds Tested as Positive Controls

Compound	IC_{50} Value (μM)	
	MDA-MB-468	MCF7
DFO	21.7*	20.8*
L1	247.5	397.2
CPT	1.7	36.8
5FU	4.4*	
TAM	66.2	58.8
PAC	0.018*	
VIN		
ETO	10.3	

* in agreement with published IC_{50} values for other cell lines (see Table 3.2).

The same compounds were also tested on other cell lines, but only in a single experiment (Fig. 3.14). Thus, IC_{50} values were not determined for these cell lines.

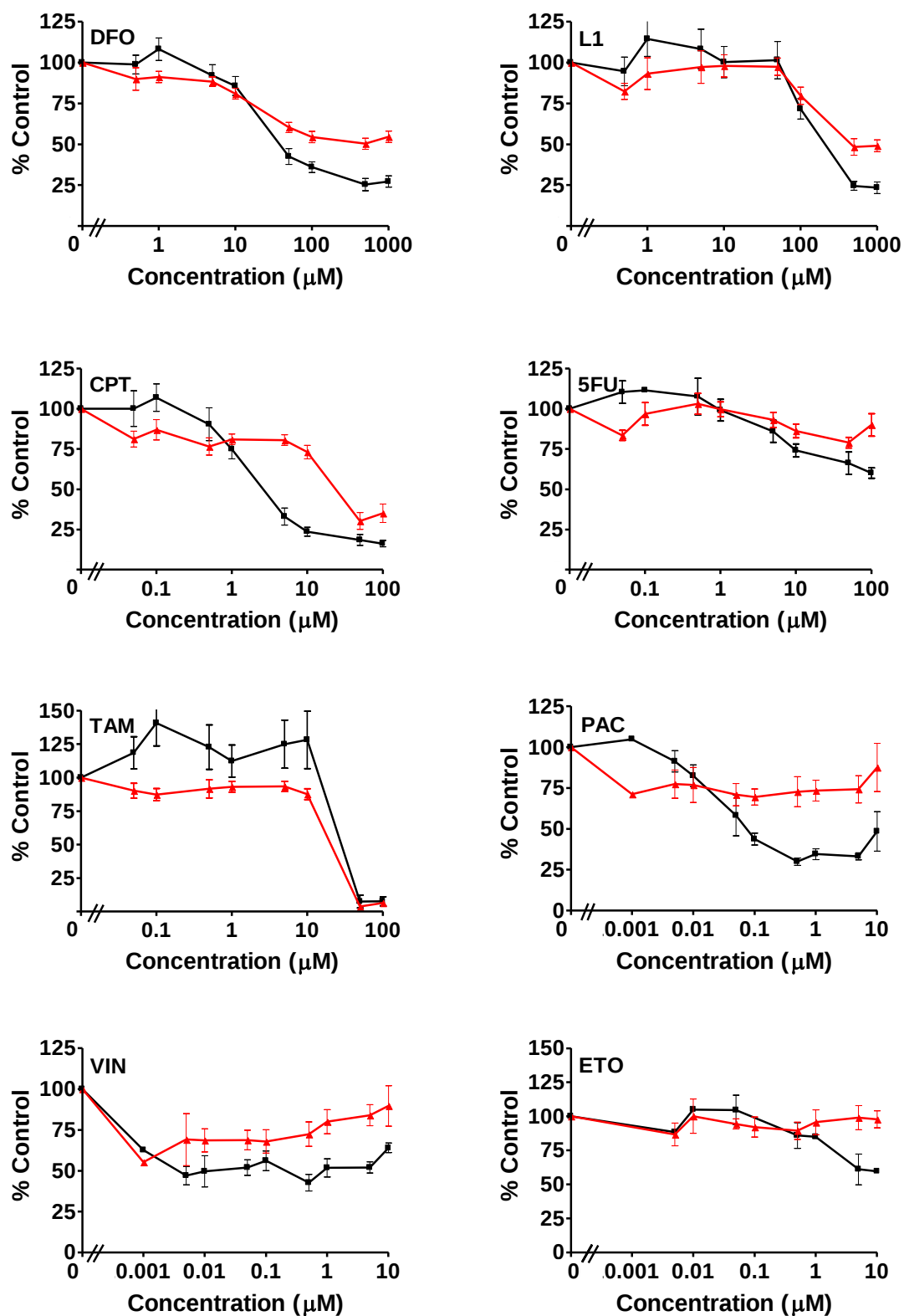


Figure 3.13 Effect of potential positive controls on breast cancer cell lines.

MDA-MB-468 (■) or MCF7 (▲) cells were incubated at 37°C with a range of concentrations of various compounds for 48 h before being subjected to the MTT assay. Results are expressed as % of control values and are the means $\pm$ SE of 3-7 separate experiments, each containing quadruplicate wells.

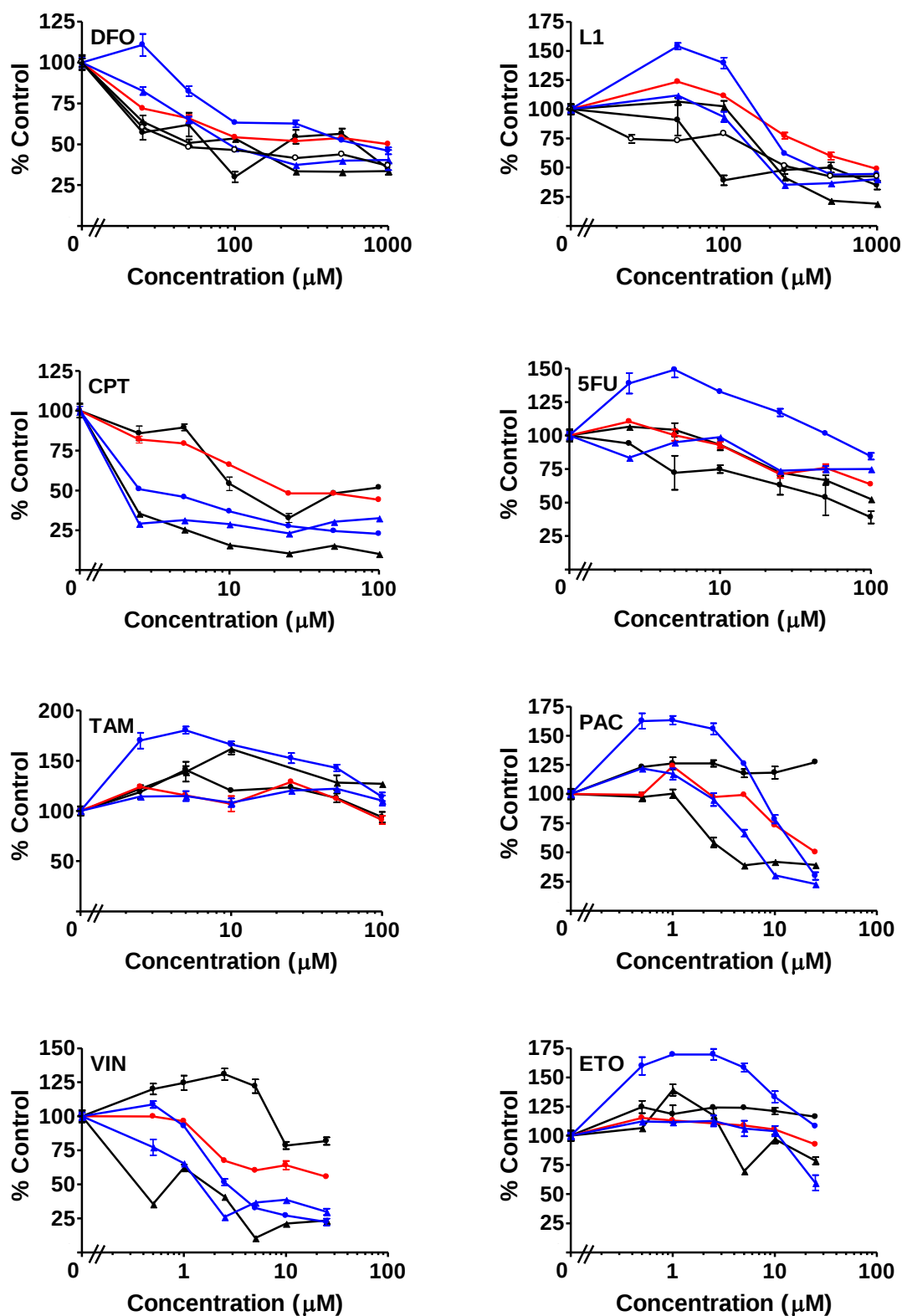


Figure 3.14 Effect of potential positive controls on other cell lines.

Caco-2 (●), MM253 (▲), A549 (●), PC3 (●) and DU145 (▲) and MRC5 (○) cells were incubated at 37°C with a range of concentrations of various compounds for 48 h before being subjected to the MTT assay. Results are expressed as % of control values and are the means $\pm$ SE of quadruplicate wells of a single experiment.

As can be seen, DFO, L1 and CPT caused a dose-dependent inhibitory effect against all cell lines tested. In contrast, ETO and 5FU had little effect on most cell lines tested over the concentration ranges used, while PAC and VIN substantially inhibited the proliferation of some, but not all, cell lines. TAM was only effective against MDA-MB-468 and MCF7 and the dose-response curve was undesirably steep between 10 and 50 μ M. In some cases, the effect of a compound was stimulatory, especially at lower doses, a phenomenon known as hormesis (Calabrese, 2005a), which will be discussed further in Sections 5.4.3, 8 2.4.3 and Appendix N.

3.4 DISCUSSION

3.4.1 Cell lines

From Table 3.4 it can be seen that the doubling times of the cell lines varied according to which method was used to assess cell proliferation. In general, doubling times derived from direct cell counts were lower than those obtained via the MTT assay which were, in turn, lower than CS-derived values. As the MTT assay values were more in keeping with the range of published values, in the main, these were given most credence. However, it must be remembered that the culture conditions, including the composition of the culture medium can vary quite considerably between research groups. In addition, the inherent genetic instability of cell lines in long-term culture and natural selection as cells are passaged under different culture conditions can account for differences in biological behaviour, including growth characteristics, between the same cell line grown in different laboratories (Osborne *et al.*, 1987). Indeed, it is for these very reasons that it was essential to assess the growth characteristics of each cell line under the conditions employed in this study.

These experiments showed that if cells were seeded at 10,000 cells per well in microtitre plates, they would be in logarithmic phase 48-72 h later. Therefore, it was determined that for later experiments in which cells were exposed to plant extracts or controls, 10,000 cells per well were allowed to adhere O/N under standard culture conditions before being exposed to test agents for 48 h. Thus, incubation periods were a maximum of 72 h. Significantly, this was the same incubation schedule employed by the NCI in routine screening (Boyd & Paull, 1995).

3.4.2 MTT assay

A reliable quantitative method of assessing cytotoxicity was a prime requirement in order to undertake screening studies. The MTT assay was chosen for its simplicity, reproducibility and because it was already in routine use in this laboratory and many others for this application (Kicic *et al.*, 2002; Edwards *et al.*, 2008). A major effort was applied to refining this technique, which resulted in various modifications to the original method. Overall, these changes resulted in a quicker system that was not any more expensive to run. The main advantage, however, was that it reduced the effects of several problems associated with the mixture of substances in the wells. The specificity and sensitivity of the MTT assay is known to be influenced by various factors, including coloured substances and cell volume (Wang *et al.*, 2006). Because the modified protocol involved discarding the contents of the wells before the MTT solution was added, the problems of chromatic interference by ingredients in the medium such as FCS and evaporation of media over time were eliminated. More importantly, there was no longer the issue of colour differences between wells containing different plant extracts with varying hues, and even wells containing the same extract, but at varying concentrations. The modified method also overcame complications resulting from possible interactions between control drugs, and

MTT, such have been shown to occur with cisplatin and paclitaxel which showed false increases in viability (Ulukaya *et al.*, 2004). However, because results were always expressed as a percentage of an internal control, previous results were still comparable to newer ones.

As expected, a strong positive relationship between absorbance and cell number was evident in all cancer cell lines. The correlation was less convincing with the non-cancerous line, MRC5, but this was most likely a function of the error introduced by counting fewer cells. Better correlations were seen for the MRC5 cell line when MTT assay data were compared to confluency values obtained using the CS. Indeed, squared correlation coefficient values for this relationship were no less than 0.850 for every cell line examined (Fig. 3.10). These observations confirm those of Viebahn *et al.*, (2006), who showed that the precision of the CS is comparable to that of the MTT assay. When calculated cell numbers were plotted against individual values generated by the CS and the MTT assay, these authors reported correlation coefficients of 0.969 and 0.971, respectively (Viebahn *et al.*, 2006). Additionally, a very good correlation between confluency values as determined by the CS system and protein content of lysed cells was demonstrated (Fig. 3.10).

Moreover, the validity of the MTT assay was also supported by high r^2 values between fluorescence values in the DNA assay and absorbance values from the MTT assay (Fig. 3.8). Similarly, a high correlation was found between cell numbers as assessed by protein content of lysed cells and MTT-derived OD₅₇₀ values (Fig. 3.8).

Overall, these results demonstrate that the MTT assay was, indeed, a very good technique for accurately determining the number of viable cells present in a culture. Therefore, we were confident this was a valid means of detecting any cytotoxic effects of plant extracts.

3.4.3 Vehicle

All plant extracts tested dissolved in DMSO at 100 mg/mL, but not all were soluble in aqueous solutions. In the interest of consistency, DMSO was therefore chosen as the vehicle for all test samples as well as the controls. However, as DMSO itself did affect the proliferation of some of the cell lines tested at the concentrations used (Fig. 3.12), it was necessary to include a control for DMSO. Cells incubated with DMSO alone (<1% final volume) were therefore included and such wells were considered the negative control, sometimes referred to as the vehicle control. All absorbance values obtained for test or positive control wells were expressed as a percentage of the absorbance reading recorded for such negative control wells.

Originally, for simplicity and to conserve resources, only the highest concentration of DMSO (1%) was used as a vehicle control. Initially, it was assumed from unpublished past studies on other cell lines in this laboratory that there was no difference in absorbance readings obtained over the range of DMSO used. Pianetti *et al.* (2002) also used only a single concentration of DMSO, equivalent to the highest dose, as a negative control in their research. However, the present study provided clear evidence of the dose-dependent effects of DMSO on the cell lines used (Fig. 3.12). Therefore, extra control wells consisting of DMSO at concentrations corresponding to the amount in wells containing different dilutions of plant extract or positive control were included in subsequent experiments.

DMSO as the vehicle control has been reported for many studies on the same cell lines used here. A list of selected references providing precedents for each cell line in this panel is provided in Table 3.7.

Table 3.7 Previous Studies Using DMSO as Vehicle Control

Cell Line	Maximum Concentrations of DMSO (v/v) Used by Others
MDA-MB-468	0.1% (Prassas <i>et al.</i> , 2008; Spink <i>et al.</i> , 1998; Krueger <i>et al.</i> , 2001)
MCF7	0.1% (Spink <i>et al.</i> , 1998; Cover <i>et al.</i> , 1999); 0.2% (Zhang <i>et al.</i> , 2003; Nguyen <i>et al.</i> , 2004; Agarwal <i>et al.</i> , 2002); 2% (Kuntz <i>et al.</i> , 1999)
Caco-2	0.1% (Wolter & Stein, 2002); 0.5% (Stormer <i>et al.</i> , 2002); 1% (Janzowski <i>et al.</i> , 2003; Gibbs <i>et al.</i> , 2000); 2% (Kuntz <i>et al.</i> , 1999)
MM253	1% (Jevtovic-Todorovic & Guenther, 1991)
A549	0.1% (Nguyen <i>et al.</i> , 2004); 0.2% (Cheng <i>et al.</i> , 2005); 3.4% (Palanee <i>et al.</i> , 2001)
PC3	0.1% (Anderson <i>et al.</i> , 1998; Oliveira <i>et al.</i> , 2008; Hafeez <i>et al.</i> , 2008); 1% (Xiao & Singh, 2002; Li <i>et al.</i> , 1995)
DU145	0.1% (Agarwal <i>et al.</i> , 2002; Li <i>et al.</i> , 1995)
MRC5	0.5% (Hadjur <i>et al.</i> , 1995); 0.3% (Cheng <i>et al.</i> , 2002)

These references are by no means exhaustive; they are merely examples of relevant articles that were featured in an online search using the name of the cell line and DMSO as keywords. However, from this limited sample of references it is clear that DMSO has been widely used as a solvent carrier in the *in vitro* evaluation of potential anticancer agents. Furthermore, a range of concentrations of DMSO has been safely used and the final concentrations employed in this study (<1%) were within that range. The maximal concentration of 1% DMSO used was a compromise between the concentration required to solubilise the extracts and that shown to have a minimal effect on cell viability. Therefore,

it was concluded that DMSO was a suitable vehicle, as long as the appropriate control, DMSO at the same concentration as the sample, was included in experiments.

3.4.4 Controls

As mentioned, the negative control for each cell line was cells incubated with DMSO alone. However, positive controls were also required to show that the assay system was working. Potential positive controls included DFO, L1, TAM, CPT, ETO, VIN, PAC and 5FU. These compounds were tested for their suitability as positive controls using the particular culture conditions and cell lines selected for use in impending screening studies. As it was intended that plant extracts would generally be tested on every cell line in every experiment, it was desirable that the same concentration of a positive control reduced the proliferation of all cell lines by a substantial amount.

From Figures 3.13 and 3.14, it is clear that some compounds were more effective than others at reducing cell numbers over the concentration ranges tested. DFO, L1 and CPT showed the most convincing dose-dependent inhibitory effect across all cell lines. Other compounds tested had little inhibitory effect, and sometimes even a stimulatory effect, on at least one cell line in the panel. Nevertheless, it was possible to generate IC_{50} values of several compounds for MDA-MB-468 and MCF7 cells. Based on calculated IC_{50} values agreeing with published values in conjunction with unanimous inhibitory activity, DFO was chosen as the most suitable positive control of the compounds tested. However, L1 and CPT were sometimes also included as positive controls in supporting roles. These were provided in 10 to 50-fold excess of their calculated IC_{50} values to ensure maximal inhibitory effect.

3.5 SUMMARY

In summary, this chapter described the validation and optimisation of the experimental design. Firstly, the panel of cell lines chosen was justified and their growth characteristics defined in order to optimise the culture conditions required for experiments involving 48 h exposure to plant extracts. Secondly, the rationale for choosing the MTT assay to monitor cytotoxic effects of test agents was provided and its validity was assessed. Additionally, the refinement of the routine procedure hitherto employed in this laboratory was recounted. Next, the suitability of DMSO as a vehicle was examined and shown to be appropriate. Finally, a fitting positive control, DFO, was chosen from a range of potentially suitable compounds. From these studies, a standard protocol was devised which was used in subsequent experiments to evaluate the cytotoxic activity of extracts of plants traditionally used as medicines by Aboriginal people. The following chapter will describe studies involved in the initial screening of these selected plant extracts.

Chapter 4: Preliminary Screening of Plant Extracts

4.1 INTRODUCTION

As already discussed extensively in Section 1.3, plants and other natural products (NPs) have been and remain a prime source of new chemical entities (NCEs), leading to the development of a myriad of novel drugs to combat a diverse range of diseases (Clardy & Walsh, 2004; Newman & Cragg, 2007). Nature has been experimenting with chemical combinations for aeons (Macilwain, 1998) and so the amount of chemical diversity within natural compounds cannot be matched by mankind (Verdine, 1996; Tulp & Bohlin, 2004). Hence, NPs provide a virtually limitless source of novel and complex chemical structures that probably would never otherwise have been the subject of a beginning synthetic programme (Fabricant and Farnsworth, 2001). Moreover, the chemical properties of NPs, such as lipophilicity and binding affinities for specific receptors, are generally superior to chemically synthesised compounds and less likely to cause detrimental interactions within the complex microenvironment of a cell (Feher & Schmidt, 2003; Dobson, 2004).

In this age of modern drug discovery, bioprospecting for potential chemotherapeutics has sometimes been dismissed as a futile exercise, bearing the negative connotations associated with such labels as “stamp collecting” and “fishing expedition” (Norvell & Cassman, 2003; Bull & Stach, 2007). It has also been criticised as an instrument responsible for exploiting the natural environment (Shiva, 1997). Arguably worse still, ethnopharmacology has been criticised for taking advantage of the indigenous cultures who provided the original knowledge about plants they have traditionally used as medicines (Shiva, 1997; Verma, 2002; Hamilton, 2006; Stone, 2008). While these criticisms may have some valid historical basis (see section 1.4.4.2.3), the fact remains that over half of all drugs, including cancer

therapies, have been derived wholly or partly from plants (Melnick, 2006a; Cragg *et al.*, 1997; Newman & Cragg, 2007). Furthermore, 80% of these compounds have had an ethnomedical use identical or related to the current use of the active elements of the plant (Fabricant & Farnsworth, 2001). Despite this impressive statistic and the fact that Australian indigenous people maintain the oldest culture on Earth, very little research has been undertaken to evaluate the therapeutic potential of any plants in the Aboriginal Pharmacopoeia (Locher & Currie, 2010). Studies that validate traditional Aboriginal medical knowledge include compounds with antibacterial (Palombo & Semple, 2001; Strobel *et al.*, 2005; Pennacchio *et al.*, 2005; Smith *et al.*, 2007) and antiviral (Semple *et al.*, 1998) activities, as well as those useful for treating inflammatory conditions like gout and headaches (Sweeney *et al.*, 2001; Li *et al.*, 2003b; Grice *et al.*, 2010). However, few published studies could be found that have specifically assessed the anticancer potential of plants traditionally used by Australian Aboriginal people as medicines (Kerr *et al.*, 1996; Mijajlovic *et al.*, 2006). For this reason, plants identified by local Aboriginals as having medicinal value were screened for their antineoplastic potential as outlined below.

4.1.1 Physical properties

At the outset it was important to establish that any observed extract-induced cytotoxicity/cytostaticity was not simply due to general physical assault such as would occur if the extracts were hypo- or hypertonic or excessively alkaline or acidic. Therefore, the osmolality and pH of selected extracts was measured. Additionally, as the extracts were coloured and the main assay used to assess changes in cell proliferation upon exposure to extracts was colourimetrically-based, spectrophotometric scans were performed to determine the extent of potential interference.

4.1.2 Initial screening

The plants chosen for assessment of their anticancer properties were those identified by local Aboriginal experts as having some medicinal qualities. These could be either general-purpose or specific to some condition, not necessarily cancer. Plants were collected in three batches from areas surrounding two different townships, Titjikala in the Northern Territory and Scotdesco in South Australia. While Aboriginal people would have traditionally steeped the plants in boiling water, effectively producing aqueous extracts, in this project it was the methanolic extracts that were evaluated. The rationale behind this decision was that target compounds are usually extremely polar, so aqueous media or strongly polar solvents, such as methanol, must be used for extraction to ensure most are recovered. However, substantial difficulties are associated with the isolation and purification of water-soluble compounds. For example, bacterial and fungal growth is an almost inevitable problem of aqueous extraction procedures. These contaminants often degrade the active components or give erroneous results in bioassays due to endotoxins produced by the microorganisms. This is an especially real problem in anticancer activity screening because many endotoxins (e.g. lipopolysaccharides) show distinct antitumor activities of their own. Additionally, concentrating aqueous extracts is also problematical due to the evaporation of water. If prolonged, the evaporation process often leads to the destruction of bioactivity and to microbial growth (Shimizu, 1985). Therefore, methanol extracts were used instead.

4.1.3 Dose-response

The correlation between the dose (or concentration) of a compound and the biological effects it produces is known as the dose-response relationship. The dose-response relationship is one of the most basic tenets of pharmacology and toxicology. It purports that, over the range of tissue sensitivity, as the dose increases, the effect increases and *vice*

versa. There are two main types of dose-response relationships: graded and quantal. The quantal dose-response relationship is also known as the “all or nothing” response- it either happens or it does not. The graded dose-response relationship, on the other hand, occurs on a continuous scale and can be determined quantitatively (Schiefer *et al.*, 1997; DiPasquale & Hayes, 2001; Walsh *et al.*, 2005).

4.1.4 Morphological changes

As unhealthy and dead cells are generally apparent under phase-contrast microscopy, cultures were viewed after exposure to extracts and compared to cells in control cultures. Moreover, one of the differences between apoptosis and necrosis is in their cell morphology. Apoptotic cells shrink without losing plasma membrane integrity and break into smaller pieces that macrophages recognise and engulf. In contrast, necrotic cells swell until their plasma membrane eventually ruptures (Liles & Sailer, 2002; Lee *et al.*, 2009). Therefore, it was hoped that by microscopically examining cells, any such responses may be obvious and hint at the mode of cell death.

4.1.5 Exposure and recovery

While the MTT assay is a very valid technique for measuring changes in cell proliferation over time, it cannot distinguish between cytotoxic and cytostatic effects *per se* (Plumb, 1999). However, if cells are exposed to treatments for periods less than their population doubling time and viable cell numbers are lower than for untreated cells, then it could be assumed that the treatment had killed the cells.

Therefore, time-course experiments were performed to assess the rate of any extract-induced inhibitory effects. It was assumed that if cell numbers were lower than control levels within short periods (less than a doubling time, ~ 24 h, see Table 3.4), then the

extracts must have been cytotoxic to that degree. If % control levels were lower in the presence of extract only after longer exposure times, then the inhibition could have been due to delayed cytotoxicity or overall cytostaticity (cells had either left the cell cycle or cell death was in equilibrium with cell proliferation).

Additionally, the reversibility of the extracts' inhibitory effects was examined in a separate experiment in which extracts were removed and the cells given an opportunity to recover. Any increase in cell proliferation within this recovery period would suggest that surviving cells were capable of growth and that any extract-induced inhibitory effects must have been due to a reversible, cytostatic mechanism. Conversely, a lack of cell proliferation during this period would suggest any surviving cells had either subsequently died, or had withdrawn from the cell cycle into G₀ (see Appendix A1.1).

Cells that die due to necrosis generally do so in an uncontrolled manner, releasing their contents into the medium (Al-Lamki *et al.*, 1998; Liles & Sailer, 2002). In contrast, cells that undergo apoptosis die more tidily and this process generally requires less time, 1-3 h (Kerr *et al.*, 1972; Gavrieli *et al.*, 1992; Al-Lamki *et al.*, 1998). Hence, some clues as to the mode of any extract-induced cell death may also have been revealed from these time-course experiments.

4.2 METHODOLOGY

4.2.1 Physical properties

The following tests were conducted on selected extracts dissolved at 100 mg/mL in DMSO and diluted to 1 mg/mL in DMEM without phenol red.

4.2.1.1 Osmolality

As the osmometer on hand (a Fiske F1-10) operates by freezing point depression and the vehicle, DMSO, has a much higher freezing point (18.5°C) than water, it was not possible to use this apparatus to measure the osmolality of all the extracts. Even when the extracts were diluted to 1 mg/mL, which corresponded to 1% final concentration (v/v) vehicle, DMSO interfered with freezing and gave erroneous values. Therefore, it was only possible to directly measure the osmolality of extracts that readily dissolved in water using this instrument.

However, the osmolality of extracts containing DMSO can be estimated by indirect determination of the specific gravity (SG). This was done by measuring changes in the refractive index of the sample using a hand-held refractometer (Atago, Japan). This procedure is routinely performed by physicians when analysing urine samples (Chadha *et al.*, 2001, Eberman *et al.*, 2009).

4.2.1.2 pH

The pH of extracts was measured using an Aqua pH cube (TPS).

4.2.1.3 Colour

As most extracts had a colour of their own, spectrophotometric scans were conducted over wavelengths ranging from 800 nm to 200 nm using a Cary UV/VIS spectrophotometer (Varian).

4.2.2 Initial screening

In order to identify plants having potential as anticancer therapeutics, crude methanolic extracts were screened for their ability to inhibit cancer cell proliferation. Various plants identified by local Aboriginal people as having medicinal properties were chosen for

assessment as described in Section 2.4.1. For simplicity and confidentiality, codes were assigned to each of the plant extracts as shown previously in Table 2.3 and reproduced here (Tables 4.1 to 4.3). Specific details of the methodology involved in extraction are described in Section 2.4.2.

Batch 1 samples were collected from Titjikala (NT) in July 2004. Batch 2 samples were also from Titjikala but collected in October 2004. Batch 3 samples were collected from Scotdesco (SA) in April 2005.

On the day of use, plant extracts were dissolved at 100 mg/mL (10% w/v) in DMSO before dilution in incubation medium to give a final concentration of 1 mg/mL. Extracts 4 and 10 proved difficult to dissolve and, thus, the final concentration of these extracts was lower (250 µg/mL). The diluted extracts were sterilised through 0.22 µm filters (Acrodisc) and quadruplicate samples of 10 µL was added to 100 µL of cells in 96-well plates. In line with other primary screening studies (Kenakin, 2003; Andreani *et al.*, 2010), a panel of four different human cancer cell lines were incubated with a single high concentration (1 mg/mL) of crude extracts for 48 h before measuring any changes in cell proliferation via the MTT assay (original method, see Sections 2.6.1 and 3.2.2).

Extracts effecting the greatest response, as measured by the relative degree of inhibition of cell proliferation (expressed as a percentage of the negative control, % Control), were selected for further testing in dose-response mode. In this way, the pool of extracts worthy of subsequent analysis was reduced from 25 to 16. The inhibitory effects of the most bioactive extracts were also compared to those of DFO and L1, compounds that have been used clinically to treat cancer (Estrov *et al.*, 1987; Donfrancesco *et al.*, 1992).

Table 4.1 Designation of Plant Extracts: Batch 1 (from Titjikala)

Code	Sample ID	Plant Species
1	1 (i)	<i>Eremophila longifolia</i>
2	2 (i)	<i>Eremophila latrobei</i>
3	3 (i)	<i>Thysanotus exiliflorus</i>
4	*5 (i)	<i>Eremophila sturtii</i> (filter paper residue)
5	5 (i)	<i>Eremophila sturtii</i>
6	15 (i)	<i>Euphorbia drummondii</i>
8	8 (i)	<i>Eremophila freelingii</i>
9	11B (i)	<i>Acacia tetragonophylla</i>
10	10 (i)	<i>Sarcostemma australe</i>
11	11A (i)	<i>Acacia tetragonophylla</i>
12	12 (i)	<i>Hakea divaricata</i>

Table 4.2 Designation of Plant Extracts: Batch 2 (from Titjikala)

Code	Sample ID	Plant Species
19	Ti 19	<i>Eremophila duttonii</i>
20	Ti 20	<i>Euphorbia tannensis</i>
21	Ti 21	<i>Eremophila duttonii</i>
22	Ti 22	<i>Hakea</i> unknown species
23	Ti 24A	<i>Hakea divaricata</i>
24	Ti 24B	<i>Hakea divaricata</i>
25	Ti 25	<i>Codonocarpus cotinifolius</i>
26	Ti 26	<i>Euphorbia tannensis</i>
27	Ti 27	<i>Eremophila freelingii</i>
28	Ti 28	<i>Acacia tetragonophylla</i>

Table 4.3 Designation of Plant Extracts: Batch 3 (from Scotdesco)

Code	Sample ID	Plant Species
A	S #1 Bag 01	<i>Eremophila alternifolia</i>
B	S #1 Bag 02	<i>Eremophila alternifolia</i>
C	S #3	<i>Scaevola spinescens</i>
D	S #7	<i>Eremophila alternifolia</i>

4.2.3 Dose-response

In these studies, graded dose-response relationships were assessed for a range of extract-cell line combinations. Firstly, the effects of different concentrations of the most promising 16 extracts from the initial screen on the same four cancer cell lines were examined. From these data, the dose-responses of the most active six extracts on more cancer cell lines and one non-cancerous cell line were assessed. Unless specified otherwise, cells were exposed to various concentrations of extracts for 48 h before being subjected to the MTT assay (original method, see Sections 2.6.1 and 3.2.2).

4.2.4 Morphological changes

After exposure to extracts for the given period (generally 48 h), cells were assessed for any changes in their morphology, indicative of viability, as well as any differences in cell numbers compared to control cells. For this qualitative assessment, cells were usually viewed under 400X magnification of a phase-contrast light microscope as described in Section 2.2.2.9. Photographs of the cells were not always taken, but when they were, the 20X objective was employed instead, meaning the total magnification of micrographs was 200X. In order to make direct comparisons between cultures, the micrographs presented herein (Fig. 4.10) were all taken on the same day.

4.2.5 Exposure and recovery

Time-course experiments were performed to determine how quickly the extracts were exerting their effects on cells and whether or not their actions were cytotoxic or merely cytostatic. This involved exposing cultured cells to extracts for various periods, instead of the standard 48 h incubation. MTT assays were performed as in the original method described in Sections 2.6.1 and 3.2.2.

The first experiment was a preliminary test to ascertain how quickly the extracts were causing their inhibitory effects in order to establish cytotoxicity or cytostaticity. In this experiment (see Fig. 4.11), cells were simply incubated with the extracts for the given periods and the MTT assay performed immediately upon the end of the exposure period. That is,

Period (h)	Growth 24	Exposure 2 - 48	Recovery 0	Total 26 - 72
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The results of this preliminary experiment led to the design of the second experiment (see Fig. 4.12), in which cells were exposed to extracts for more time points, concentrating on the shorter periods. Additionally, at the end of the exposure period, extracts were removed by aspiration and the medium replaced with fresh medium to allow the cells to recover for varying periods until MTT assays were performed in synchrony at the end of the experiment (at the end of the 72 h exposure period). That is,

Period (h)	Growth 24	Exposure 0.5 - 72	Recovery 71.5 - 0	Total 96
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In this way it was possible to assess whether the surviving cells were capable of proliferation in the absence of extract.

4.3 RESULTS

4.3.1 Physical properties

4.3.1.1 *Osmolality*

The osmolality of extracts was estimated by measuring the SG, which is, in turn, estimated by measuring the refractive index. Table 4.4 summarises the refractive indices of selected extracts (the most extensively studied in this project) compared to various controls.

Table 4.4 Refractive Indices of Selected Extracts and Controls

Extract Code	Refractive Index (=SG) (units)	Estimated Osmolality (mosmol/kg)*
5	1.011	440
6	1.011	440
8	1.011	440
21	1.012	480
27	1.011	440
28	1.011	440
1% DMSO	1.011	440
DMEM	1.007	280
DDW	1.000	0

* Based on 40 mosmol/kg per 0.001 unit above 1.000 (Chadha *et al.*, 2001).

As can be seen, while water had a SG of 1.000, as expected, the SG of culture medium, DMEM (without phenol red), was 1.007. As the measured osmolality of this DMEM was 280 mosmol/kg, these measurements substantiate the published relationship (for urine analysis) that states that, generally, SG rises by 0.001 unit for every 40 mosmol/kg increase in osmolality (Chadha *et al.*, 2001). However, as the refractive index is influenced by the proportion of glucose, protein and other heavy molecules in a sample as well as the ambient temperature (Chadha *et al.*, 2001), it must be noted that these measurements are fundamentally estimates of osmolality. Nevertheless, the technique was useful in discerning any differences in osmolality between samples and controls. Indeed, it was shown that (except for Extract 21 which had a SG of 1.012) the SG of the negative control (1% DMSO in DMEM) was the same as that of the extracts (1.011). Hence, as the extracts themselves did not increase the SG above that of the control, it can be inferred that there was no difference in osmolality either.

4.3.1.2 *pH*

The pH of selected extracts was measured and compared to that of the control. As can be seen from Table 4.5, pH values all lay within 0.1 unit of the negative control (1% DMSO), which was no different to that of the medium (DMEM) alone.

Table 4.5 pH Values of Selected Extracts and Controls

Extract Code	Recorded pH
5	7.64
6	7.56
8	7.52
21	7.67
27	7.58
28	7.73
DMEM	7.67
1% DMSO	7.64

4.3.1.3 *Colour*

The powdered extracts had characteristic colours that changed very little upon dissolution in DMSO at 100 mg/mL. After dilution in DMEM without phenol red to 1 mg/mL, the colour was correspondingly diluted. A list of selected extracts and descriptions of their corresponding colours is presented in Table 4.6.

The 1 mg/mL samples in DMEM were spectrophotometrically scanned over wavelengths ranging from 800 to 200 nm. Charts of these scans can be found in Appendix D. Table 4.7 summarises the absorbance readings recorded at 595 nm and 570 nm, the wavelengths of

the two different microplate readers used in this project at which solubilised MTT-formazan is optimally absorbed. The wavelength at which the absorbance reading first reached 2.0 is also presented.

Table 4.6 Colours of Selected Extracts

Extract Code	Colour of powdered extract	Colour @100 mg/mL in DMSO	Colour @1 mg/mL in DMEM
5	Brown	Brown	Yellow
6	Brown	Reddish brown	Murky yellowish brown
8	Brown	Dark brown	Tan, orangey brown
21	Dark brown	Dark reddish brown	Light murky yellowish brown
27	Light brown	Light reddish brown	Tan, orangey brown
28	Rust	Reddish brown	Salmon, light pinkish brown

Table 4.7 Absorbance Readings of Extracts at Relevant* Wavelengths and Wavelength at which Absorbance >2.0

Extract Code	Absorbance @ 595 nm	Absorbance @ 570 nm	λ at which Absorbance > 2.0 (nm)
5	0.108	0.120	395.0
6	0.470	0.523	432.1
8	0.276	0.312	404.4
21	0.686	0.775	438.1
27	0.276	0.310	404.4
28	0.087	0.108	320.7
DMEM	0	0	234.0
1% DMSO	0	0	235.5
DDW	0	0	-

* Wavelength at which solubilised MTT-formazan crystals are absorbed optimally. For old microplate reader (Bio-Rad) λ = 595 nm; new microplate reader (Biotek) λ =570 nm.

It is evident from the scans presented in Appendix D that extracts were, for the most part, absorbing in the ultraviolet range of the light spectrum. However, most also absorbed strongly in the visible spectrum around the yellow and orange regions (570-620 nm). Extract 21 followed by Extract 6 recorded the highest absorbance readings at both 570 and 595 nm. The absorbance readings were higher at 570 nm than 595 nm.

4.3.2 Initial screening

In order to obtain a general picture of the potential effectiveness of each of the plant extracts against cancer, cells were exposed to a single high concentration (1 mg/mL) of extract for 48 h and evaluated using the MTT assay as described in Section 2.6.1.

Batch 1 samples (Table 4.1, Fig. 4.1) were the first to be assessed for bioactivity. For each cell line, extract-induced effects on cell proliferation, as measured by the MTT assay, were determined and expressed as a percentage of the negative control value (i.e. cells exposed to DMSO alone). These data are depicted in Figure 4.1. Similarly, Batch 2 samples (Table 4.2) and Batch 3 samples (Table 4.3) were evaluated against four cell lines and these data comprise Figures 4.2 and 4.3, respectively.

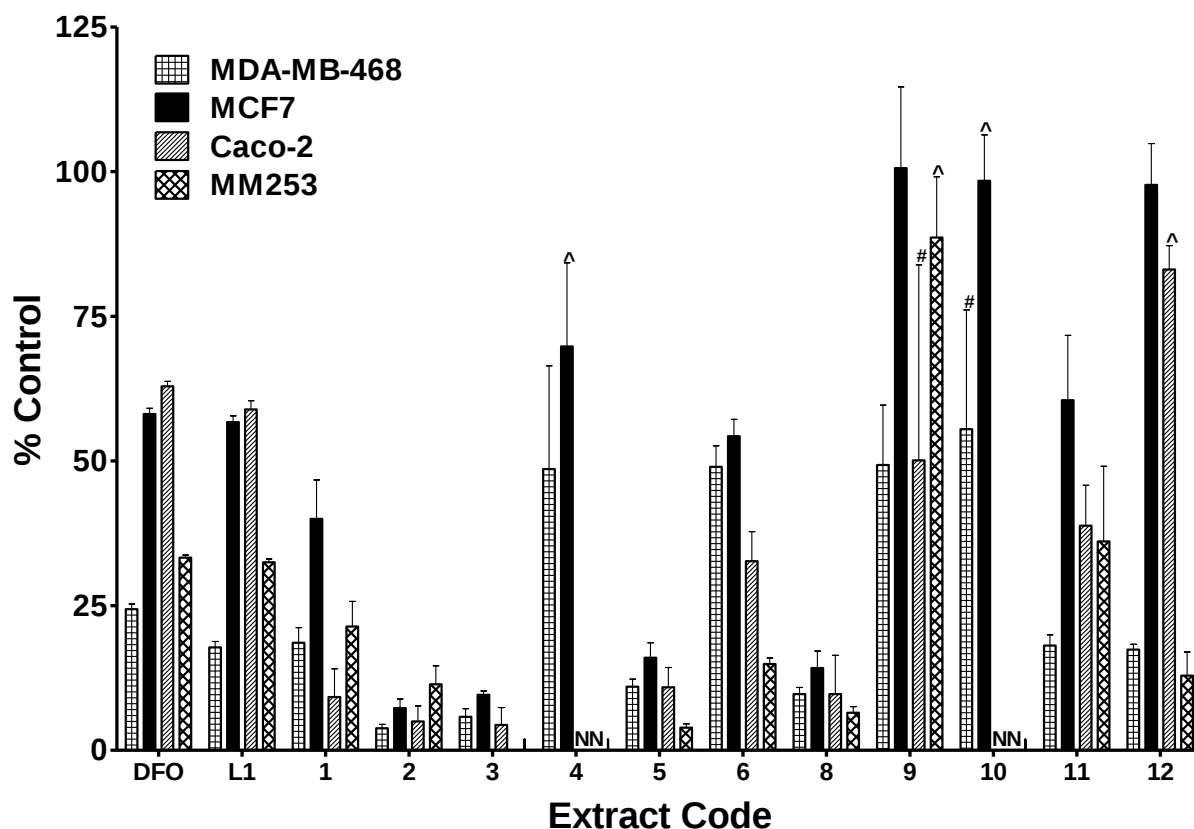


Figure 4.1 Inhibition of cancer cell proliferation by Batch 1 plant extracts at 1 mg/mL.

Results are the means $\pm$ SE of at least 3 experiments, except for Extracts 1, 2, 3, 8, 9, 11 and 12 against Caco-2 cells and 5 and 12 against MM253 cells, which are the means of only two replicate experiments. Those extracts not tested against a particular cell line are designated "N".

ANOVAs were performed using Dunnet's Multiple Comparisons Test to determine which extracts significantly inhibited cancer cell proliferation compared to the negative control (1% DMSO).

Unless designated otherwise, extracts significantly inhibited cell proliferation at $P < 0.01$. # denotes $P < 0.05$; ^ denotes non-significance.

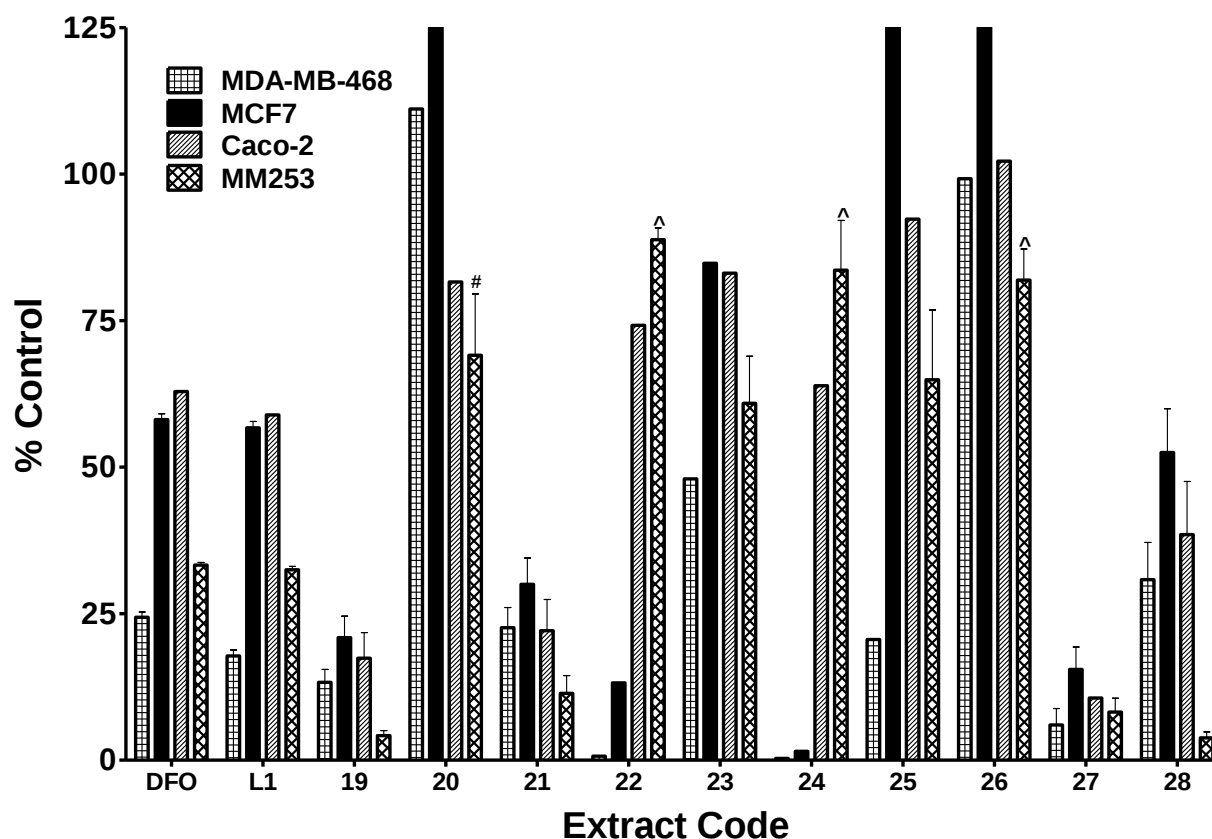


Figure 4.2 Inhibition of cancer cell proliferation by Batch 2 plant extracts at 1 mg/mL.

Results are the means $\pm$ SE of at least 3 experiments, except for Extract 27 against MDA-MB-468 and MCF7 cells which are the means of only two replicate experiments and against Caco-2 cells in which case $n=1$. Only one experiment was performed with Extracts 20, 22, 23, 24, 25 and 26 against MDA-MB-468, MCF7 and Caco-2 cells.

ANOVAs were performed using Dunnet's Multiple Comparisons Test to determine which extracts significantly inhibited cancer cell proliferation compared to the negative control (1% DMSO).

Unless designated otherwise, extracts significantly inhibited cell proliferation at $P<0.01$.

denotes $P<0.05$; ^ denotes non-significance.

4.3.2.1 *Extracts versus negative control (DMSO)*

As can be seen from Figures 4.1 to 4.3, many extracts were highly effective in inhibiting cell proliferation when compared to the negative control (1% DMSO). All extracts from Batch 1 (Table 4.1) significantly ($P<0.01$) inhibited MDA-MB-468 cell proliferation, while only Extracts 1, 2, 3, 5, 6 and 8 significantly ($P<0.01$) inhibited proliferation of the other breast cancer cell line, MCF-7. Of the Batch 1 extracts tested against Caco-2, all but Extracts 9 and 12 significantly ($P<0.01$) inhibited cell proliferation, and Extract 9 was significantly inhibitory at $P<0.05$. For MM253 cells, all but Extract 9 were significantly inhibitory at $P<0.01$.

For Batch 2 extracts (Table 4.2, Fig. 4.2), only Extracts 19, 21 and 28 were significantly ($P<0.01$) inhibitory against all cell lines tested. Extract 27 significantly ($P<0.01$) inhibited proliferation of MDA-MB-468, MCF7 and MM253 cells. Extract 27 also appeared to be effective against Caco-2 cells, but statistics could not be performed on these data as the experiment was only performed once. This was because these experiments were simply screening studies and Extract 27 had already qualified for further investigation based on its inhibitory effects observed against the other cell lines tested. Similarly, the effects of Extracts 20, 22, 23, 24, 25 and 26 against MDA-MB-468, MCF7 and Caco-2 could not be statistically validated as $n=1$ in these preliminary studies. However, statistical assessment could be made in MM253 cells, and Extracts 23 and 25 were significantly inhibitory at $P<0.01$ and Extract 20 was inhibitory at $P<0.05$. Extracts 22, 24 and 26 were not significantly active in the cells tested statistically.

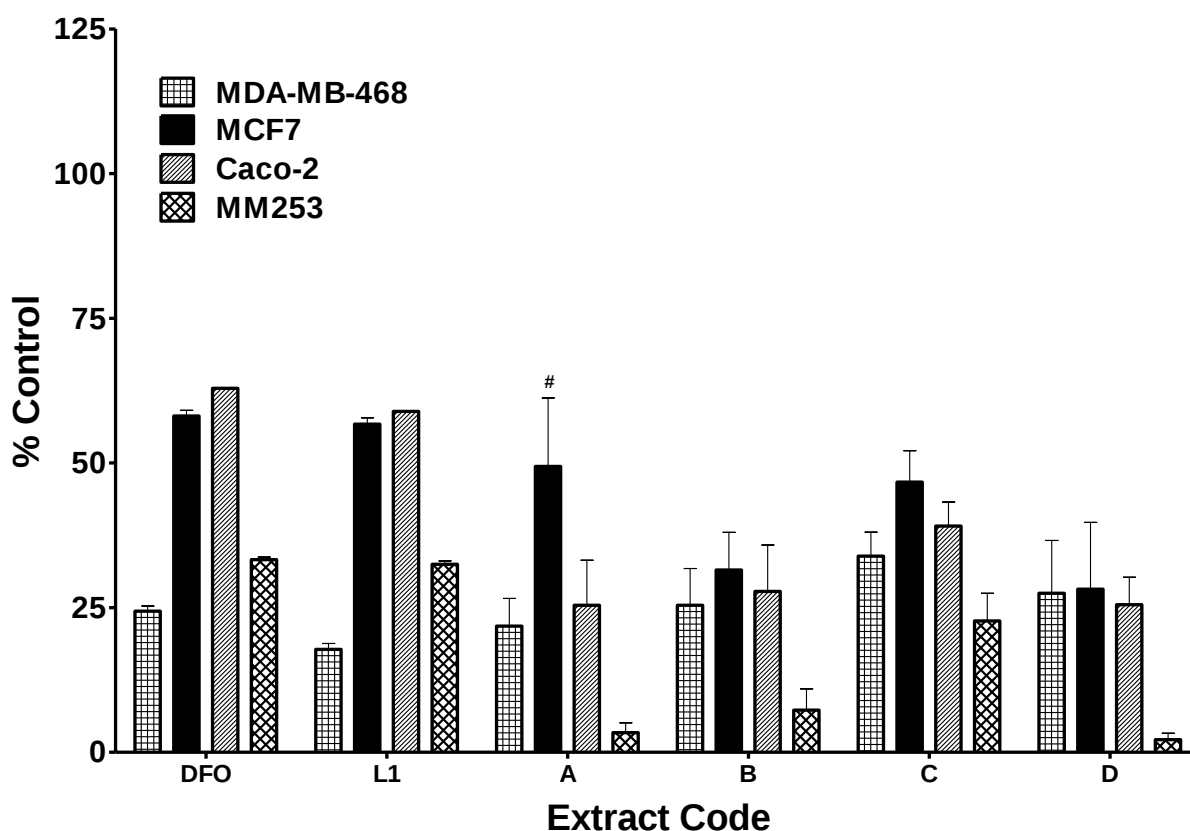


Figure 4.3 Inhibition of cancer cell proliferation by Batch 3 plant extracts at 1 mg/mL.

Results are the means $\pm$ SE of at least 3 experiments.

ANOVAs were performed using Dunnet's Multiple Comparisons Test to determine which extracts significantly inhibited cancer cell proliferation compared to the negative control (1% DMSO).

Unless designated otherwise, extracts significantly inhibited cell proliferation at $P < 0.01$.

denotes $P < 0.05$; ^ denotes non-significance.

Batch 3 extracts (Table 4.3, Fig. 4.3) were all highly active against all four cell lines, inhibiting cell proliferation compared to the negative control at the 99% significance level, with the exception of Extract A against MCF7 cells, which was only statistically inhibitory at $P < 0.05$.

In these experiments, bioactivity, as evidenced by inhibition of cell proliferation (% of negative control), was assessed. Thus, the most consistently bioactive extracts against all four cell lines were Extracts 1, 2, 3, 5, 6 and 8 from Batch 1, Extracts 19, 21, 27 and 28 from Batch 2 and all Extracts, A, B, C and D, from Batch 3. From these preliminary screening data, the most potent appeared to be Extract 3 against MM253 cells, in particular, but also in the other cell lines tested. The next most potent extracts were Extracts 2, 5, 19, 28, A and D, all of which, at 1 mg/mL, restricted the proliferation of at least one cell line to less than 5% of the control. Extracts effecting an inhibition >75% of the negative control levels (<25% control) in at least one cell line were selected for further studies.

4.3.2.2 *Extracts versus positive controls (DFO and L1)*

These data were also used to get a general idea of the effectiveness of the most bioactive extracts in comparison to the positive controls, DFO and L1. For simplicity, only those extracts effecting an inhibition of >75% compared to negative control (DMSO) levels in at least one cell line are depicted in Figure 4.4. For inclusion in this figure, more than one experiment ($n \geq 2$) had to be performed. Hence Extracts 22, 24 and 25 are not included in the graph, despite registering >75% inhibition of proliferation of some cell lines. Asterisks (*) and hash symbols (#) indicate a level of inhibition significantly better than the positive control, DFO, at $p < 0.01$ or $p < 0.05$, respectively, as determined by Dunnett's One-way ANOVA.

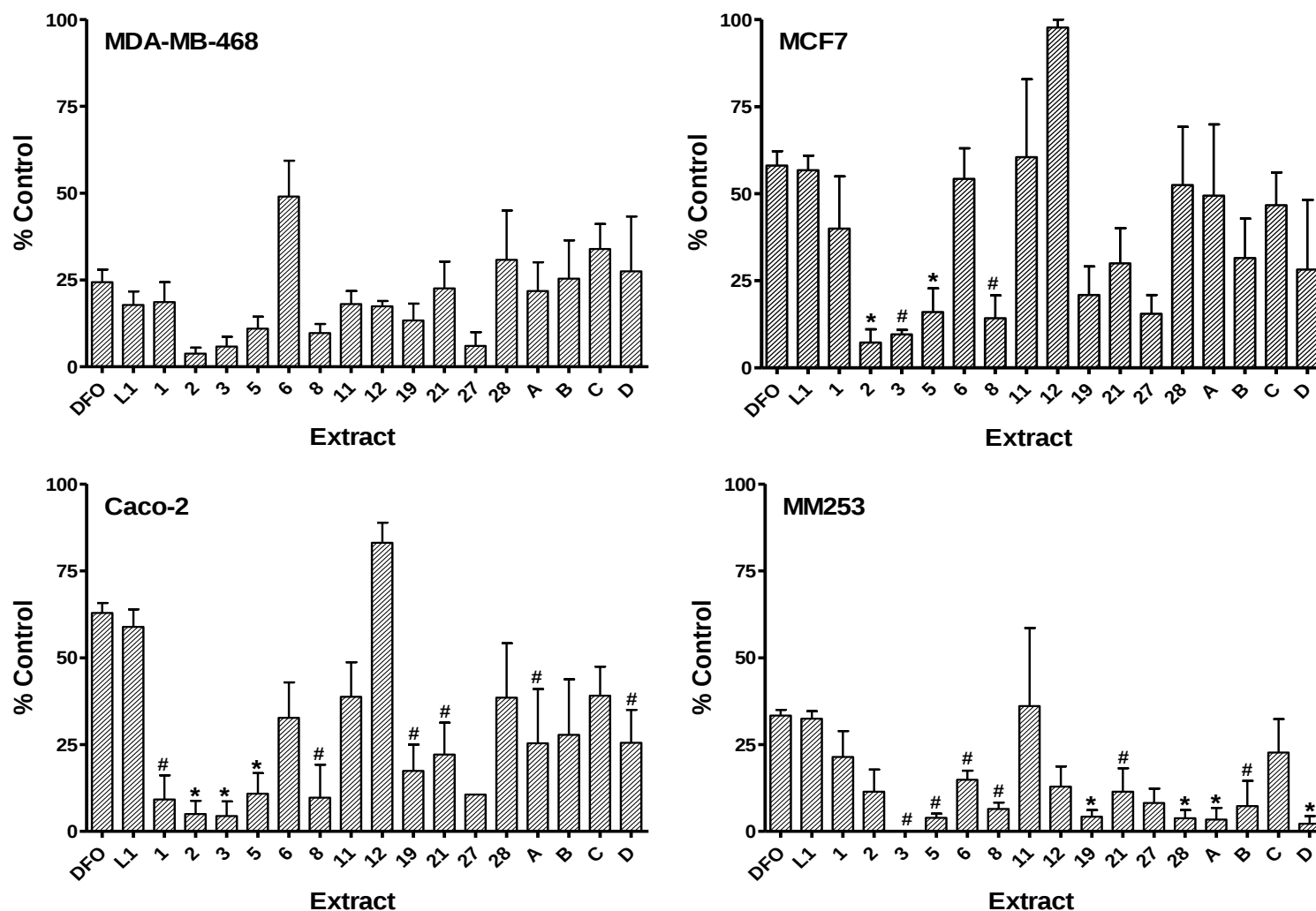


Figure 4.4 Comparison of inhibition of proliferation of cancer cell lines by selected plant extracts at 1 mg/mL.

Results are the means $\pm$ SE of at least 3 experiments, except for Extracts 1, 2, 8 and 11 against Caco-2 cells, Extracts 3 and 12 against Caco-2 and MM253 cells and Extract 27 against MDA-MB-468, MCF7 and Caco-2 cells, which are the means of only two replicate experiments. ANOVAs were performed using Dunnet's Multiple Comparisons Test to determine which extracts significantly inhibited cancer cell proliferation compared to the positive control (DFO). * denotes $P < 0.01$; # denotes $P < 0.05$.

As can be seen, out of all the samples tested, no extract was significantly better than either positive control, DFO or L1, in effecting inhibition of MDA-MB-468 cell proliferation. However, it must be noted that both DFO and L1 caused significant inhibition themselves in these cells. For MCF-7 cells, Extracts 2 and 5 caused significantly greater than control inhibition at the $p < 0.01$ level, while Extracts 3 and 8 were significantly better than the DFO control at $p < 0.05$. These four active extracts, all from Batch 1, were also significantly more effective than DFO in inhibiting the proliferation of Caco-2 and MM253 cells, with varying degrees of significance. Another Batch 1 sample, Extract 1, also caused inhibition of Caco-2 cell proliferation significantly more so than DFO at $P < 0.05$. Batch 2 extracts displaying significantly better inhibitory effects than DFO were Extracts 19 and 21 in Caco-2 and MM253 cells and Extract 28 in MM253 cells. From Batch 3, Extracts A and D were more inhibitory than DFO against MM253 cells ($P < 0.01$) and Caco-2 cells ($P < 0.05$), while Extract B was also significantly ($P < 0.05$) better than DFO in MM253 cells.

Similar results were obtained when the antiproliferative effects of extracts were compared to those of the other positive control, L1. The only differences were that for Caco-2 cells, Extracts 2 and 3 were only significantly better than L1 at $P < 0.05$, while Extracts 21, A and D were not significantly more inhibitory in this line. Additionally, in MM253 cells, Extract A was only significantly better than L1 at $P < 0.05$, while Extracts 3, 8 and 21 were not significantly different.

Additionally, by presenting these data grouped according to cell line (Fig.4.4), it is more apparent that there were differences between the effects of the various extracts on the four cancer cell lines tested. These differential antiproliferative activities are the first suggestion of some selectivity of the extracts.

Taking all results together, the experiments of the primary screen show that some crude extracts were more inhibitory than others (compared to each other and to positive controls) and some displayed selectivity. In the first instance, selectivity was not a criterion for selection for further study. Extracts were chosen for dose-response analysis based solely on their relative inhibitory responses at 1 mg/mL. Thus, extracts causing >75% inhibition compared to the negative control were identified and the pool of plant extracts to be tested was reduced from 25 to 16.

4.3.3 Dose-response

Preliminary dose-response curves were generated for the 16 plant extracts featured in Figure 4.4. Based on these screening data, the same four cancer cell lines, MDA-MB-468, MCF7, Caco-2 and MM253, were exposed to a range of concentrations of the selected extracts. It was not possible to average the data for each extract-cell line combination as sometimes the extracts were only tested once against a particular cell line due to insufficient sample being available and/or low sensitivity shown in the initial screen. Additionally, the concentrations of extracts selected for use sometimes varied from experiment to experiment. This was done intentionally in order to optimise the concentration range and reduce the chance of sudden changes in proliferation rates. Thus, the graphs presented in Figures 4.5 to 4.7 are “typical” curves obtained from numerous experiments. For simplicity, only curves obtained for Caco-2 and MM253 cells exposed to the extracts are presented. Nonlinear regression (sigmoidal dose-response, variable slope) curves were fitted for each extract-cell line pair using GraphPad Prism (Section 2.9.1). These curves, along with their corresponding IC_{50} values (where calculable using the default 4-Parameter model) and related statistical data, can be found in Appendix E.

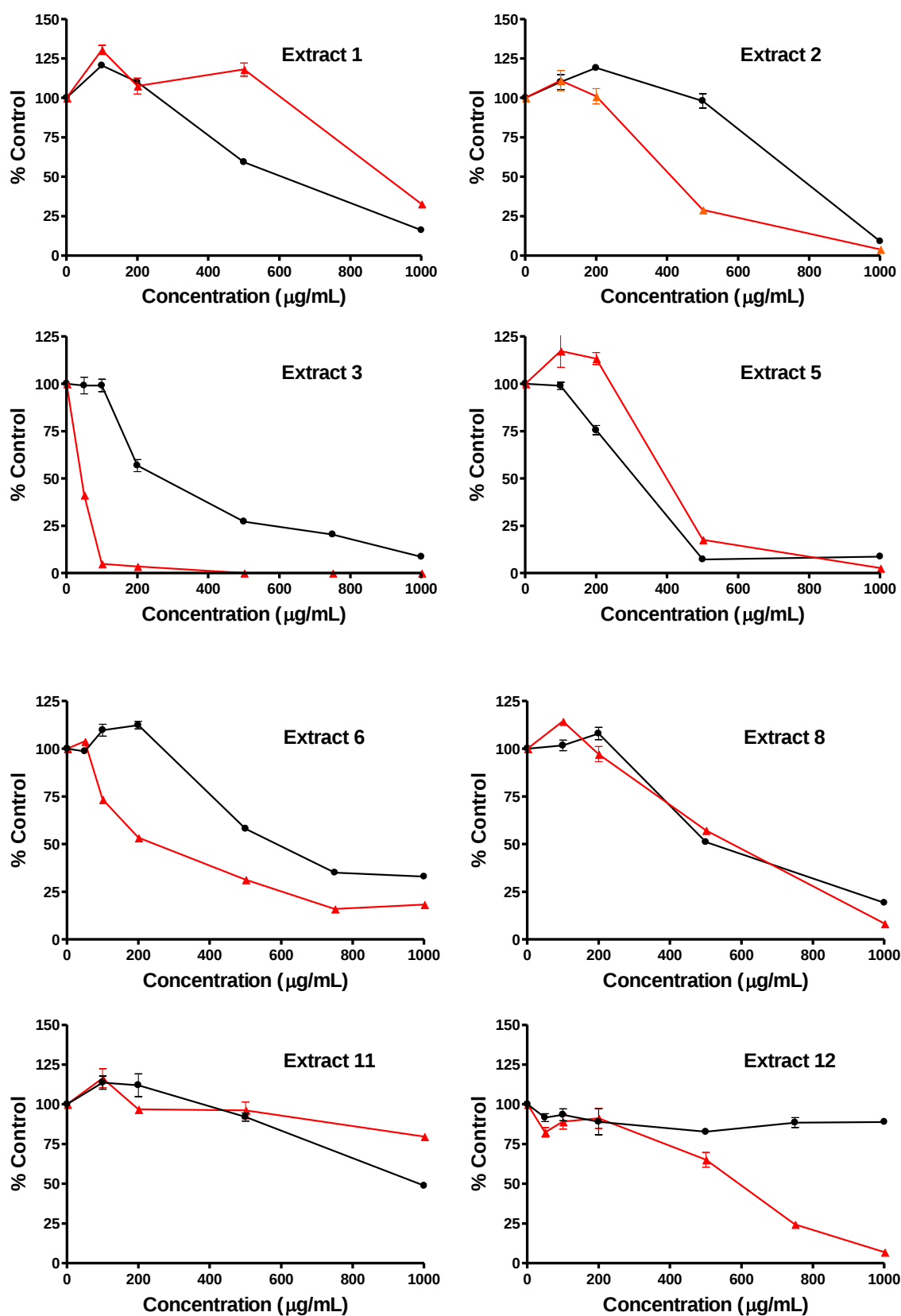


Figure 4.5 Typical dose-response curves for promising Batch 1 extracts.

Caco-2 (●) and MM253 (▲) cells were exposed to extracts over a range of concentrations for 48 h before being subjected to the MTT assay. Results are expressed as % of control values and are the means $\pm$ SE of quadruplicate wells.

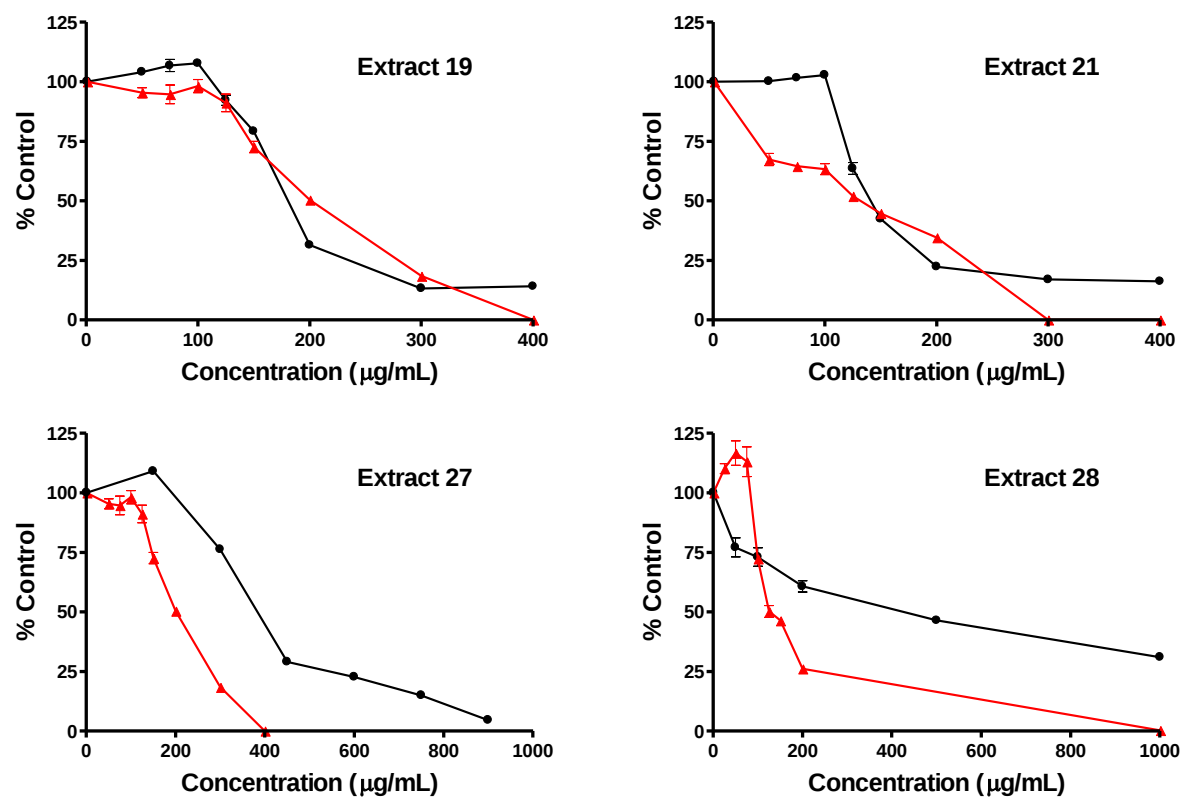


Figure 4.6 Typical dose-response curves for promising Batch 2 extracts.

Caco-2 (●) and MM253 (▲) cells were exposed to extracts over a range of concentrations for 48 h before being subjected to the MTT assay. Results are expressed as % of control values and are the means $\pm$ SE of quadruplicate wells.

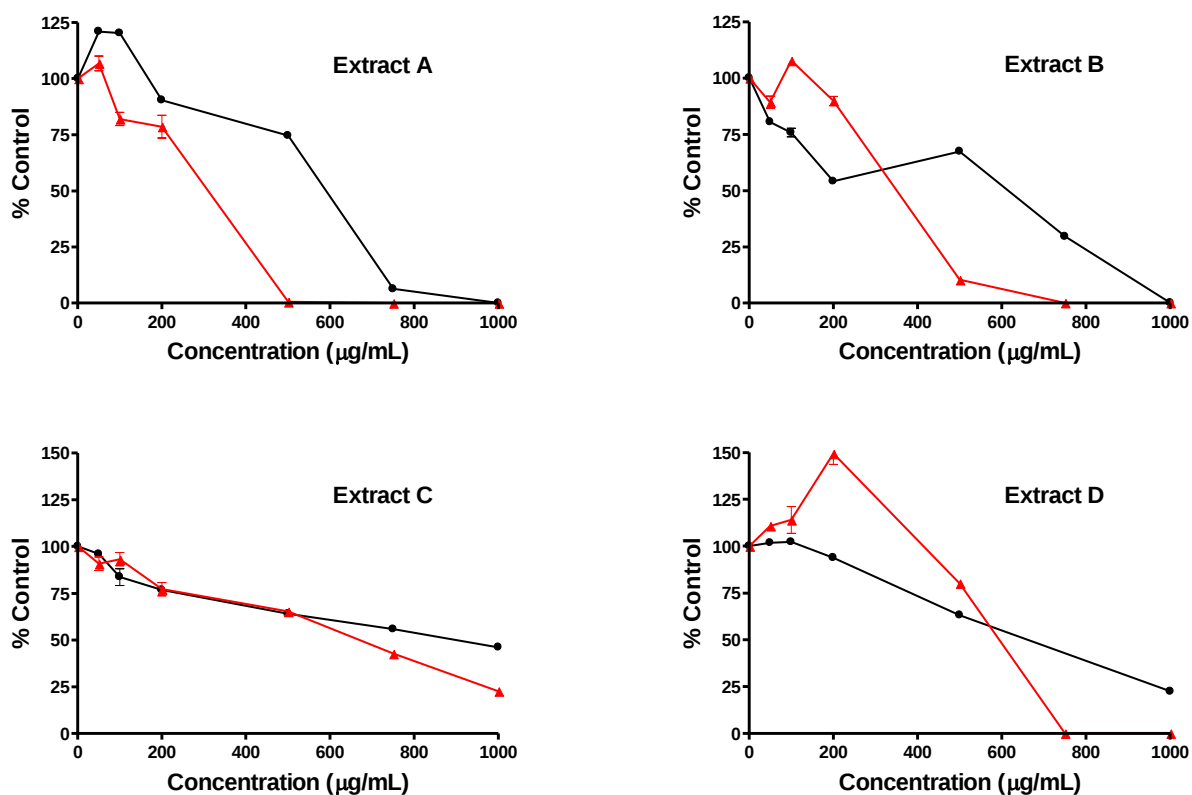


Figure 4.7 Typical dose-response curves for promising Batch 3 extracts.

Caco-2 (●) and MM253 (▲) cells were exposed to extracts over a range of concentrations for 48 h before being subjected to the MTT assay. Results are expressed as % of control values and are the means $\pm$ SE of quadruplicate wells.

From these data, the six most promising extracts, based on a process of elimination, were chosen for further study. Selection criteria included potency (relatively high levels of inhibition at low concentrations and/or low IC₅₀ values) in conjunction with suitably shaped dose-response curves, as well as guaranteed supply of the extract. At the end of the reductive process, Extracts 5, 6, 8, 21, 27 and 28, were identified as the six most worthy of further evaluation. The others were eliminated as outlined below.

Extracts 1 and 2 were rejected because of high (above 350 $\mu\text{g/mL}$) IC_{50} values (see Appendix E). However, Extract 3 was eliminated despite recording the lowest IC_{50} value of all the featured curves. While Extract 3 showed great potential as an anticancer agent against all four cell lines in primary screening, the limited amount of sample available precluded this extract from further study. Extract 11 was deemed not worthy of further study based on its relatively flatter dose-response curves compared to the other extracts available (Fig. 4.5). Like Extract 3, there was an insufficient supply of Extract 12 and so it, too, was eliminated from the pool of extracts subject to further investigations.

Only one extract from Batch 2 was rejected. As Extract 19 was originally from the same plant species as Extract 21 and their dose-responses and screening profiles were similar, just one of these was chosen for further study. Despite Extract 19 recording antiproliferative activity in the 1 mg/mL screening assays against all four cell lines (Fig. 4.2) and a lower IC_{50} value than Extract 21 against MM253 cells in the featured dose-response curves (Fig. 4.6), it was eliminated as Extract 21 was more potent at lower concentrations.

All extracts from Batch 3 were rejected. Extracts A, B and D (all the same species) were eliminated as they all showed relatively low levels of inhibition at low concentrations (Fig. 4.7) and correspondingly high IC_{50} values (Appendix E) against Caco-2 cells. Moreover, the regularly occurring sudden drops to zero in % control values over a narrow concentration range in MM253 cells exposed to these extracts were also not desirable (see Section 4.4.3). Extract C's dose-response curves were generally relatively flat, as evidenced by the featured curves (Fig. 4.7).

Thus, Extracts 5, 6, 8, 21, 27 and 28 were selected for further evaluation of their bioactivities against a wider range of cell lines. In addition to MDA-MB-468, MCF7, Caco-2 and MM253 cells, the lung cancer cell line, A549, two prostate cancer cell lines, PC3 and DU145, and a non cancerous cell line, MRC5, were exposed to a consistent range of concentrations of these six extracts for 48 h. After this time, changes in cell proliferation, as measured by the MTT assay, and hence bioactivity, were assessed. The dose-response curves obtained are presented in Figure 4.8. As can be seen, higher concentrations of extract generally resulted in greater inhibition of cell proliferation with many extracts generating the classic sigmoidal dose-response curves for each cell line over the range of concentrations used.

In toxicology, data from dose-response experiments are frequently analysed by a logistic model and summarised in the form of the IC_{50} , the concentration or dose which causes a 50% inhibitory effect. In these studies it was desirable to calculate IC_{50} values in order to directly compare the activities of various extracts against all the cell lines tested. The concentrations of extracts used were transformed into logarithmic values and the data subjected to four different models of nonlinear regression in order to achieve the most accurate curve fits for each extract-cell line combination. However, for some extract-cell line combinations this was problematical due to reasons to be discussed in Section 4.4.

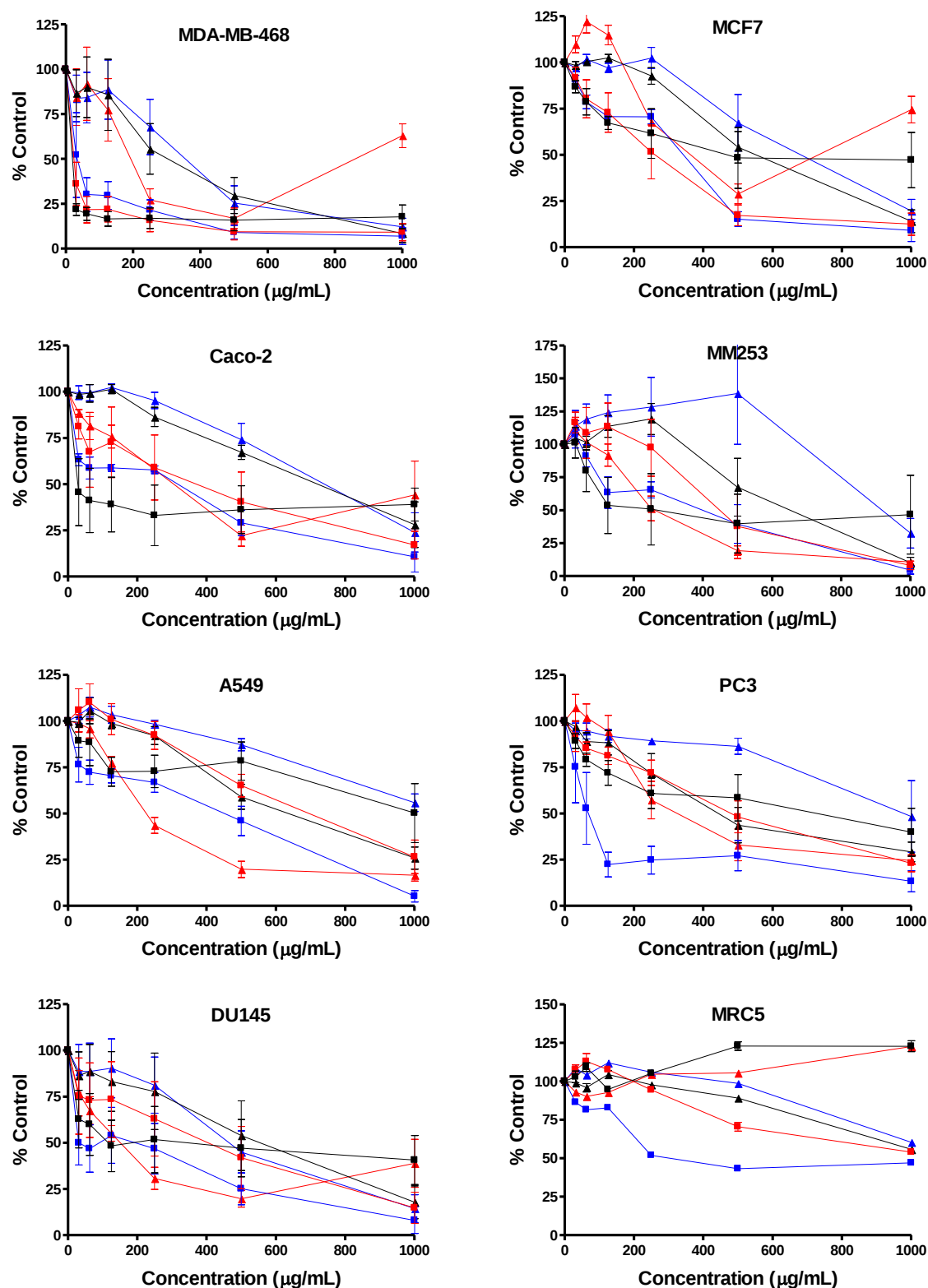


Figure 4.8 Dose-response curves for the 6 most active extracts against all cell lines.

Cell lines were exposed to six different Extracts, 5 ($\blacktriangle$), 6 ($\blacktriangle$), 8 ($\blacktriangle$), 21 ($\blacksquare$), 27 ($\blacksquare$), 28 ($\blacksquare$), over a range of concentrations for 48 h before being subjected to the MTT assay. Results are expressed as % of control values and are the means $\pm$ SE of at least 3 separate experiments performed in quadruplicate wells, except for MRC5 cells, which are the data from only one experiment (means $\pm$ SE of quadruplicate wells).

Nevertheless, attempts were made to calculate these data using GraphPad Prism software. Four different models were applied to each extract-cell line combination: the default 4-Parameter (4P) model in which no constraints are set, the Top-fixed model (T) in which the upper limit is a constant equal to 100%, the Bottom-fixed model (B) in which only the 0% level is set and the Top and Bottom-fixed model (TB) in which both the 100% and 0% constraints are set. The optimum method for each extract-cell line pair was chosen by employing a function of the GraphPad Prism programme which enabled two models at once to be directly compared and the preferred model identified. This was done for two models at a time, the non-preferred equation being successively rejected until only one “optimum” method remained. Usually, the optimum method selected by the software coincided with that which any reasoned judgment would adopt based on the highest squared correlation coefficient (r^2 value) and the lowest confidence interval as well as the shape of the curve fitted. All fitted curves are shown in Appendix F.

For simplicity, just the optimum method used for each extract-cell line pair is depicted in Figure 4.9 and the IC_{50} values generated using this optimum method are presented in Table 4.8. Blank spaces mean that the IC_{50} values were incalculable for those particular extract-cell line combinations, regardless of which model was applied. As the x- axis was a logarithmic scale, it was not possible to calculate meaningful SEs. Instead, variation between experiments is reported as a range, expressed as the 95% confidence interval (CI) in $\mu\text{g/mL}$.

Table 4.8 IC₅₀ Values Calculated Using Optimum Model* for Individual Cell Line and Extract Combinations**MDA-MB-468**

Extract Code	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Fraction of MRC5 (%)	Optimum Model	r ²
5 ▲	324.1	225.4 – 466.2	29.9	TB	0.937
6 ▲	301.1	58.8 – 1547		TB	0.553
8 ▲	291.7	230.4 – 369.3	26.8	TB	0.977
21 ■	33.6	21.0 – 53.6	6.6	TB	0.979
27 ■	0.902	0.002 – 422.8	0.1	B	0.995
28 ■	9.56	4.09 – 22.4	3.5	T	0.999

MCF7

Extract Code	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Fraction of MRC5 (%)	Optimum Model	r ²
5 ▲	638.5	573.7 – 710.6	58.9	TB	0.988
6 ▲	903.3	116.7 – 6990		TB	0.428
8 ▲	535.7	504.3 – 568.9	49.3	TB	0.997
21 ■	259.3	154.9 – 434.0	51.2	TB	0.911
27 ■	225.3	179.5 – 282.9	22.4	TB	0.982
28 ■	113.2	58.8 – 217.6	41.7	T	0.992

Caco-2

Extract Code	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Fraction of MRC5 (%)	Optimum Model	r ²
5 ▲	694.7	666.4 – 724.1	64.1	TB	0.998
6 ▲	340.8	162.1 – 716.3		TB	0.849
8 ▲	666.7	608.3 – 730.6	61.3	TB	0.993
21 ■	146.7	65.4 – 329.3	28.9	TB	0.878
27 ■	275.8	168.1 – 452.7	27.4	TB	0.934
28 ■	11.7	3.46 – 39.5	4.3	T	0.992

MM253

Extract Code	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Fraction of MRC5 (%)	Optimum Model	r ²
5 ▲					
6 ▲	272.9	219.8 – 338.9		TB	0.979
8 ▲	585.6	428.6 – 800.2	53.9	TB	0.926
21 ■	304.3	188.7 – 490.5	60.0	TB	0.928
27 ■	425.6	349.3 – 518.5	42.2	B	0.984
28 ■	74.8	57.5 – 97.4	27.6	T	0.983

A549

Extract Code	IC₅₀ (μg/mL)	IC₅₀ Range (95% CI) (μg/mL)	Fraction of MRC5 (%)	Optimum Model	r²
5 ▲	1091	902.7 – 1319	100.6	TB	0.957
6 ▲	189.4	178.3 – 201.2		T	0.999
8 ▲	613.3	544.8 – 690.3	56.4	TB	0.989
21 ■	285.6	132.0 – 617.7	56.3	TB	0.845
27 ■	654.3	542.6 – 789.0	64.9	TB	0.972
28 ■	1667	391.3 – 7106	614.0	TB	0.822

PC3

Extract Code	IC₅₀ (μg/mL)	IC₅₀ Range (95% CI) (μg/mL)	Fraction of MRC5 (%)	Optimum Model	r²
5 ▲	1018	880.8 – 1177	93.9	B	0.970
6 ▲	232.4	192.1 – 281.2		T	0.990
8 ▲	468.4	397.0 – 552.6	43.1	TB	0.988
21 ■	71.0	37.4 – 134.8	14.0	TB	0.925
27 ■	445.3	349.5 – 567.4	44.2	TB	0.976
28 ■	605.2	434.5 – 842.8	222.9	TB	0.977

DU145

Extract Code	IC₅₀ (μg/mL)	IC₅₀ Range (95% CI) (μg/mL)	Fraction of MRC5 (%)	Optimum Model	r²
5 ▲	453.6	349.8 – 588.2	41.8	TB	0.958
6 ▲	148.2	70.6 – 310.9		TB	0.882
8 ▲	480.3	330.9 – 697.2	44.2	TB	0.931
21 ■	75.7	26.8 – 214.0	14.9	TB	0.865
27 ■	294.1	161.9 – 534.1	29.2	TB	0.903
28 ■	19.7	8.14 – 47.8	7.3	T	0.972

MRC5

Extract Code	IC₅₀ (μg/mL)	IC₅₀ Range (95% CI) (μg/mL)	Fraction of MRC5 (%)	Optimum Model	r²
5 ▲	1084	914.2 – 1286	100	B	0.956
6 ▲					
8 ▲	1087	960.9 – 1230	100	TB	0.976
21 ■	506.9	251.6 – 1021	100	TB	0.891
27 ■	1008	605.6 – 1677	100	TB	0.859
28 ■	271.5		100	4P	0.848

*Four different models were applied to each extract-cell line combination: the default 4-Parameter (4P) model in which no constraints are set, the Top-fixed model (T) in which the upper limit is a constant equal to 100%, the Bottom-fixed model (B) in which only the 0% level is set and the Top and Bottom-fixed model (TB) in which both the 100% and 0% constraints are set. Where possible, IC₅₀ values are expressed as a percentage of those of the normal MRC5 cells to aid in scanning for differential toxicity between the cell lines and extracts.

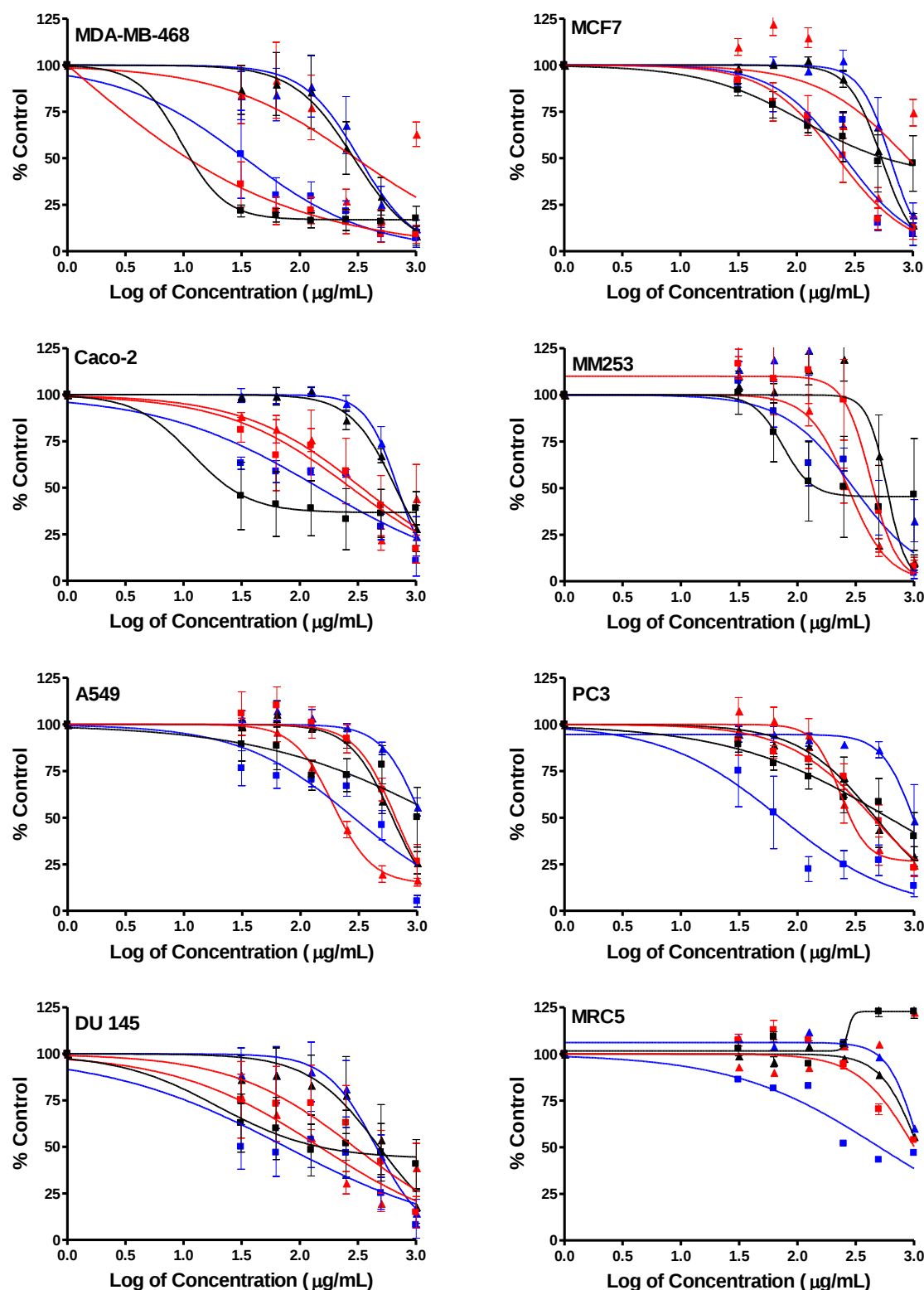


Figure 4.9 Nonlinear regressions of six most active extracts using the optimum model for each extract-cell line pair.

Cell lines were exposed to six different Extracts, 5 (▲), 6 (▲), 8 (▲), 21 (■), 27 (■), 28 (■), over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values (Fig. 4.8) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) according to the optimum method for each extract-cell line combination.

Based on IC_{50} values, it would appear that Extract 27 was the most active of all, inhibiting MDA-MB-468 cell proliferation with an IC_{50} of 0.902 $\mu\text{g/mL}$. However the range of error for this value was very large (95% CI = 0.002 – 422.8 $\mu\text{g/mL}$). Extract 28 was also very active, recording IC_{50} values of 9.56, 11.7 and 19.7 $\mu\text{g/mL}$ in MDA-MB-468, Caco-2 and DU145 cells, respectively. The next most potent extract according to these data was Extract 21, with IC_{50} values below 80 $\mu\text{g/mL}$ in three cancer cell lines. Extract 6 also recorded IC_{50} values of less than 200 $\mu\text{g/mL}$ in two cell lines, A549 and DU145. The highest IC_{50} value recorded for any extract against the non-cancerous MRC5 cells was 271.5 $\mu\text{g/mL}$ for Extract 28. However, the shape of the curve fitted in this case is clearly not appropriate as MRC5 cell proliferation was actually stimulated by higher concentrations of this extract (Fig. 4.9).

It was possible to calculate valid IC_{50} values for most extract- cell line pairs. However, as will be addressed in the Discussion (Section 4.4.3), despite using the optimum method of curve-fitting, IC_{50} values could not be calculated for some extract-cell line pairs (e.g. Extract 5 against MM253 cells). Furthermore, in some cases there was a mismatch between the IC_{50} value and the shape of the dose-response curve. For example, Extract 28 had limited effect on reducing the proliferation of MCF7 cells (Fig. 4.8), barely reducing the proliferation of these cells to less than 50% of the control, and yet it recorded a relatively low IC_{50} value of 113.2 $\mu\text{g/mL}$ (Table 4.8). Additionally, confidence intervals were sometimes very large and correlation coefficients small. For example, the 95% CI for Extract 6 against MDA-MB-468 cells ranged from 58.8 to 1,547 $\mu\text{g/mL}$ and the corresponding correlation coefficient of the curve fit was only 0.553. Other inconsistencies

between dose-response curves and IC_{50} values were also apparent and reasons for and the implications of these discrepancies are discussed in Section 4.4.3.

4.3.4 Morphological changes

Micrographs of selected extract-cell line combinations are presented in Figure 4.10. These micrographs were taken of cells cultured under the same conditions, except for the presence or absence of the extract under scrutiny.

From these selected micrographs it is clear that there were differences between the effects of different extracts on different cell lines. For example, Extract 21 caused MDA-MB-468 cells to round up and lose adherence to the culture surface. Moreover, there were fewer cells in this scenario than in the DMSO control. The effect was even more pronounced in MM253 cells. However, the same extract had very little effect on A549 cells with DMSO- and Extract 21-treated cultures appearing very similar.

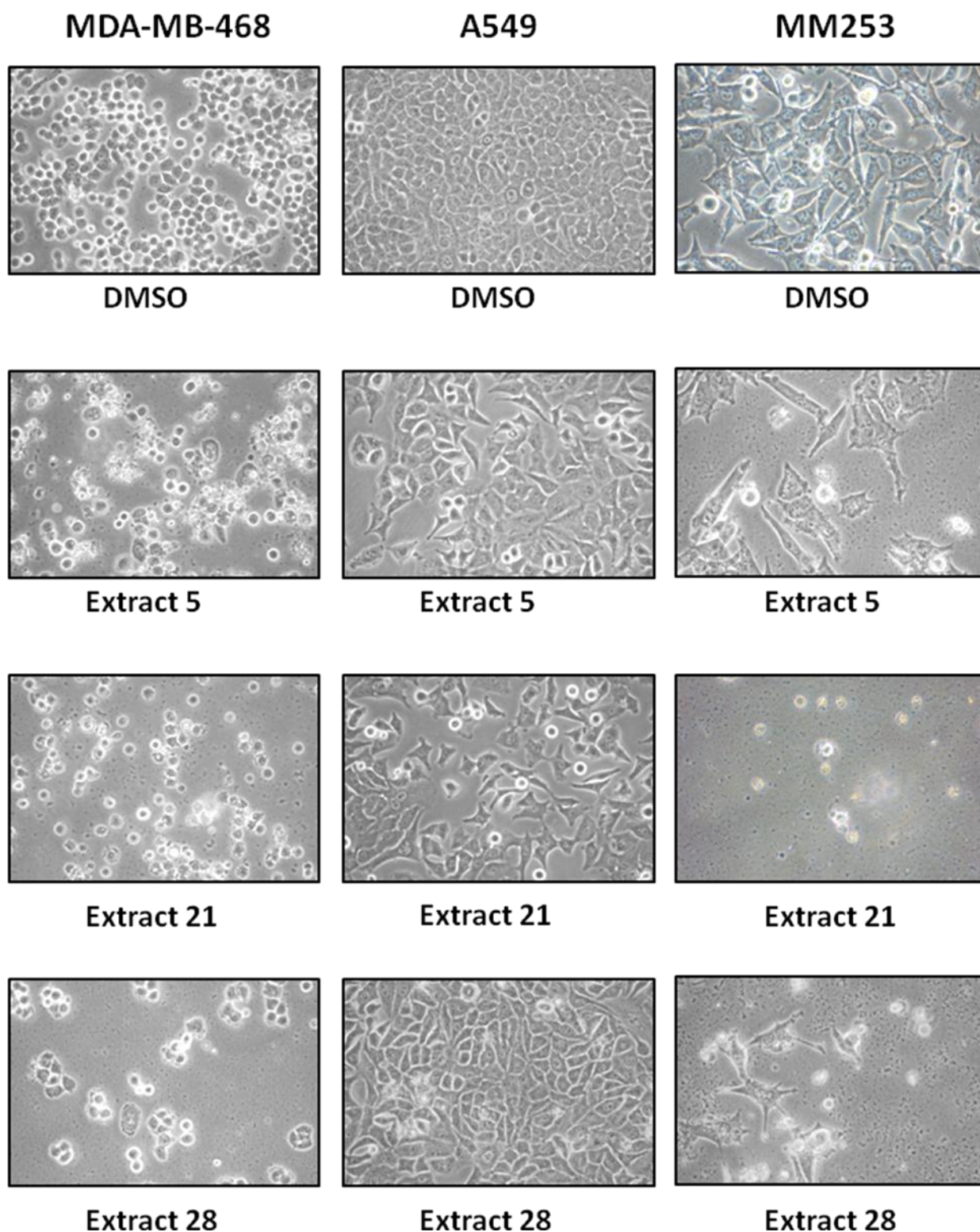


Figure 4.10 Micrographs of selected extract-cell line combinations.

Cells were exposed to 500 $\mu\text{g/mL}$ of selected extracts for 48 h and examined under a phase-contrast light microscope (200X) for confluency and morphological differences to the control (DMSO).

4.3.5 Exposure and recovery

In order to obtain some insight into how the extracts were causing inhibition of cell proliferation, experiments were undertaken in which cancer cells were exposed to the six most active extracts for varying periods of time. Schematics summarising the design of the experiments are provided here to remind the reader of variations in the periods of exposure and recovery between experiments. The schematics can be found below, or on the page opposing, the appropriate figure to optimise space available for the figures themselves.

In the schematics, each point represents 30 min (each block is 24 h) of either growth, exposure to extract, or recovery after the removal of extracts according to the following colour coding scheme.

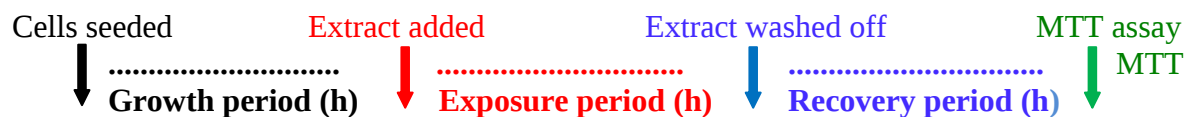


Figure 4.11 is a typical graph depicting what happened when MDA-MB-468 and MCF7 cells were exposed to 1 mg/mL concentrations of Extracts 5, 6, 8, 21, 27 or 28 for various periods. For interest, the effects of the positive controls, DFO and L1, both at 1 mM final concentration are also shown on the graph. Cells were exposed to the extracts or controls for either 2, 24 or 48 h after which time they were immediately assessed by the MTT assay.

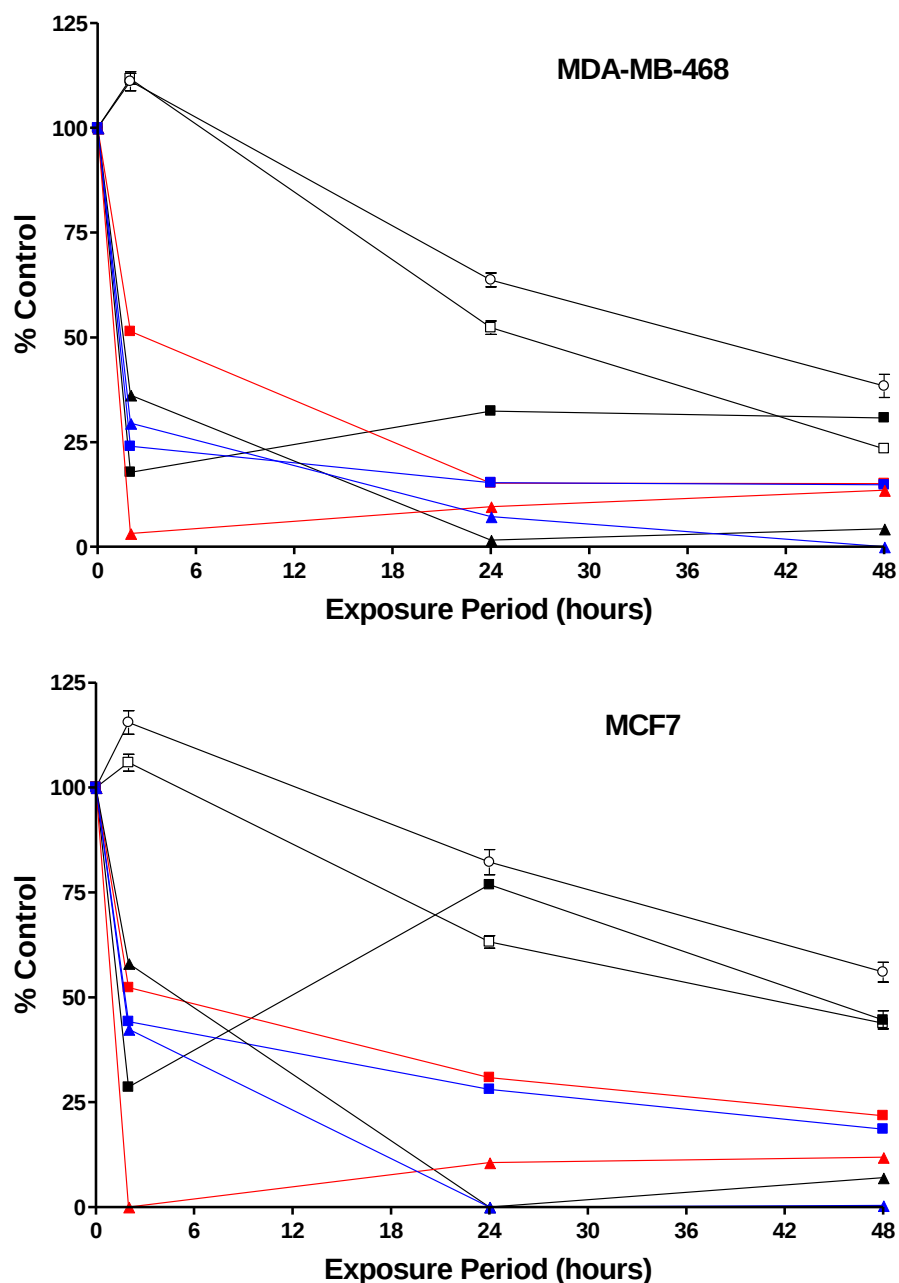
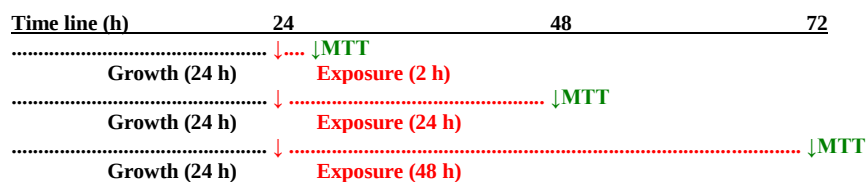


Figure 4.11 Effect of exposure time of most active six extracts on MDA-MB-468 and MCF7 cell proliferation.

Cell lines were exposed to 1 mg/mL of one of six different Extracts, 5 (▲), 6 (▲), 8 (▲), 21 (■), 27 (■) or 28 (■), or 1 mM of controls, DFO (○) or L1 (□), for various periods before being subjected to the MTT assay. Results are expressed as % of control values and are the means $\pm$ SE of a representative experiment performed in triplicate wells.



As can be seen from Figure 4.11, in both cell lines all extracts exerted most of their inhibitory effects within the first 2 h of exposure, most recording >50% inhibition compared to control levels within this time. Extract 8 against MCF7 cells and Extract 27 against both cell lines were only slightly less inhibitory than this, but even these extracts still registered inhibition of >40% within 2 h. This initial marked drop within 2 h indicates that the effects of the extracts were cytotoxic to some cells, as any difference in the number of viable cells present could not be due to proliferation of control cells within such a short period. In contrast, both positive controls, DFO and L1, required longer to elicit a (smaller) response in these cell lines, suggesting their inhibitory actions were via cytostatic mechanisms.

Extract 6 caused the quickest effect of all the extracts and controls in both cell lines, causing >95% inhibition within 2 h in both cases. This indicates that the extract was cytotoxic to at least 95% of the cell population. However, after 24 h exposure to Extract 6, the % of control values had increased in both MDA-MB-468 cells and MCF7 cells, and even more by 48 h, indicating the surviving cell populations were capable of proliferation. This also appeared to be the case for Extract 28, although the levels of inhibition were not as great as with Extract 6. In contrast, longer exposure periods to other extracts caused increased inhibition of cell proliferation, suggesting either cytostaticity of the surviving cells, or cytotoxicity that required longer to manifest.

Therefore, it was of interest to clarify how extracts were affecting the surviving cells. Were the effects of extracts reversible and cells able to return to the cell cycle (see Appendix A.1.1), or did the extracts irreversibly affect the cells such that they left the cell cycle and were either static or dead? To answer this question, cells were exposed to the extracts for various time periods before the extracts were withdrawn and the medium

replaced so the cells may be given a chance to recover. The effects of the six extracts and two positive controls were assessed after nine exposure periods. It is important to note that the subsequent MTT assays were performed at the same time on the same day such that the recovery period differed for each exposure time point. Since the MTT assays were performed just after the 72 h exposure time point, the recovery periods for each exposure time point were effectively 72 h minus the exposure period. For example, cells exposed to an extract for 24 h had 48 h to recover before being assayed and cells exposed for 4 h had an additional 20 h (=68 h) to recover before they were assayed. The results of a representative experiment in which the reversibility of the inhibitory effects of the six most active extracts on MDA-MB-468 and MCF7 cells were assessed are depicted in Figure 4.12. Similar results were obtained from other experiments, but because the ranges of exposure periods used were not identical, mean values could not be calculated.

The results depicted in Figure 4.12 echo those of Figure 4.11 in that the inhibitory effects of all the extracts were relatively rapid, indicating cytotoxic effects. Within just 30 min, two extracts, 5 and 21, had caused more than 90% inhibition of both MDA-MB-468 and MCF7 cell proliferation, and within only 1 h all extracts had caused >40% inhibition of MDA-MB-468 cell proliferation, with Extract 27 also effecting marked inhibition (>85%) of MCF7 cell growth. By 2.5 h all extracts had caused more than 40% inhibition of proliferation of both cell lines, and by 6 h all extracts had caused greater than 60% inhibition. This is a different result than what is depicted in Figure 4.11 in which Extract 6 caused almost total inhibition of both MDA-MB-468 and MCF7 cell growth within just 2 h. However, in the second experiment, the cells were given an opportunity to recover as the MTT assay was not performed until nearly three days later (for the shortest exposure periods). While the magnitude of the inhibitory effects of Extract 6 may not have been as large (as in Fig. 4.11), the pattern was still similar in that the inhibitory effects of this

extract were rapid, with >50% inhibition achieved within the first 30 min in MDA-MB-468 cells and within 4 h in MCF7 cells.

Figure 4.12 conveys that the inhibitory effects of at least three of the extracts, 5, 21 and 27, were irreversible within 24 h of exposure. That is, even after the extracts were removed from the cells, proliferation, as measured by the MTT assay, did not increase after exposure to these extracts. If the treated cells had been able to recover, it would be expected that the % control values of cells would be higher for short exposure periods than longer exposure periods. For example, cells exposed to extracts for less than 6 h would have at least 66 h in which they could proliferate, if they were capable of doing so, and so should have higher % control values than cells given less time to recover. This appears to be the case for Extracts 6, 8 and 28. However, as lower % control values could simply be a function of extract-induced cytostaticity when compared to control cells that were able to proliferate normally over the same period, it was helpful to compare the results of Figure 4.12 with those of Figure 4.11.

Experimental design (Fig. 4.12):

Time line (h)	24	48	72	96
.....	↓ ↓			↓ MTT
Growth (24 h)	Exposure (0.5 h)			Recovery (71.5 h)
.....	↓ ↓			↓ MTT
Growth (24 h)	Exposure (1 h)			Recovery (71 h)
.....	↓ ↓			↓ MTT
Growth (24 h)	Exposure (2.5 h)			Recovery (69.5 h)
.....	↓ ↓			↓ MTT
Growth (24 h)	Exposure (4 h)			Recovery (68 h)
.....	↓ ↓			↓ MTT
Growth (24 h)	Exposure (6 h)			Recovery (66 h)
.....	↓ ↓	↓		↓ MTT
Growth (24 h)	Exposure (24 h)			Recovery (48 h)
.....	↓ ↓		↓	↓ MTT
Growth (24 h)	Exposure (48 h)			Recovery (24 h)
.....	↓ ↓			↓ ↓ MTT
Growth (24 h)	Exposure (72 h)			No recovery

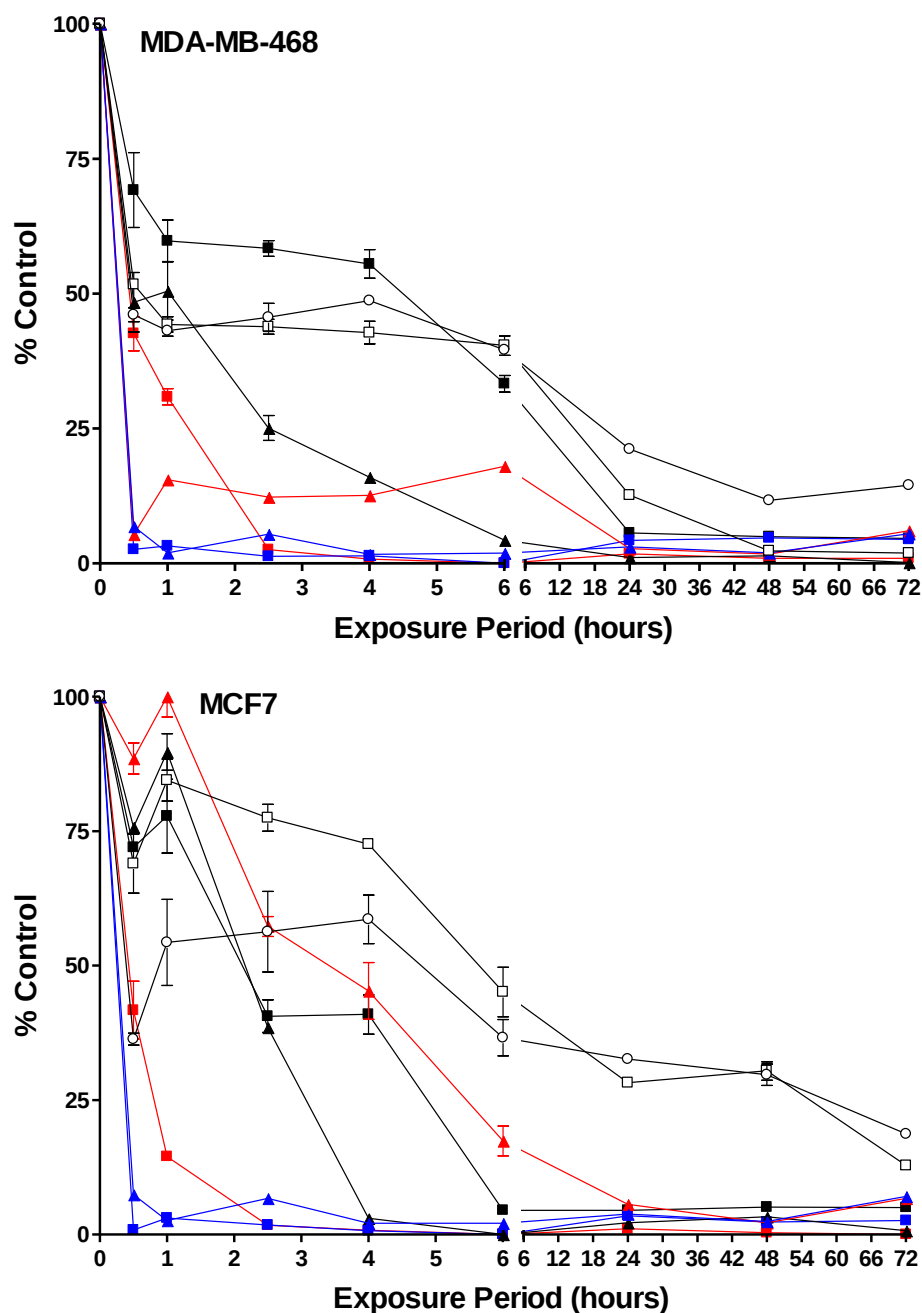


Figure 4.12 Reversibility of inhibitory effects of the six most active extracts on MDA-MB-468 and MCF7 cell proliferation.

Cell lines were exposed to 1 mg/mL of one of six different Extracts, 5 (▲), 6 (▲), 8 (▲), 21 (■), 27 (■) or 28 (■), or 1 mM of controls, DFO (○) or L1 (□), for the various periods indicated. The extracts were then washed off, the medium replaced and the cells allowed to recover before being subjected to the MTT assay at the 72 h time point. Results are expressed as % of control values and are the means $\pm$ SE of triplicate wells of a representative experiment.

To further ascertain if the inhibitory effects of Extracts 6, 8 and 28 were reversible or just required longer to manifest, % control values were compared to the corresponding time points in the experiment presented in Figure 4.11. For Extracts 6 and 28 (but not 8), the % control in both MDA-MB-468 and MCF7 cells after 2 h was lower when the MTT assay was performed immediately after the exposure period ended (Fig. 4.11) than when the cells were allowed to recover (Fig. 4.12). This suggests the inhibitory effects of Extracts 6 and 28 were reversible, while those of Extract 8 were irreversible.

Conversely, fewer viable cells present for a given time point in Figure 4.12 compared to Figure 4.11 would have been suggestive of cell death (cytotoxicity), or at least complete growth arrest (cytostaticity) of surviving cells, within the recovery period. Extracts 5 and 21 irreversibly arrested the growth of both cell lines within 30 min, while Extract 27 required 1 h exposure. However, it is not possible to say whether the lower % control values in Figure 4.12 were because surviving cells had died during the recovery period, or simply cell-cycle arrested. Nevertheless, after at least 6 h exposure to these extracts, cells did not recover even when the recovery period was extended to almost six days (data not shown).

4.4 DISCUSSION

4.4.1 Physical properties

While many physical properties of the extracts could possibly influence their effects on cells, just three were examined as part of this study. Osmolality and pH were chosen as they were simple to measure and yet of fundamental physiological significance. Extract colour was significant as cytotoxicity was assessed primarily via a colourimetric method.

4.4.1.1 Osmolality

According to refractive indices (SG measurements), none of the extracts were particularly hypertonic. However, it must be remembered that while there is generally a very good correlation between SG and osmolality, SG is only an estimate of osmolality (Voinescu *et al.*, 2002). This is because SG is the ratio of a sample's density to water and as such it depends on both the number and mass of the solute particles, whereas osmolality is a measure of the number of solute particles only (Pradella *et al.*, 1988; Dorizzi & Caputo, 2005).

Given that the refractive index of Extract 28 (and others) was no different to that of the control (1% DMSO) and cells exposed to this concentration of DMSO were viable and readily proliferated (see Figs. 3.11 and 3.12 and 6.8), this finding supports the inferences made, based on measured refractive indices, that the extracts did not have particularly high osmolalities. Therefore, it was concluded that, except for the possible exception of Extract 21 (which had a slightly higher refractive index than the control), high osmolality was not the reason behind the extracts' cytotoxic properties.

4.4.1.2 pH

The observed cytotoxicity of the extracts was also not due to excessive acidity or alkalinity as the pH values of the most effective extracts were similar to that of the negative control, 1% DMSO.

4.4.1.3 Colour

The fact that the extracts all had some colour is significant as this could potentially interfere with the results of the MTT assay, which is, of course, spectrophotometrically based. From Table 4.7 it is obvious that all extracts tested, but 6 and 21 in particular, absorbed at 570 and 595 nm, the same wavelength range as MTT-formazan. While wells containing extract and medium only were used as controls for such background

absorbance, very high values still caused complications for the MTT assay. This problem was the driving force behind changing to the modified assay of Fox *et al.* (2005) in latter experiments.

4.4.2 Initial screening

The constraints of this project meant it was not logistically possible to further evaluate all 25 samples for their anticancer potential. Hence, the preliminary screening data were used to narrow down those extracts to those that showed the greatest potential as anticancer agents. The theory was that if an extract did not display much bioactivity at the highest concentration tested (1 mg/mL), it would show less activity at a lower dose. This is the same principle behind the NCI's initial screening studies in which compounds are tested at a single high concentration against the all cell lines in the NCI-60 panel and only compounds meeting pre-determined threshold inhibition criteria in a minimum number of cell lines progress to the full five-concentration assay (Andreani *et al.*, 2010). While it is acknowledged that this well-established theory does have its flaws, at the time this seemed to be the most logical method of discriminating between the different extracts. The weaknesses of this approach, such as possible interactive effects between individual components of mixtures causing lower doses to display higher bioactivities than higher doses, will be discussed further in Sections 5 and 8.

Nevertheless, the initial screening data established that some extracts were more potent than others at 1 mg/mL (Figs 4.1 – 4.3). They also hinted at a degree of cell line selectivity. Some extracts were even more inhibitory than the positive control (DFO) in one or more cell lines. The fact that no extract was significantly more inhibitory than DFO in the MDA-MB-468 cell line could probably be attributed to the fact that DFO itself was very inhibitory in this line, rather than any lack of sensitivity to the various extracts.

From these screening data, the 16 extracts showing the greatest inhibition of cancer cell proliferation at this high dose, as assessed by the MTT assay, were chosen for preliminary dose-response evaluation. Unfortunately, it was not possible to further evaluate one of the most active extracts, Extract 3, due to insufficient material being available.

4.4.3 Dose-response

The 16 extracts exhibiting the greatest bioactivity in the initial screen were subjected to preliminary dose-response studies (Figs 4.5 – 4.7). The most promising extracts were chosen from this group based on the calculated IC_{50} values and the general shapes of the fitted curves (4P model only). From these results, it was concluded that the six most promising extracts were 5, 6, 8, 21, 27 and 28. The others were eliminated from the pool for further study.

The six most promising extracts were subjected to more intensive dose-response studies, using a panel of eight cell lines in total, including one non-cancerous line, MRC5, in order to evaluate selectivity for cancer cells. It is clear from the dose-response curves in Figure 4.8 that the extracts did, indeed, have differential effects on different cell lines. For example, Extract 28 markedly inhibited MDA-MB-468 cell proliferation compared to the control. However, the same extract had much less effect on A549 cells. Similar low activity was demonstrated in MCF7, MM253 and PC3 cells exposed to Extract 28, while Caco-2 cells and DU145 cells displayed intermediary responses. In MRC5 cells, Extract 28 caused no inhibition whatsoever. If anything, it stimulated proliferation of these cells. Other similarities and differences can be easily discerned between the dose-response curves of different extracts and between cell lines, indicating selectivity.

This selectivity is confirmed by the range of different IC_{50} values obtained for different cell lines exposed to the same extract (Table 4.8). In toxicology, IC_{50} values are a

generally accepted way of directly comparing dose-responses of various substances.

Unfortunately, despite using several models to calculate IC_{50} values, it was not possible to determine these values for all extract-cell line combinations (i.e. Extract 5 on MM253 cells and Extract 6 on MRC5 cells). Moreover, when IC_{50} values were determined, they were not always reliable measures of an extract's effectiveness. For some extract-cell line combinations the 95% confidence interval was extremely large or incalculable and the correlation coefficient was small (e.g. Extract 6 on MDA-MB-468 and MCF7 cells). In other cases there was a mismatch between the IC_{50} value and the shape of the dose-response curve (e.g. Extract 28 on MCF7 cells).

But why was there such a discrepancy between what is evident from the dose-response curves and what was determined mathematically? In some cases the answer lies in the fact that the curves do not reach the 50% inhibition level over the concentration range tested. This was true for Extract 5, for example, where even the highest concentration tested, 1,000 $\mu\text{g/mL}$, only inhibited proliferation of A549 cells to 55.8% of the control level. The only way to overcome this problem would have been to perform repeat experiments using a higher range of concentrations of extract. However, a limiting factor of the concentrations chosen was the solubility of the extracts. By dissolving the extracts in DMSO at 100 mg/mL , the highest achievable final concentration of extracts in the cultures was 1,000 $\mu\text{g/mL}$. This corresponded to a final DMSO concentration of 1% (v/v). As discussed in Chapter 3, DMSO levels above this concentration were inhibitory on cell proliferation themselves and would have resulted in skewed IC_{50} values anyway. Besides, as the point of the study was to identify extracts with high potency, using concentrations of extract above the screening concentration was considered something of limited interest to be done only if time permitted.

In other instances the sigmoidal dose-response (variable slope) nonlinear regression model was probably not a suitable model in the first place. This would be true in the case of Extract 6 on MDA-MB-468 (and MCF7, Caco-2 and DU145) cells where the curve obviously “kicks up” at 1,000 $\mu\text{g/mL}$. Removing this high concentration data point results in a lower IC_{50} value with improved CI and r^2 values. However, it was believed that in order to fairly compare the effects of each individual extract on the different cell lines, honesty in data reporting was fundamental. Therefore, the same general dose-response (variable slope) model was applied to each extract-cell line pair; only the equations used to define the top and bottom parameters were altered as described.

As discussed above, the dose-response curves of some extract- cell line pairs (e.g. Extract 28 against DU145 cells) were flatter than a classical sigmoidal dose-response model would predict. Conversely, for other extract-cell line combinations, the dose-response curve was steeper than predicted. For example, just 31.2 $\mu\text{g/mL}$ of Extract 28 caused more than 75% inhibition of proliferation of MDA-MB-468 cells followed by a flattening of the curve. As this was the lowest concentration tested, the dose-response curve dropped suddenly from 100% at 0 $\mu\text{g/mL}$ to 21.7% at 31.2 $\mu\text{g/mL}$. Similarly, 250 $\mu\text{g/mL}$ of Extract 27 did not cause significant inhibition of MM253 cells, while 500 $\mu\text{g/mL}$, the next concentration tested, and only twice as much, was enough to cause over 60% inhibition of proliferation of these cells. Usually, this order of change is achieved over a much greater concentration range, with classical single-site inhibitor increases from 10% to 90% inhibition recorded over an 80-fold concentration range (Shoichet, 2006). Steep dose-response curves are very prevalent in early screening studies but the underlying physical events responsible are poorly understood and beyond the scope of this discussion (Shoichet, 2006).

While both particularly flat and particularly steep dose-response curves both pose problems for fitting sigmoidal curves, the especially steep scenario is more likely to be misinterpreted. In screening studies, extracts causing flat dose-response curves are generally ignored and eliminated from further investigation. On the other hand, extracts showing any marked bioactivity are tagged as interesting, warranting further study. While a steep change in response may be due to extreme potency, the high proportion of this occurrence in screening studies would suggest otherwise (Shoichet, 2006). In fact, one common indication of an artifactual hit in screening is a steep dose-response curve, often signalled by a high Hill slope coefficient (Shoichet, 2006). The general interpretation is that high Hill slopes indicate more inhibition of a target is being observed than would be expected if the compound had a simple binding mechanism. Practically, high Hill slopes often portend compounds less likely to be easily optimised and, therefore, pharmaceutical companies (e.g. Vertex) use this parameter to eliminate compounds from further consideration (Walters & Namchuk, 2003). If extracts in the current study had shown universally steep dose-response curves against all cell lines tested, they would have been similarly eliminated from the pool for further evaluation.

While Hill coefficients are a valid means of measuring the steepness of a curve, the values calculated for the dose-response curves featured in Figure 4.9 were not reported in Table 4.8. This is because Hill coefficients are dependent on the parameters used in a curve fit and, therefore, there is a danger the values could be over interpreted (Shoichet, 2006). However, regardless of whether the steep dose-responses observed in these studies are due to artifactual inhibition or are a function of very potent inhibitory properties of the relevant extracts, the fact remains that the cells' sensitivities to small changes in extract concentrations presented difficulties in accurate curve fitting and therefore the generation of accurate IC_{50} values.

Nevertheless, while the calculated IC_{50} values are possibly not accurate, there can be little argument that they are of some use in providing a way of directly comparing the dose-responses of different extract-cell line combinations. Irrespective of their exact values, what can be undeniably inferred from the calculated IC_{50} values is that some of the plant extracts exhibited differential bioactivity against different cancer cell lines. Additionally, the cell lines displayed heterogeneity of cell sensitivities to the various extracts which was not a simple function of population doubling time.

4.4.4 Morphological changes

The dose-response data quantitatively reveal the differential bioactive effects of extracts on the various cell lines. However, these differences were also readily apparent by eye. From the sample micrographs presented (Fig. 4.10), it is quite evident that some cell lines are more susceptible to certain extracts than others. For example, Extract 5 clearly caused MDA-MB-468 and MM253 cells to round up and lift off the culture dish whilst having little effect on A549 cells. Similarly, MDA-MB-468 cells appeared necrotic after exposure to Extract 21, as evidenced by their shrivelled appearance, as did MM253 cells to an even greater extent. However, A549 cells exposed to the same extract appeared no different to the control (DMSO only). The effects of Extract 28 on MDA-MB-468 cells were more pronounced than either of the other two extracts featured, whereas it was Extract 21 that caused the greatest change to the number and morphology of MM253 cells.

These observations highlight the specificity of some extracts for various cell lines. They can also offer valuable insights, but ultimately only clues, as to their modes of action. For example, it may be inferred from the above micrograph that there were fewer MDA-MB-468 cells present after they were exposed to Extract 28, for example, because this extract was causing the cells to die, either by apoptosis or necrosis, and lift off the culture dish.

However, it is more likely that the observed differences in cell number were due to this extract inducing inhibition or retardation of cell proliferation (cytostaticity) as these treated cells appeared bright and rounded (implying viability) and were also noticeably smaller (suggesting G₀ arrest). The case for cell death is more compelling for MM253 cells exposed to Extract 21, the amount of extracellular debris and distorted shapes of the cells suggesting they were necrotic and the reduced number is because the rest, as they died, had become non-adherent and been washed away or simply shrivelled up to virtually nothing. Shrinkage was unlikely to have been due to osmotic effects as the extracts were not hypertonic (see Section 4.3.1.1). Neither were the observed effects on cells related to alkalinity or acidity as all extracts were pH neutral (Section 4.3.1.2). The specificity of the extracts for various cell lines confirms that the effects were not due to generalised cytotoxicity caused by non-physiological pH or osmolalities.

However, in order to make judgments regarding necrosis, corroborating evidence from further experiments is necessary. Other ways to detect necrosis are discussed later in Appendix O. However, a major disadvantage of many of these methods of determining cell viability by measuring losses in membrane integrity is that, while they are commonly used as markers for necrosis, they cannot distinguish between primary necrosis resulting from a physical or chemical insult to the cell and secondary necrosis that eventually follows apoptosis of cells *in vitro* (Riss *et al.*, 2006; Krysko *et al.*, 2008). Similarly, in the current study, it was not possible to discern microscopically whether the perceived necrosis was primary or secondary as cells were viewed only at a single, late-stage time point when even cells that died via an apoptotic pathway would have displayed many of the morphological features of primary necrosis (Krysko *et al.*, 2008; see Section 8.2.4.4).

4.4.5 Exposure and recovery

Although the MTT assay is often described as a cytotoxicity assay (Sgouras & Duncan, 1990; Fotakis & Timbrell, 2006; Bopp & Lettieri, 2008), all it can really do is provide information about whether there are differences between the numbers of metabolically active cells present in treated and untreated cultures. If there are differences in the numbers of viable cells present, the MTT assay does not allow inferences to be made regarding whether there are fewer cells because the treatment has arrested proliferation or growth of cells, or whether the treatment has actually killed the cells (Plumb, 1999).

Thus, to gain more insight into the modes of action of the extracts, time-course experiments were performed in which cancer cells were exposed to the most active extracts for different durations. In one experiment, the extracts were removed and the cells cultured for longer periods in order to distinguish between cells that were viable and able to proliferate and those that remained viable but were incapable of proliferation. The results of this experiment, depicted in Figure 4.12, suggested that the inhibitory effects of the extracts were generally irreversible within the time periods tested. Even after recovery periods of up to six days (data not shown), the numbers of viable cells did not increase at all compared to control cultures, suggesting, therefore, that the actions of the extracts were cytotoxic or irreversibly cytostatic.

It was also clear from these experiments that the inhibitory effects of all the extracts were very rapid. Where cytotoxicity was evident, the relative speed of the response implied an apoptotic mechanism, as opposed to necrosis which is generally a longer process (Kerr *et al.*, 1972; Gavrieli *et al.*, 1992; Al-Lamki *et al.*, 1998). From Figure 4.11, it appears that Extract 6 exhibited the quickest response of all the extracts tested, with >95% of both

MDA-MB-468 and MCF7 cells dying within just 2 h. However, after 24 h exposure to this extract, the number of cells in both cell lines had increased and by 48 h exposure they were even higher. These increases in cell numbers suggest that, in contrast to the effects of some other extracts, the inhibitory effects of Extract 6 were only short-term as surviving cells were capable of proliferation. Similarly, Extract 28 registered a higher number of viable cells present after 24 and 48 h compared to 2 h exposure. With Extract 28 there was a primary increase in cell numbers between 2 and 24 h and a subsequent decrease at the 48 h time point, suggesting a biphasic response of initial cytostaticity followed by secondary cytotoxicity. This biphasic pattern in which cytostatic effects become cytotoxic after prolonged exposure (and/or at higher concentrations) is frequently observed in studies of inhibition of cell proliferation (Krischel *et al.*, 1998; Lori *et al.*, 2005; Obajimi *et al.*, 2009; Pappa *et al.*, 2007). All other extracts tested, 5, 8, 21 and 27, and positive controls inhibited cell proliferation of MDA-MB-468 and MCF7 cells in a time-dependent manner, with Extract 5 being the most inhibitory after both 24 and 48 h exposure.

The results depicted in Figure 4.12 indicate that Extracts 5 and 21 elicited the fastest inhibitory responses of all the extracts and controls tested, recording greater than 90% inhibition of both MDA-MB-468 and MCF7 cell proliferation after just 30 min exposure. While this is not the same result as that shown in Figure 4.11, where the actions of Extract 6 were the most rapid, the general data presented in both figures are similar in that they reflect the overall speed of the inhibitory effects of the extracts. Differences in the magnitudes of the responses of the individual extracts in both figures can be attributed to several factors. Firstly, in the second experiment (Fig. 4.12), cells were given an opportunity to recover and proliferate, before being assayed. Hence, a lower % control value in the first experiment (Fig. 4.11) than for the corresponding time point in the

second experiment (Fig. 4.12), suggested its inhibitory effects were reversible. On the other hand, lower cell numbers in the second experiment were indicative of additional cell death (cytotoxicity) or irreversible cytostaticity.

A second reason for observed discrepancies between the data depicted in Figures 4.11 and 4.12 is to do with the fact that the extracts were present in the wells when the MTT assay was performed in the first experiment, but not in the second. While colour interference was controlled for by subtracting background absorbance values, it is possible that the extracts themselves may have interfered with MTT reduction (see Section 8.2.3.2). This is the main reason why the MTT assay was overhauled to remove extracts before the addition of MTT (see Chapter 3).

An alternative explanation for higher cell numbers after longer exposure periods is that the half-life of the extracts (or at least their active constituents) in solution may be short. Perhaps the extracts were simply degraded within this period. Alternatively, maybe the cells were able to metabolise the active compounds. Regardless, the possibility that the extracts were somehow eliminated or otherwise unavailable within the experimental time frame cannot be excluded and could explain some cell recovery. Certainly, many anticancer agents, including important chemotherapeutics like paclitaxel, vinblastine and fluorouracil, have short biological half-lives and yet are still clinically very useful (Galijatovic *et al.*, 1999; Shalaby, 2004; Yang *et al.*, 2007). Moreover, drug delivery techniques exist (and more are being developed) that allow the controlled, slow and sustained release of drugs over prolonged periods. These systems include coating drugs with various biomaterials, incorporating them into biodegradable copolymer films, encapsulating them inside hydrogels, nanoparticles or nanotubes, or using nanofibre scaffolds (Farb *et al.*, 2001; Haider *et al.*, 2004; Nagai *et al.*, 2006; Cheng & Lim, 2009;

Hilder & Hill, 2009; Numata & Kaplan, 2010). Therefore, while it was desirable that the inhibitory effects were cytotoxic, extracts that exerted potent effects via reversible, cytostatic mechanisms were also of interest.

In conclusion, from these screening studies, the four most promising extracts, in terms of their potency and selective cytotoxicity or cytostaticity against cancer cells, were 5, 6, 21 and 28. These were obtained from *Eremophila sturtii*, *Euphorbia drummondii*, *Eremophila duttonii* and *Acacia tetragonophylla*, respectively. This is in support of other research in which four of the same *Eremophila* species tested in the current study were screened for bioactivity against three common cancer cell lines (including DU145) and normal fibroblasts and all, but *E. duttonii* and *E. sturtii* in particular, were found to display substantial and differential cytotoxic activities (Mijajlovic *et al.*, 2006).

4.5 SUMMARY

In summary, a total of 25 plants from two distinct areas were chosen for evaluation of their anticancer potential based on traditional Indigenous medical knowledge of those species. Relevant plant parts were harvested and crude methanolic extracts derived. These plant extracts were assessed for their bioactivity against four different human cancer cell lines using the MTT assay to measure changes in cell proliferation. Preliminary screening narrowed the field to a pool of 16 promising extracts which were assessed for their dose-responses against the same four cell lines. Based on these data, six extracts were chosen for more thorough assessment of their effects, including morphological changes, the kinetics of their actions and their dose-response relationships against a panel of several more cancer cell lines and one non-cancerous cell line. These six extracts exhibited differences between each other and between cell lines, indicating a degree of selectivity. The most promising four of these methanolic Extracts, 5, 6, 21 and 28 were chosen for further evaluation, the results of which constitute Chapter 5.

Chapter 5: Evaluation of Most Promising Plant Extracts

5.1 INTRODUCTION

5.1.1 Sample collection and extract preparation

Of the original 25 plant samples collected, four (5, 6, 21 and 28) were chosen for further evaluation, based on the bioactivity of their methanolic extracts, as assessed by the MTT assay (see Chapter 4). However, as these methanol samples were aging (over 2 years old) and some were almost depleted, it was necessary to collect fresh samples of the relevant plants in order to perform chemical analysis. For two of the species, two samples from different locales were collected, giving six plant samples altogether. To ensure the recovery of more active constituents, these six plant samples were extracted not only with methanol, but with water or ethyl acetate too. This extraction procedure resulted in three fractions for each sample and a total of 18 extracts.

5.1.2 Chemical analysis of extracts

The 18 extracts were analysed by High Performance Liquid Chromatography (HPLC) coupled to UV detection in order to identify the chemical components and provide clues as to those likely to be responsible for any cytotoxic effects. Additionally, volatiles of the four plant species were analysed by Gas Chromatography-Mass Spectrometry (GC-MS).

5.1.3 Extract bioactivity

The 18 extracts were subjected to the MTT assay as well as a lethality assay using brine shrimp. The results of these tests highlighted several extracts worthy of further study as potential anticancer agents.

5.2 METHODOLOGY

5.2.1 Sample collection and extract preparation

The collection, harvesting and transport of fresh plant samples was carried out as described in Section 2.4.1, except that the quarantine facility was at the Chemistry Centre of Western Australia (Hay Street, East Perth) and this was also where the extractions were performed.

Plant parts were ground up thoroughly into a powder using a café grinder and extracted into ethyl acetate (EA), methanol (M) and aqueous (A) fractions. The extraction procedure is summarised in the flowchart presented as Figure 5.1.

Extracted samples were given further identifier codes such that each sample (1 to 6) was classified accordingly. For example, the ethyl acetate extract of Sample 3 was called EA3 and the aqueous extract of Sample 6 was A6.

Extracts were prepared and chemically analysed by a collaborator, Dr Shao Fang Wang (ChemCentre, WA). HPLC-UV for chemical profile analysis was performed on all 18 extracts. GC-MS analyses for volatiles were performed only on the essential oils of samples with strong aromas.

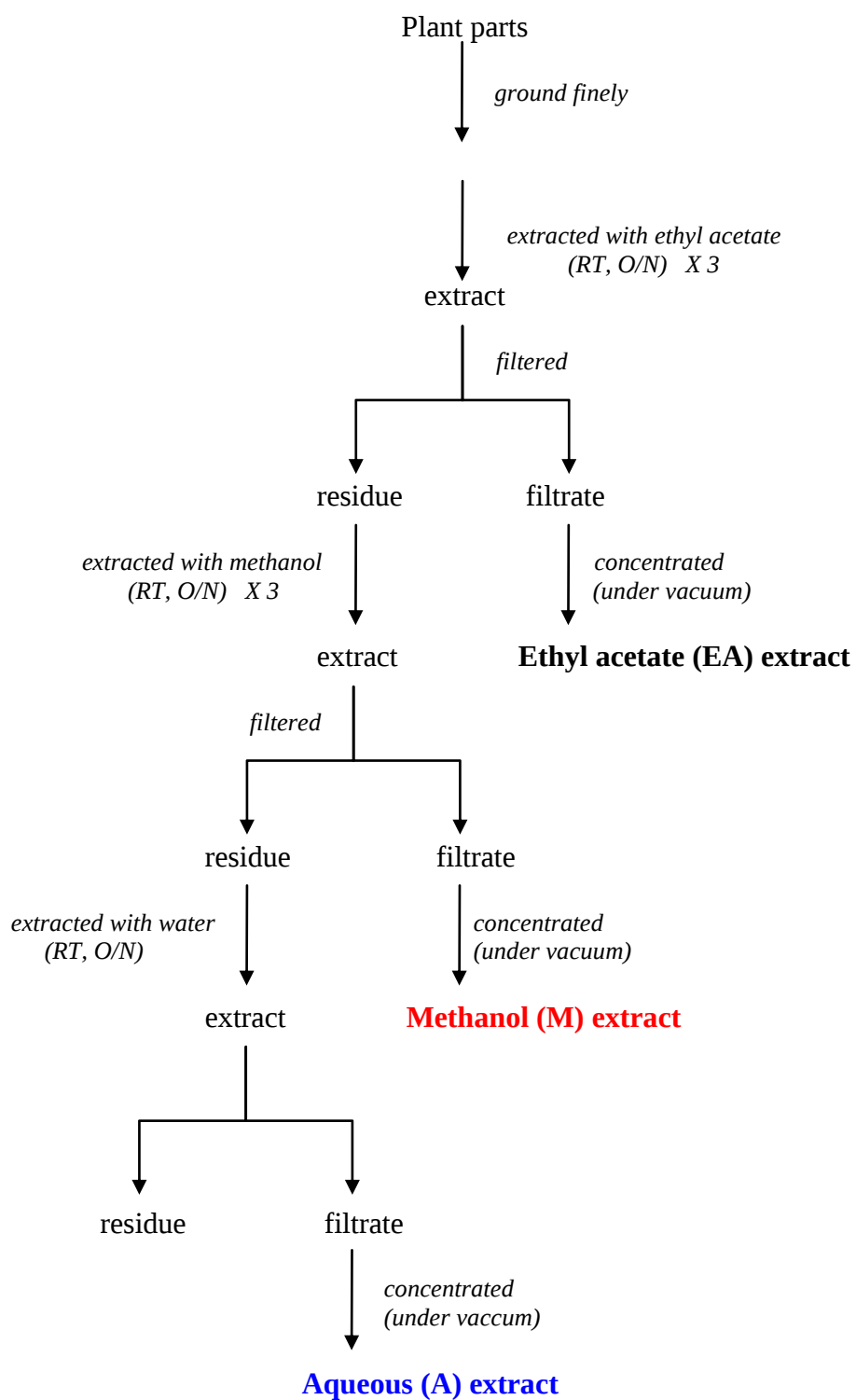


Figure 5.1 Flowchart showing steps in preparation of extracts.

5.2.2 Chemical analysis of extracts

5.2.2.1 HPLC-UV

HPLC-UV for chemical profile analysis and identification of compounds was performed on an Alliance Waters 2695 HPLC separation module pump with a Waters 2996 Photodiode Array Detector (UV detector). Empower software was used for the HPLC instrument. The chromatography was performed using an Apollo C18, 5 μ m 250 x 4.6 mm column (Alltech) at 30°C. The analyte was eluted by a gradient mobile phase system consisting of solvent A (acetonitrile) and solvent B (0.6% H₃PO₄ in water). As an example, the solvent gradient used for HPLC-UV analysis of the ethyl acetate fraction of Sample 3 (EA3) is presented (Table 5.1).

Table 5.1 Solvent Gradient for HPLC-UV Analysis of EA3

Time (min)	Acetonitrile	Water:H ₃ PO ₄
initial	10	90
1.00	10	90
25:00	25	75
40:00	90	10
45:00	90	10
46:00	10	90
55:00	10	90

UV absorption spectra at wavelengths of 254 nm, 280 nm and 350 nm allowed for optimal detection of all phenolic compounds, simple phenolics and flavonoids, respectively.

5.2.2.2 GC-MS

For identification of essential oils by GC-MS analysis, samples were first extracted with a solvent mixture (hexane: ether 1:1) and filtered. The organic solution was then concentrated under N₂ and hexane was added to dissolve the residue. Volatiles were identified based on comparison to standard compounds in the GC-MS library.

5.2.3 Extract bioactivity

All extracts were dissolved at 100 mg/mL in DMSO on the day of use, diluted and filtered, as described previously (section 4.2.2).

5.2.3.1 *MTT assay*

The MTT assay was again employed to screen for bioactivity. This procedure was identical to the alternative method of Fox *et al.* (2005) as outlined in section 2.7.1.1. Importantly, extracts were removed before starting the assay. As the four samples had already been selected on the basis of their potency, all extracts were initially screened against cancer cells at a lower concentration (250 µg/mL) than in the primary screen. The most active eight were chosen for dose-response studies at concentrations ranging from 25 to 500 µg/mL.

5.2.3.2 *Brine shrimp lethality assay (BSLA)*

The brine shrimp lethality assay (BSLA) described in section 2.6.3 was used to assess general toxicity of the eight most bioactive extracts. Briefly, nauplii in artificial sea water (ASW) in 96-well plates were exposed to various concentrations of extracts and scored for immobility, equating to death, over time. The effects of eight extracts were tested at a higher range of concentrations, 125 to 1,000 µg/mL at three time points (2, 24 and 48 h) and compared to control wells containing just the vehicle (DMSO) at the same final concentration. Total numbers of brine shrimp in each well were determined after sacrifice with methanol (~ 15% v/v final concentration). Extracts were filtered before use through a 2 µm membrane in order to reduce debris that interfered with visibility and therefore accurate counting of total nauplii. Due to the labour intensiveness of transferring swimming nauplii to wells, each experiment was performed with duplicates only, although experiments were repeated up to five times. Other controls used were deferoxamine mesylate (DFO) and camptothecin (CPT) at 1 mM and 0.1 mM final concentrations, respectively.

5.3 RESULTS

5.3.1 Sample collection and extract preparation

Samples labelled 1 to 6 were collected from sites near the township of Titjikala as indicated in Table 5.2. As can be seen, Samples 5 and 6 were from the same species, *Acacia tetragonophylla*, collected from different trees located approximately 40 km apart. Similarly, Samples 3 and 4 were from different specimens of the same plant, *Eremophila duttonii*, approximately 12 km distant from each other. As these samples were fresh collections of those displaying bioactivity in the initial screen (see Chapter 4), the original identifier codes are also presented in parentheses for reference.

Table 5.2 Plant Sample Collection Details

Sample Code (= original code)	Species	Details
1 (=6)	<i>Euphorbia drummondii</i>	14 plants collected at Titjikala town site at top of sand hill near water tank
2 (=5)	<i>Eremophila sturtii</i>	10 plants collected at Titjikala rubbish tip site
3 (=19 and 21)	<i>Eremophila duttonii</i>	Collected 12 km from Titjikala down Chambers Pillar Rd
4 (=19 and 21)	<i>Eremophila duttonii</i>	10 plants collected near Titjikala rubbish tip site
5 (=28)	<i>Acacia tetragonophylla</i>	4 roots from 3 trees collected 25 km west of Titjikala (root bark and root shavings)
6 (=28)	<i>Acacia tetragonophylla</i>	7 small roots from 3 trees collected 30 km south of Titjikala (root scrapings)

5.3.2 Chemical analysis of extracts

5.3.2.1 HPLC-UV

Representative HPLC analyses performed by Dr Shao Fang Wang are presented in Appendix G. A spreadsheet outlining some of the more important differences and similarities between the methanol and ethyl acetate fractions of the various samples (in terms of the major chromatographic peaks) is also provided. According to Dr Wang's

expert interpretations of the chromatograms and UV spectra, the following summary of the composition of each of the extracts is offered (Fang, S.-F., personal communications, 31st May 2010 and 13th June, 2010). The methanol extract of Sample 1 (M1) contained fewer flavonoids than all other methanolic samples. Sample M2 had a very high flavonoid content, as did M3 and M4 whose chromatograms were very similar to each other and to M2. M5 contained fewer flavonoids, but more than M6 (>M1). There was a particular flavonoid (unidentified, RT = 24.07 min, 350 nm) that was more concentrated in M5 than any other sample. Peaks corresponding to the retention times (RTs) typical of catechins and similar UV spectra at 254 nm suggested the presence of this class of compound in Samples M1, M5 and M6, while cinnamic acid-like compounds were detected in M2, M3 and M4.

The ethyl acetate fraction of Sample 1 (EA1) did not contain many flavonoids, but there were some other unidentified polyphenols and perhaps catechins. EA2, EA3 and EA4 contained many flavonoids and other polyphenols, including compounds that may have been catechins (or similar). The chemical profile of EA2 was much more complex than that of EA3 or EA4, which were similar to each others. There was not much difference between the chemical make-ups of EA5 and EA6, which both contained a few flavonoids and little else.

All aqueous fractions contained some tannins (Fang, S.-F., personal communications, 31st May 2010 and 13th June, 2010). There were some flavonoids in A1, but at a lower concentration than was present in any of the ethyl acetate fractions. A1 contained the highest proportion of phenolics, with one major peak at a RT of 18.298 min (254 nm), which corresponded to a compound that could not be identified due to a complex UV spectrum. All other aqueous fractions also contained some phenolics, with A2 having a

similar distribution of polyphenolics as A3 and A4. The chromatograms for Samples A5 and A6 were very similar, except that for A5 there was a small peak at a RT of 10.235 min that was not detected in A6. Other small differences were also noted in the chromatograms at 205 nm, but the corresponding compounds could not be identified because the UV spectrum was not easily interpreted.

Overall, for every fraction, the chemical profiles of Samples 3 and 4 (both *E. duttonii*) were very similar to each other, as were those of Samples 5 and 6 (both *A. tetragonophylla*).

5.3.2.2 GC-MS

GC-MS data, also obtained by Dr Wang, are presented in detail in Appendix H. Briefly, Sample 1 contained elemol, phytol, caryophyllene and a group of compounds with similar mass spectrometry. The major constituent of Sample 2 was elemol, which accounted for more than 80% of the volatiles present, although α -, β - and γ -eudesmol and caryophyllene were also detected. The chemical profiles of volatiles from Samples 3 and 4 (both *E. duttonii*) were very similar, with a number of mono-terpenes and sesquiterpenes found. The major compounds were α -pinene and guaiol, although other monoterpenes, including camphene, β -pinene and m-cymol were also detected. Other major constituents were the sesquiterpenes bulnesol, α -caryophyllene, β -caryophyllene, α -eudesmol, β -eudesmol and spathulenol. As Samples 5 and 6 did not have strong aromas indicative of the presence of essential oils, GC-MS was not performed on these.

5.3.3 Extract bioactivity

5.3.3.1 *MTT assay*

Cancer cell proliferation was inhibited by many of the 18 samples tested at the lower screening concentration of 250 µg/mL (Figure 5.2). The ethyl acetate extracts of Samples 3 and 4 (EA3 and EA4) were the most potent agents tested at this concentration. Other extracts that inhibited cell proliferation of at least two cell lines by more than 75% were M5 and A5 in MM253, PC3 and DU145 cells. Extracts that reduced cell proliferation of at least one cell line by at least 25% were M1, M5, M6, EA3, EA4, EA5, EA6 and M5.

The least active extracts were M2, M3, M4, EA1, EA2, A1, A2, A3, A4 and A6, which did not reduce cancer cell proliferation of any line tested, and, in fact, actually caused increased cell proliferation in some cases.

From these data, the most promising eight extracts were chosen for further inhibition studies over a range of concentrations from 25 to 500 µg/mL. Dose-response curves were obtained for extracts EA3, EA4, EA5, EA6, A5, M1, M5 and M6, and these are presented in Figure 5.3.

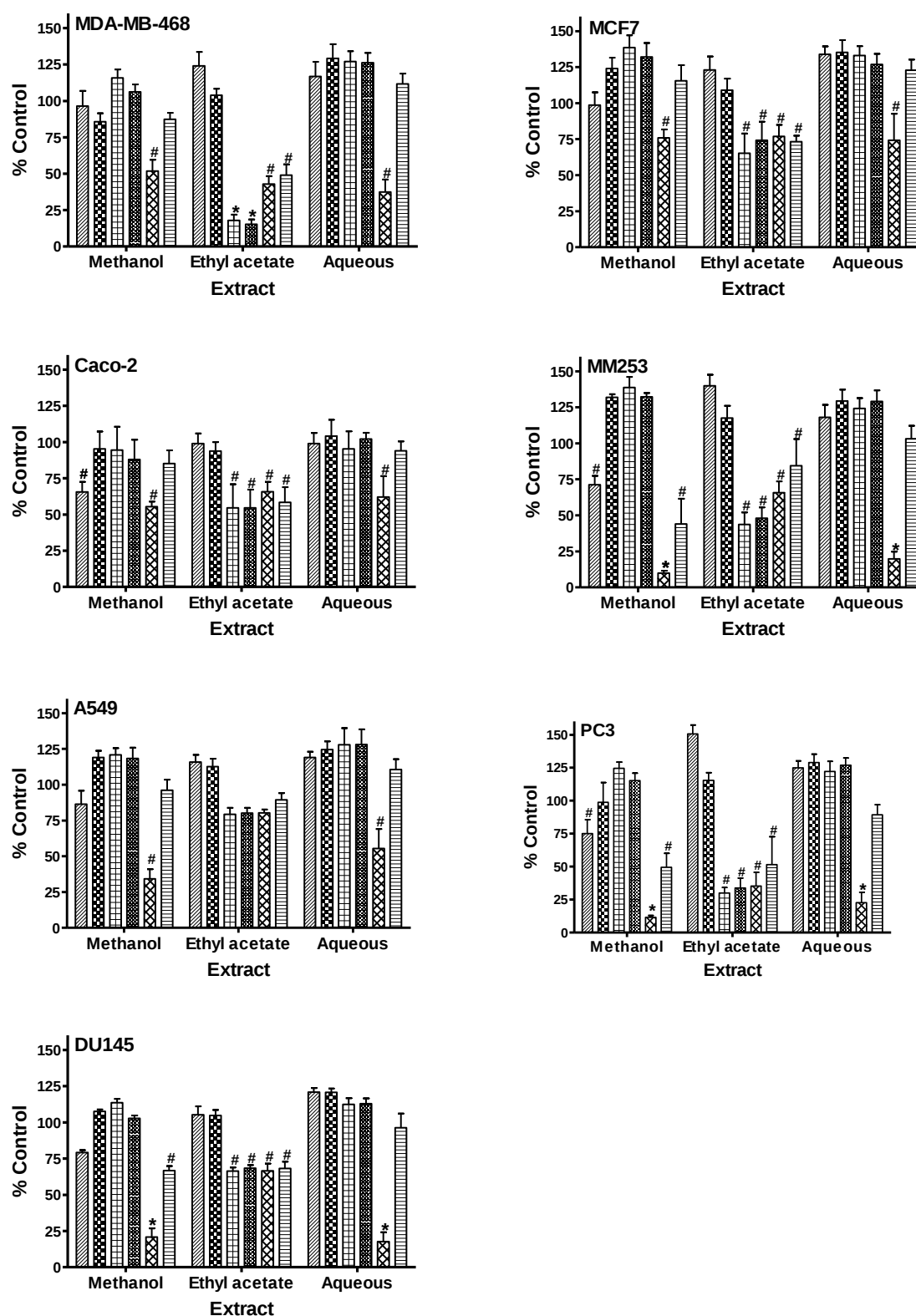


Figure 5.2 Screening of 18 extracts

Cancer cell lines were exposed to various extracts, methanol, ethyl acetate or aqueous fractions of Samples 1, 2, 3, 4, 5, 6, at 250 $\mu\text{g/mL}$ for 48 h before being subjected to the MTT assay. Results are expressed as % of control values and are the means $\pm$ SE of 4 separate experiments.

denotes <75% of control; * denotes <25% of control.

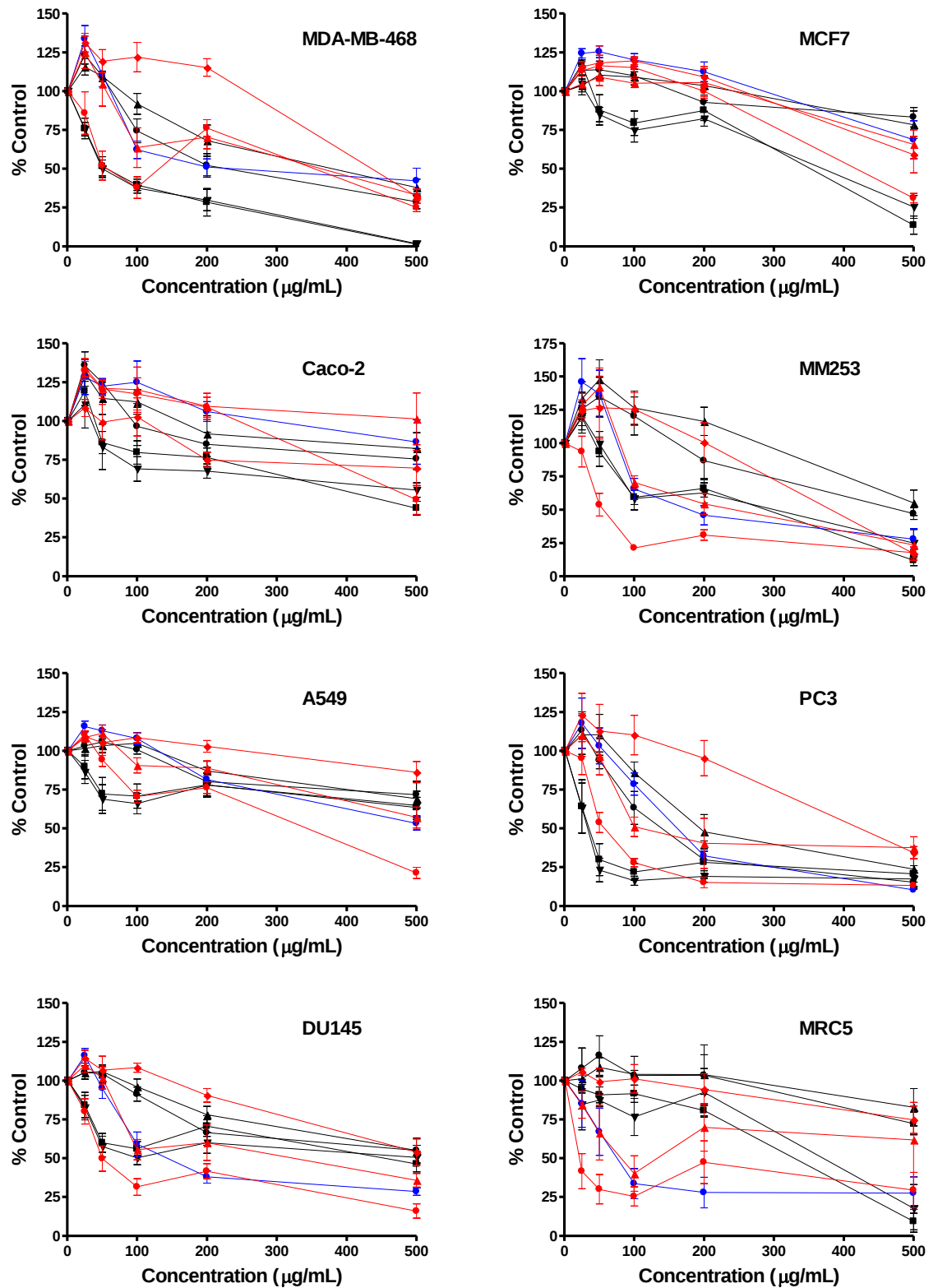


Figure 5.3 Dose-response curves for 8 most active extracts.

Cell lines were exposed to eight different extracts, EA3 (■), EA4 (▼), EA5 (●), EA6 (▲), A5 (●), M1 (◆), M5 (●), M6 (▲), over a range of concentrations for 48 h before being subjected to the MTT assay. Results are expressed as % of control values and are the means $\pm$ SE of at least 5 separate experiments performed in quadruplicate wells.

It is apparent from Figure 5.3 that extracts EA3 and EA4 were bioactive against most cell lines, even at the low concentrations. At 25 $\mu\text{g/mL}$, the lowest concentration trialled, the only other extract to cause more than 10% growth inhibition of any cancer cell line tested was M5 in DU145 cells and MDA-MB-468 cells. However, this extract also resulted in relatively high reduction in growth of the non-cancerous cell line, MRC5.

Higher concentrations of extracts generally resulted in increased inhibition of proliferation of most cell lines. At the highest concentration tested, 500 $\mu\text{g/mL}$, all eight extracts inhibited the growth of MDA-MB-468 and PC3 cells by more than 50%, while all but EA6 caused greater than 50% inhibition of MM253 cells. DU145 cells were almost as susceptible to every extract, with 500 $\mu\text{g/mL}$ of each enough to cause greater than 45% inhibition of growth in each case. Proliferation of MCF7 cells was inhibited by over 50% by 500 $\mu\text{g/mL}$ EA3, EA4 and M5. At this concentration, only extracts EA3 and M5 reduced proliferation of Caco-2 cells by more than 50%. Except for M5, no extract caused more than 50% reduction in growth of the lung cancer cell line, A549.

Most extracts generated classic dose-response curves for each cell line over the range of concentrations used (Fig. 5.3). It was desirable to calculate IC_{50} values in order to directly compare the activities of various extracts against all the cell lines tested.

However, for some extract- cell line combinations this was problematical due to reasons already discussed in Section 4.4 (e.g. not dropping below 50% level). Another factor, the existence of hormesis, also complicated curve fitting. Hormesis, to be discussed further in Sections 5.4.3 and 8.2.4.3, is the phenomenon of a stimulatory response at low doses of an inhibitor. Nevertheless, attempts were made to calculate these data using GraphPad Prism software.

As in Section 5.2.2, four different models were applied to each extract-cell line combination: the default 4-Parameter (4P) model in which no constraints are set, the Top-fixed model (T) in which the upper limit is a constant equal to 100%, the Bottom-fixed model (B) in which only the 0% level is set and the Top and Bottom-fixed model (TB) in which both the 100% and 0% constraints are set. The optimum method was chosen based on the highest correlation coefficient (r^2 value) and the lowest confidence interval as well as the shape of the curve fitted. All fitted curves are shown in Appendix I. For simplicity, just the optimum method used for each extract-cell line pair is depicted (Fig. 5.4) and the IC_{50} values $\pm$ 95% CI generated using this optimum method presented (Table 5.3). The presence of hormetic effects is indicated by asterisks in the last column of Table 5.3. Those extracts that caused stimulation of more than 20% of the control values (Fig. 5.3) are denoted by two asterisks (**), while those causing at least 10% stimulation of growth are denoted by *.

Table 5.3 IC₅₀ Values Calculated Using Optimum Model[#] for Individual Cell Line and Extract Combinations**MDA-MB-468**

Extract Code	IC₅₀ (µg/mL)	IC₅₀ Range (95% CI)	Optimum Model	r²	Hormesis present
EA3	63.8	45.2 - 90.0	TB	0.987	
EA4	61.6	42.2 – 90.0	TB	0.981	
EA5	237.2	111.9 – 502.8	TB	0.939	**
EA6	355.0	207.3 – 607.9	TB	0.963	*
A5	79.8	18.0 – 353.4	4P	0.910	**
M1					**
M5	158.6	21.2 - 1183	TB	0.624	
M6	300.8	110.3 – 820.4	TB	0.826	**

MCF7

Extract Code	IC₅₀ (µg/mL)	IC₅₀ Range (95% CI)	Optimum Model	r²	Hormesis present
EA3	313.0	175.3 – 558.8	TB	0.868	
EA4	314.8	168.2 – 589.2	TB	0.866	
EA5					*
EA6					
A5					**
M1					*
M5	381.9	270.1 – 539.9	B	0.966	
M6					

Caco-2

Extract Code	IC₅₀ (µg/mL)	IC₅₀ Range (95% CI)	Optimum Model	r²	Hormesis present
EA3	418.6	173.2 - 1012	TB	0.811	*
EA4	530.5	132.4 - 2126	TB	0.785	
EA5					**
EA6					**
A5					
M1	861.8	218.1 - 3405	TB	0.759	**
M5					
M6					**

MM253

Extract Code	IC₅₀ (µg/mL)	IC₅₀ Range (95% CI)	Optimum Model	r²	Hormesis present
EA3	209.1	96.2 – 454.4	TB	0.853	*
EA4	231.6	98.3 – 545.8	TB	0.826	
EA5					**
EA6					**
A5	143.3	37.6 – 547.1	B	0.786	**
M1					**
M5	45.3	35.4 – 58.0	T	0.984	
M6	172.6	48.7 – 611.5	B	0.808	**

A549

Extract Code	IC ₅₀ (µg/mL)	IC ₅₀ Range (95% CI)	Optimum Model	r ²	Hormesis present
EA3	28.2	13.4 – 59.1	T	0.883	
EA4	25.3	Not given	T	0.889	
EA5	165.7	97.5 – 281.5	4P	0.984	
EA6	760.2	409.0 - 1413	TB	0.900	
A5	201.0	112.9 – 357.8	4P	0.951	*
M1	861.0	144.8 - 5121	B	0.850	
M5	279.3	155.8 – 500.9	TB	0.892	
M6	575.4	330.0 - 1004	TB	0.865	

PC3

Extract Code	IC ₅₀ (µg/mL)	IC ₅₀ Range (95% CI)	Optimum Model	r ²	Hormesis present
EA3	25.8	22.1 – 30.2	T	0.993	
EA4	26.4	25.1 – 27.7	T	0.999	
EA5	138.8	95.7 – 201.2	TB	0.955	*
EA6	161.2	96.6 – 268.8	T	0.963	
A5	145.4	99.7 – 212.2	B	0.977	*
M1	373.3	242.6 – 574.2	B	0.947	**
M5	49.4	40.5 – 60.4	T	0.993	
M6	79.8	53.0 – 119.9	T	0.981	

DU145

Extract Code	IC ₅₀ (µg/mL)	IC ₅₀ Range (95% CI)	Optimum Model	r ²	Hormesis present
EA3	27.8	12.1 – 63.7	T	0.846	
EA4	28.2	19.8 – 40.0	T	0.969	
EA5	150.4	99.3 – 227.9	T	0.990	
EA6	205.5	126.7 – 333.3	T	0.968	
A5	91.8	56.5 – 149.2	T	0.955	*
M1	492.8	331.8 – 731.9	B	0.944	*
M5	37.2	17.6 – 78.5	T	0.938	
M6	75.8	16.0 – 359.6	TB	0.927	

MRC5

Extract Code	IC ₅₀ (µg/mL)	IC ₅₀ Range (95% CI)	Optimum Model	r ²	Hormesis present
EA3	284.4	220.3 – 367.1	TB	0.973	
EA4	342.3	184.8 – 633.8	TB	0.793	
EA5					*
EA6					
A5	50.5	38.7 – 66.1	T	0.991	
M1	828.5	466.4 – 1472	TB	0.994	
M5	27.8	3.45 – 224.6	TB	0.715	
M6	689.3	8.3 – 57238	TB	0.505	

(*at least one concentration > 110% of control; ** > 120% of control)

Four different models were applied to each extract-cell line combination: the default 4-Parameter (4P) model in which no constraints are set, the Top-fixed model (T) in which the upper limit is a constant equal to 100%, the Bottom-fixed model (B) in which only the 0% level is set and the Top and Bottom-fixed model (TB) in which both the 100% and 0% constraints are set.

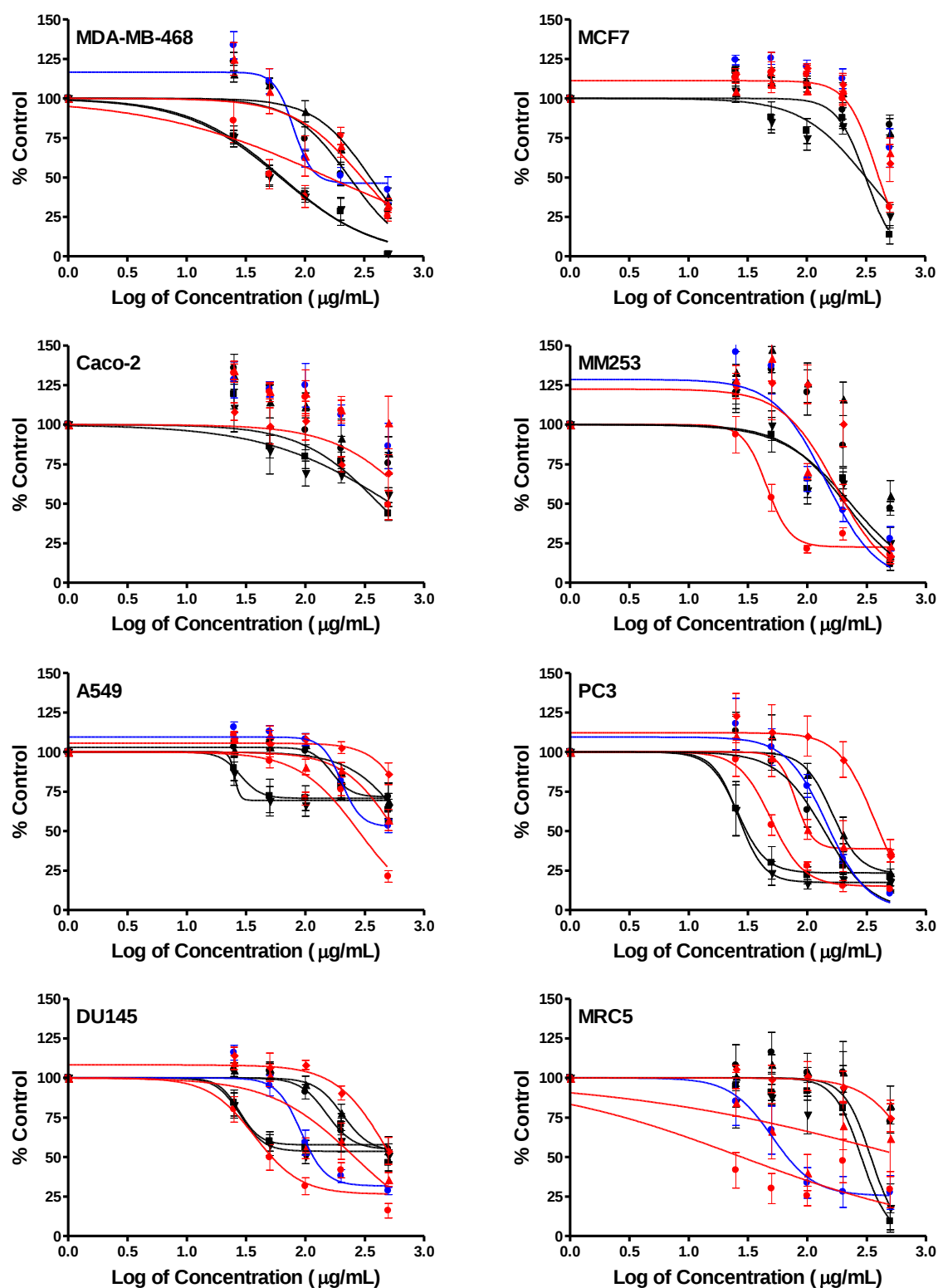


Figure 5.4 Nonlinear regression of the 8 most active extracts using the optimum model for each extract-cell line pair.

Cell lines were exposed to eight different extracts, EA3 (■), EA4 (▼), EA5 (●), EA6 (▲), A5 (●), M1 (◆), M5 (●), M6 (▲), over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values (Fig 5.3) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) according to the optimum method for each extract- cell line combination.

As can be seen from Table 5.3, the six lowest IC_{50} values obtained for cancer cells, (25 - 30 $\mu\text{g/mL}$) were all for extracts EA3 and EA4 for their activities against A549, PC3 and DU145 cells. Interestingly, the Top-fixed model was used to generate the data in each of these cases. However, a low IC_{50} value was also calculated for M5 against the non-cancerous cell line, MRC5, this time using the model by which both the top and bottom constraints were set. Other extracts with IC_{50} values under 100 $\mu\text{g/mL}$ were EA3 and EA4, A5, M5 and M6.

5.3.3.2 *Brine shrimp lethality assay (BSLA)*

General toxicity of the extracts was assessed using the BSLA and the results after 2, 24 and 48 h exposure are depicted in Figure 5.5.

As can be seen from Fig. 5.5, after just 2 h exposure no tested extract had any effect on brine shrimp lethality. Although after 24 h 500 $\mu\text{g/mL}$ of EA6 recorded a slight degree of toxicity towards brine shrimp, it is clear that M5 was the most toxic of all the extracts tested. After 24 h, M5 had registered >40% lethality at 1,000 $\mu\text{g/mL}$ and >10% at 500 $\mu\text{g/mL}$. More extracts caused shrimp to die after 48 h exposure, although M5 remained the most potent with 1,000 $\mu\text{g/mL}$ causing >65%. A5 was the next most lethal extract with 1,000 $\mu\text{g/mL}$ resulting in >20% shrimp death. EA4, EA5 and M1 also recorded some lethality, but their levels of lethality were under 10% even at the highest concentration tested. Only the toxic effects of M5 was demonstrably dose-dependent with a 50% maximal effective concentration (EC_{50}) value of 627.3 $\mu\text{g/mL}$ (95% CI = 475.3 to 828.0 $\mu\text{g/mL}$; $r^2 = 0.984$) after 48 h (Figure 5.6).

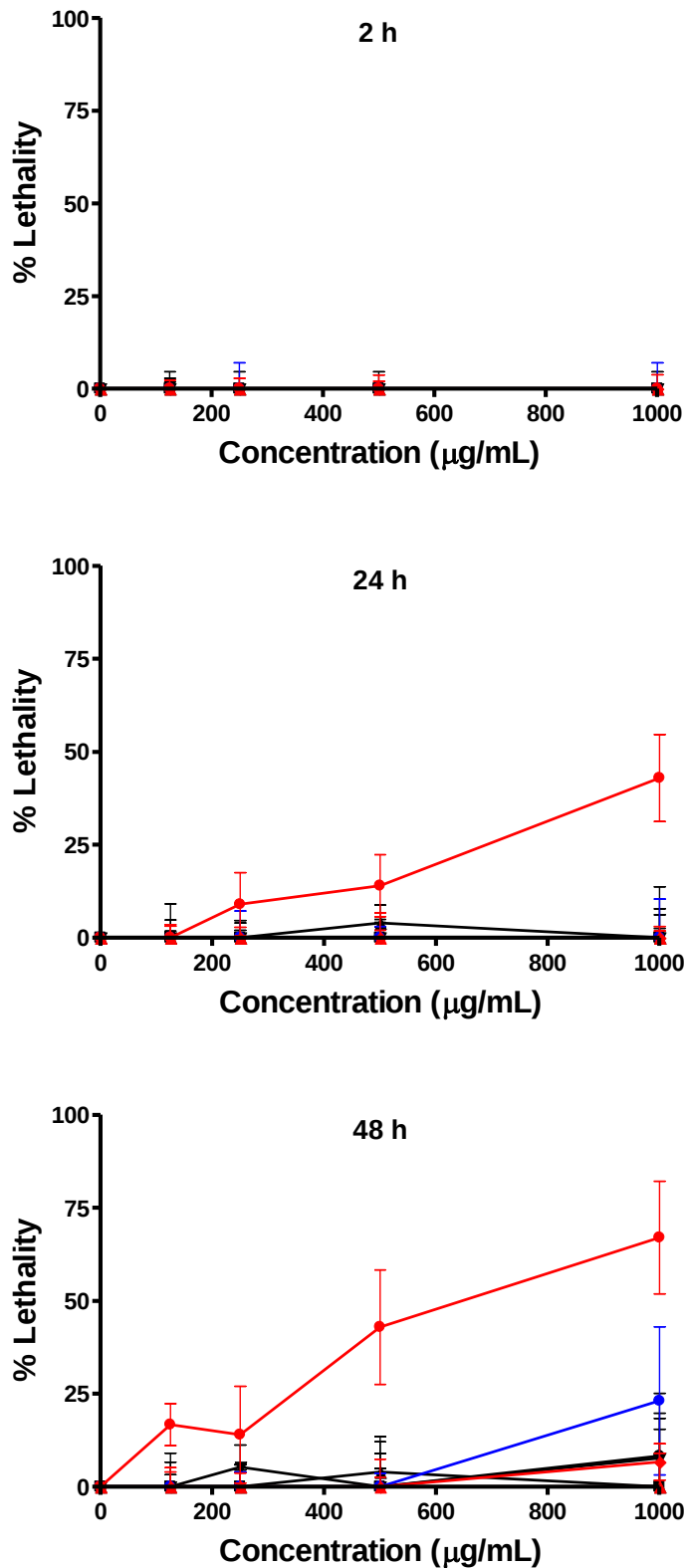


Figure 5.5 General toxicity of best 8 extracts over time

Brine shrimp were exposed to eight different extracts, EA3 (■), EA4 (▼), EA5 (●), EA6 (▲), A5 (●), M1 (◆), M5 (●), M6 (▲), over a range of concentrations for 2, 24 and 48 h. Results are expressed as a % of total shrimp death and are the medians $\pm$ SE of at least 4 separate experiments, performed in duplicate wells.

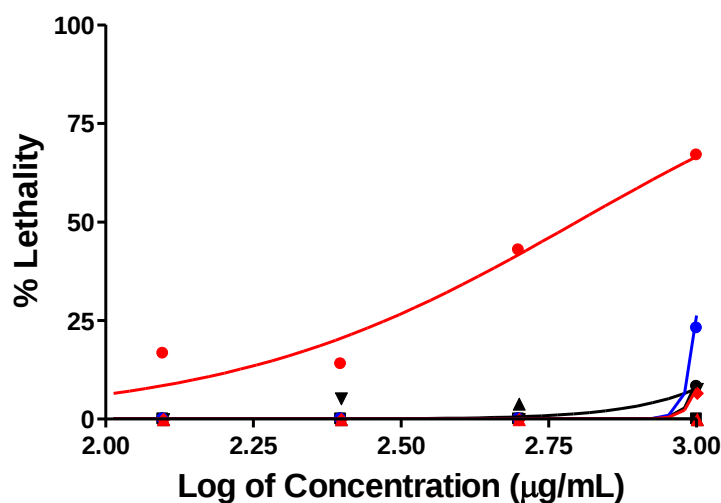


Figure 5.6 Nonlinear regression of general toxicity of best 8 extracts over time

Brine shrimp were exposed to 8 different extracts, EA3 (■), EA4 (▼), EA5 (●), EA6 (▲), A5 (●), M1 (◆), M5 (●), M6 (▲), over a range of concentrations for 48 h. Results are expressed as a % of total shrimp death and are the medians $\pm$ SE of at least 4 separate experiments, performed in duplicate wells. Dose values (Fig 5.5) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) with the bottom parameter fixed at 0% and the top parameter set at 100% (TB model).

Table 5.4 summarises the effects of some controls on brine shrimp survival. The positive controls used, DFO (1 mM) and CPT (0.1 mM), caused >30% and 4.0% lethality, respectively, after 48 h. The negative control, DMSO at 1% final concentration (v/v), the concentration corresponding to 1,000 $\mu\text{g/mL}$ of extract, induced just 2.0% toxicity after 24 h with no further deaths up to 48 h. For interest, 10 mg/mL concentrations of extracts were also tested, along with the corresponding DMSO control (10% v/v). In all but one case (A5), this high concentration caused 100% lethality after 48 h, with varying degrees of toxicity after 2 and 24 h. However, >60% of this toxicity was due to the high DMSO content alone.

Table 5.4 General Toxicity of Controls and High Concentrations of Plant Extracts (Mean % Lethality $\pm$ SE)

Sample	Concentration	2 h	24 h	48 h
DFO	1 mM	2.2 $\pm$ 2.2	27.0 $\pm$ 13.5	31.4 $\pm$ 17.9
CPT	0.1 mM	0.0 $\pm$ 0.0	2.0 $\pm$ 1.8	4.0 $\pm$ 3.4
DMSO	1% v/v	0.0 $\pm$ 0.0	2.0 $\pm$ 2.0	2.0 $\pm$ 2.0
DMSO	0.25% v/v	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.8 $\pm$ 0.8
DMSO	0.125% v/v	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
EA3	10 mg/mL	1.5 $\pm$ 1.5	72.0 $\pm$ 16.1	100.0 $\pm$ 0
EA4	10 mg/mL	10.6 $\pm$ 6.7	77.7 $\pm$ 14.2	100.0 $\pm$ 0
EA5	10 mg/mL	3.7 $\pm$ 3.7	84.5 $\pm$ 9.8	100.0 $\pm$ 0
EA6	10 mg/mL	0.0 $\pm$ 0.0	72.5 $\pm$ 17.5	100.0 $\pm$ 0
A5	10 mg/mL	4.2 $\pm$ 4.2	31.0 $\pm$ 5.8	88.1 $\pm$ 7.8
M1	10 mg/mL	4.6 $\pm$ 3.0	97.6 $\pm$ 2.4	100.0 $\pm$ 0
M5	10 mg/mL	4.2 $\pm$ 4.2	97.2 $\pm$ 2.8	100.0 $\pm$ 0
M6	10 mg/mL	0.0 $\pm$ 0.0	86.3 $\pm$ 7.1	100.0 $\pm$ 0
DMSO	10% v/v	0.0 $\pm$ 0.0	11.1 $\pm$ 5.3	60.0 $\pm$ 20.7

5.4 DISCUSSION

5.4.1 Sample collection and extract preparation

As discussed in Section 1.3.5, natural products, especially secondary metabolites, have been and continue to be a prime source of new drugs or drug leads (Newman & Cragg, 2007). There exists a high diversity of secondary metabolites with the structures of over 200,000 having already been elucidated (Hartmann *et al.*, 2005). The three main categories of small molecule secondary metabolites are alkaloids, phenols and terpenoids (Liu *et al.*, 1998). Several major secondary metabolites are commonly accompanied by many minor components. In general, a set of related compounds is found in each plant, frequently a few main metabolites and several minor components, differing in the position of their substituents. The chemical profile can vary between plant tissues, within developmental stages and, sometimes, even diurnally (Wink & Witte, 1984). Moreover, great differences can be observed between members of different populations and often even between individual plants of a single population too (Wink, 1999). However, the types of secondary metabolites present in plants of the

same species is generally the same; it is the concentration of these particular compounds that varies (Dufault *et al.*, 2001; Orians *et al.*, 2003).

In order to limit the effects of individual variation between plants of the same geographical population, plant parts were collected from a few different plants in the same area. The choice of which particular plant parts were used for each species was based on local Aboriginal traditional knowledge (TK) of the medicinal value of each part. To account for geographical variation, different samples of the same plant species were collected from different areas. Thus, Samples 3 and 4 were both collections of *Eremophila duttonii*, gathered from numerous plants from two distinct areas 12 km apart. Similarly, Samples 5 and 6 were roots of *Acacia tetragonophylla*, each collected from 3 separate trees in areas approximately 40 km distant.

These different locales differed in their soil make-up as well as, quite probably, other environmental parameters too. It is important to realise that none of these environmental factors were actually quantified in any way, and, in fact, may not have differed measurably at all. The point is that the different sampling areas were qualitatively dissimilar and remote from each other, so variation between the secondary metabolite compositions of the plants was distinctly possible. However, there was essentially no purpose in artificially manipulating the soil conditions, altitude, light intensity or other parameters in order to discern the effects of such differences. This was because one of the main objectives of this study was to evaluate the medicinal properties of plants naturally occurring within the local area with a view to validating the traditional Aboriginal knowledge of the community. In other words, any variation between samples had to be natural.

While most extracts contained predominantly flavonoids, there were differences observed in their chemical profiles. Sample 1, from *Euphorbia drummondii*, was quite different from every other sample. However, extracts of Sample 2 (*Eremophila sturtii*) shared similarities with Samples 3 and 4, but both these were from the related *Eremophila duttonii*. As might be expected from samples of the same species, extracts of Sample 3 differed from Sample 4 only in the concentrations of compounds present. Similarly, the chemical make-up of extracts of Samples 5 and 6 (both *Acacia tetragonophylla*) were very similar, differing only in the amounts of particular compounds. These differences in the secondary metabolites probably account for the observed variation in bioactivities. For example, except for the ethyl acetate fraction of Sample 6 (EA6) against Caco-2 cells (Fig. 5.2), every extract of Sample 5 was more bioactive than its Sample 6 equivalent. This was especially noticeable in the BSLA where M5 killed many more brine shrimp than M6, or, indeed, any other extract tested (Fig. 5.10). This may have been something to do with the relatively higher levels of an unidentified flavonoid it contained (see Appendix G). The bioactive effects of all extracts of Samples 3 and 4 were very similar, paralleling their similar chemical profiles.

5.4.2 Chemical analysis of extracts

5.4.2.1 HPLC-UV

The ethyl acetate fractions of Samples 3 and 4 (EA3 and EA4) were the most potent in terms of their inhibitory effects on cancer cell growth (Fig. 5.2). This fraction generally contains flavonoids, many of which, such as quercetin and luteolin are known antitumour agents (Middleton *et al.*, 2000; Kuntz *et al.*, 1999; Lin *et al.*, 2008; Xavier *et al.*, 2009).

The methanol fractions of Samples 5 and 6 also displayed marked antiproliferative properties in some cell lines. As methanol is a more polar solvent than ethyl acetate, a larger range of compounds is extracted (Houghton & Raman, 1998; Fang, S.-F., personal communication, 31st May 2010). Hence, more polyphenolics would probably present in this fraction and some of these, such as caffeic acid and resveratrol, also have reported anticancer properties (Hudson *et al.*, 2000; Li *et al.*, 2002; Cecchinato *et al.*, 2007). Additionally, the methanol fraction may contain more flavonoids as this class of compounds has a wide polarity range (Yuan, 2005).

Apart from Sample 5 (A5), the aqueous extracts of the samples tested were the least inhibitory in terms of their effects on cancer cell proliferation. This fraction generally contained lower concentrations of flavonoids and more tannins compared to the corresponding ethyl acetate and methanol fractions. Many tannins can have antitumour activity (Fong *et al.*, 1972; Jain *et al.*, 2009), but the constituent of A5 responsible for its cytotoxicity was not identified as part of the present study as the effect seemed to be non-specific and therefore not worth pursuing. This could have been due to tannins, which were prevalent in this fraction. Tannins can interact with biological assays and they are also considered to be non-specific inhibitors (Ayehunie *et al.*, 1998; Marcy *et al.*, 2006). Additionally, since the almost ubiquitous presence of tannins in plant extracts makes the isolation and characterization of other compounds from these sources very difficult, many researchers choose to remove them prior to screening (Collins *et al.*, 1998). However, there are arguments that this practice may mean bioactive compounds with specificity of action may be missed (Zhu *et al.*, 1997). As this study was interested in validating ethnomedical data, it was important to test the whole extracts.

Future directions will involve the removal of polyphenolic compounds to assess if the extracts are still bioactive. This will be the first step in elucidating whether the observed cytotoxicity is mainly due to known compound/s or a NCE.

5.4.2.2 GC-MS

The chemical profiles of volatiles, as assessed by GC-MS, for Samples 3 and 4 were very similar. This is not unexpected given that these samples were prepared from the same species of plant, *Eremophila duttonii*. However, it does suggest that the variation that existed between the two different locales from which the samples were sourced was not enough to significantly influence the secondary metabolite profile of this species. Unsurprisingly, distinct differences were observed between the different species, *E. drummondii* (Sample 1), *E. sturtii* (Sample 2) and *E. duttonii* (Samples 3 and 4).

5.4.3 Extract bioactivity

5.4.3.1 MTT assay

Figure 5.2 depicts how different cell lines responded to the extracts to varying degrees. Although some researchers continue to report significant antiproliferative effects of plant extracts at concentrations above 200 µg/mL (Lee *et al.*, 2008), 10 mg/mL (Rao *et al.*, 2009) and even over 100 mg/mL (De Martino *et al.*, 2006), these values are physiologically quite meaningless and the implementation of a standard extract concentration cut-off of 50 µg/mL has been recommended (Gertsch, 2009). Based on this, it is clear that the ethyl acetate extracts of Samples 3 and 4 (EA3 and EA4) were potent against some cell lines (e.g. MDA-MB-468 and PC3), but less so against others (e.g. Caco-2, A549 and DU145) (Fig. 5.2).

However, according to the IC₅₀ values (Table 5.3) calculated from the dose-response curves (Fig. 5.3), it is the A549, PC3 and DU145 cell lines that are most susceptible to these two extracts. Possible reasons for this discrepancy are addressed below.

Nevertheless, regardless of which method was used to assess potency, it is clear that there were definite differences between the cell lines. These differences indicate specificity, a very important factor in drug discovery. In fact, according to the NCI, specificity is one of the defining factors in deciding if an agent showing antitumour potential in the initial screen should progress to secondary testing or not (Grever *et al.*, 1992; Boyd & Paull, 1995).

Despite using several models to calculate IC₅₀ values, it was not possible to determine these values for all extract-cell line combinations. Moreover, when IC₅₀ values were determined, they were not always reliable measures of an extract's effectiveness. For example, from Figure 5.3 it is clear that EA4 had limited effect on reducing the proliferation of A549 cells, and yet, it recorded the lowest IC₅₀ value of any extract in any cell line tested (Table 5.3). While a 95% confidence interval was inexplicably incalculable by the GraphPad Prism programme, the correlation coefficient of the curve fit was not low ($r^2 = 0.890$) and the results for EA3 (from the same plant) were very similar. As explained in Section 4.4.3, sometimes the reason for imperfect curve fits was because a level of 50% inhibition was never reached over the concentration range tested. This was true for the above situation where even the highest concentration of EA4 tested, 500 µg/mL, only inhibited proliferation of A549 cells to 64.8% of the control level. Such flat dose-response curves would suggest that the classic sigmoidal dose-response model was not appropriate in these cases. However, it was thought that the same model should be applied to every extract- cell line pair so as direct comparisons could be made between them. It was not possible to perform repeat experiments using a higher range of concentrations due to the limited solubility of the extracts and the toxicity of DMSO at high concentrations. Therefore, the IC₅₀ values generated for flat dose-response curves were interpreted with some scepticism.

Similarly, steeper dose-response curves (than the classic sigmoidal dose-response model would predict) suggested that this model was not appropriate in these cases either and so their IC₅₀ values were also accepted with some diffidence.

However, another complicating factor in calculating IC₅₀ values in this study was the existence of the intriguing phenomenon of hormesis. A biphasic dose-response in which opposite effects are displayed at high and low doses, researchers have typically reported low-dose stimulation and high-dose inhibition (Calabrese, 2005a). In fact, according to Anderson (2005), *Stedman's Medical Dictionary* defines hormesis as “the stimulating effect of subinhibitory concentrations of any toxic substance”. In other words, if high doses of a compound have an inhibitory effect on growth, for example, low doses of that compound would cause increased growth. Hormesis has been described for many dose-response relationships, including antineoplastic agents tested against cell lines in the NCI screen (Calabrese, 2005a; Calabrese *et al.*, 2006b). The prevalence of hormesis will be discussed further in the General Discussion (Chapter 8) and Appendix N, along with its controversial history in gaining acceptance as a concept, further characterisation of the model and the implications that hormesis has in medicine.

In toxicology many experiments are performed to determine the dose-response relationship between a certain compound and a living entity (e.g. group of cells or whole organism). Data from these experiments are frequently analysed by a logistic model and summarised in the form of the IC₅₀. However, when hormesis is present, it is not appropriate to use the standard log-logistic model to fit dose-response curves and calculate IC₅₀ values (Schabenberger *et al.*, 1999; Vanewijk & Hoekstra, 1993).

Common practice is to drop some of the data or to use the log-logistic model anyway.

Formulae have been developed to calculate IC₅₀ values for biphasic relationships, such

as when hormesis is present (Brain & Cousens, 1989; Vanewijk & Hoekstra, 1993; Schabenberger *et al.*, 1999; Beckon *et al.*, 2008). However, without access to computer software based on these models, the adoption of these formulae was beyond the capabilities of this PhD candidate. Nevertheless, it is acknowledged that it can be dangerous to simply ignore the presence of hormetic effects which cannot be adequately captured by a log-logistic function. Fitting a log-logistic model when hormesis is present can lead to serious bias and erroneous inferences (Schabenberger *et al.*, 1999). Therefore, the IC₅₀ values obtained in this study when hormesis was present (Table 5.3) were also taken with some reservation.

Hormesis is one possible explanation for the wide range of IC₅₀ values calculated on different days. However, Alley *et al.* (1988) also reported large variations from the mean IC₅₀ value for each cell line when the MTT assay was used to measure sensitivity to a “standard” agent, doxorubicin. One third of the cell lines tested exhibited greater than a 5-fold range in IC₅₀ values, and these large deviations occurred randomly over time. These authors could offer no explanation for most of the variations, instead claiming that the fact that they occurred at all “prompted the development and inclusion of several biological and pharmacological quality assurance criteria in the performance of subsequent screening assays”, although they did not elaborate on what those may have been (Alley *et al.*, 1988, p596). Thus, in the current study, every effort was made to ensure that controllable experimental conditions, such as incubation times and preparation of all reagents and extracts (which were stored optimally) were consistent and internal controls were included so as to facilitate reproducibility and ensure quality control as much as possible. Therefore, any variability between experiments was taken to be due to differences in the sampling of extracts, which were prepared fresh on the day of use.

While acceptable IC_{50} values were considered when choosing extracts for further study, due to hormetic effects and other concerns discussed above, more credence was placed on a certain level of growth inhibition obtained at a given concentration. The concentration chosen was 250 $\mu\text{g/mL}$ and a mean score of less than 50% of the control was chosen as a cut-off threshold. Thus, any extracts exhibiting less than 50% inhibition of proliferation of any cell line at 250 $\mu\text{g/mL}$ (Fig. 5.3) were eliminated from the pool of promising extracts. This method of reporting bioactivity is not without precedent. In fact, it is quite common in preliminary drug discovery studies that effects are reported for discrete concentrations only, especially when the test agent is not a pure compound and is subject to the interactions and complications inherent of mixtures (discussed in Section 8.2.6.2). For example, Argarwal *et al.* (2000), Opoku *et al.* (2000), Russo *et al.* (2005) and Paschke *et al.* (2009) all used the MTT assay to assess the antiproliferative effects of different concentrations of plant extracts against various cancer cells and reported the results in terms of their % of control values at specified concentrations, rather than calculated IC_{50} value. Additionally, Hunt and Rai (2005), applied a score test of a random effects hormetic-threshold dose-response model. In their study, the investigators were not primarily interested in completely analysing the data, but instead sought to develop an inference procedure for a threshold effect.

5.4.3.2 ***Brine shrimp lethality assay (BSLA)***

The BSLA is commonly employed as an initial screen for broad spectrum bioactivity. It has been used to detect the presence of bioactive compounds in crude extracts and has been shown to be predictive of cytotoxicity in many studies. For example, a positive correlation has been shown to exist between BSLA lethality and cytotoxicity toward the 9KB cell line (Meyer *et al.*, 1982 as cited by Turker & Camper, 2002).

However, in this study, a high level of bioactivity in the BSLA meant that promising plant extracts were eliminated from further evaluation, despite their strong antiproliferative effects in the MTT assay. The logic behind this decision was that as selective bioactivity against cancer cells was being sought, any extracts that killed brine shrimp were doing so by some nonspecific mechanism, possibly via arresting protein synthesis as is the case with some lectins (Lord, 1987) or perhaps by damaging the membrane integrity of all cells. In such a case, they would have little potential as new antineoplastic agents.

Unfortunately, the BSLA was rather inaccurate, as seen in the high standard error values obtained. This high degree of error was due to the necessarily small numbers of nauplii in each well, a byproduct of the labour intensiveness of the assay. While it must be remembered that the SEs were of median values, which are 25% times higher than those of means, these high SE values obtained highlighted the inherent shortcomings of the assay and the absolute necessity of the repeat experiments performed. Nonetheless, useful information was garnered from these experiments, including the fact that the methanol fraction of Sample 5 (M5) caused more nauplii to die than any other extract tested. This was interpreted to mean that it displayed a high level of nonspecific cytotoxicity. In corroboration of this was the fact that, while M5 showed bioactivity against most cancer cell lines in the antiproliferation studies (Fig. 5.2), it also inhibited the proliferation of the non-cancerous cell line, MRC5 with an IC_{50} value of 27.8 $\mu\text{g/mL}$. These inferences marked M5 inappropriate as a drug candidate, resulting in its exclusion from further investigations.

On the other hand, the ethyl acetate extracts of Samples 3 and 4 (EA3 and EA4) displayed relatively low levels of cytotoxicity towards brine shrimp. This, along with

their comparatively high IC_{50} values against non-cancerous MRC5 cells (284.4 and 343.3 $\mu\text{g/mL}$, respectively), suggests that their potent bioactivity against some cancer cells in the antiproliferation studies was due to a more specific mechanism than simply a direct cell poison. As the objective of chemotherapeutics is to eliminate cancer cells while causing minimal damage to normal cells, this finding was exactly the type of scenario sought, meaning that further investigation of EA3 and EA4 as potential anticancer agents was warranted. Since EA3 and EA4 were both ethyl acetate extracts from the same plant species and had similar chemical profiles, EA3 was chosen for further study on the simple basis that there was more of it available.

5.5 SUMMARY

In summary, more plant samples of those with the most bioactive methanolic extracts from the initial screen were collected and extracted to give aqueous, methanol or ethyl acetate fractions. These were subjected to various chemical analyses and assessed for their specificity and selectivity against cancer cells. From these data, the extract with the greatest potential as an anticancer agent, the ethyl acetate extract of *Eremophila duttonii* (EA3), was chosen for further study, the basis of which forms Chapter 6.

Chapter 6: Further Evaluation of Plant Extract, EA3

6.1 INTRODUCTION

Previous chapters describe the bioactivity screening of extracts of various native plants identified by Aboriginal people as having medicinal qualities. Several of these extracts were shown to have varying inhibitory effects on different cancer cell lines. However, due to time and budget constraints, only the extract with the most promising anticancer potential was chosen for further evaluation. The ethyl acetate fraction of Sample 3 (EA3) was selected, based on data suggesting it induced cytotoxicity (cell death) or cytostaticity (cell cycle arrest) in several, but not all, cancer cell lines while having minimal effect on normal cells. The focus of this chapter is to describe the further characterization of this extract's chemical composition and studies undertaken to elucidate its mechanism of action.

6.1.1 Chemical composition

Since all metabolomic detection techniques have unavoidable intrinsic bias towards certain metabolite groups, no single technique is adequate for determining the complete chemical make-up of an extract. Hence, multiparallel technologies are necessary to obtain the desired broad metabolic picture (Hall, 2006). HPLC coupled to a UV photodiode array detect (HPLC-UV) has been widely used for the primary chemical analysis of crude plant extracts (Moco *et al.*, 2006; De Vos *et al.*, 2007). The UV spectra of natural products obtained by HPLC-UV gives useful information on the type of constituents present in an extract. Liquid chromatography coupled to mass spectrometry (LC-MS) is a newer and more expensive technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. Mass spectrometry is one of the most sensitive methods of molecular analysis and provides information on the molecular weight as well as on the structure of the individual compounds. LC-MS is the preferred technique

for the separation and detection of the large and often unique group of semipolar secondary metabolites in plants. LC-MS enables the detection of large numbers of parent ions present in a single extract and can yield valuable information on the chemical composition and hence the putative identities of many metabolites at once (Moco *et al.*, 2006; De Vos *et al.*, 2007). It is very useful for answering the questions “what is it?” and “how much is there?” (Lee & Kerns, 1999). Therefore, LC-MS was used to identify and establish the relative proportions of the main non-volatiles present in EA3. Subsequent HPLC-UV analysis of EA3 compared to known standards was performed to quantify the absolute amounts of some of the main constituents.

6.1.2 Identification of active constituents

One of the specific objectives of this study was to try to identify plant compounds that may be of use in the treatment of cancer. Hence, it was important to establish if the cytotoxic effects of EA3 on cancer cells were due to a novel compound or a known one. Therefore, three of the major components of EA3, as determined by LC-MS, were tested for their individual cytotoxic effects and compared with those of the whole extract.

6.1.3 Exposure and recovery

As the experimental work of this project was drawing to a close, access to a new system capable of assessing the growth of cell cultures became available. This new Cellscreen (CS) system (see Sections 2.2.2.10 and 2.6.4) measures the percentage confluence of cells grown in 96-well microtitre plates. As discussed in Chapter 3, the precision of the CS system is comparable to that of the MTT assay (Viebahn *et al.*, 2006). However, the CS holds the major advantage of being non-invasive, meaning the proliferation of the same population of cells can be monitored over time.

Therefore, the CS was used to study the kinetics of exposure of cancer cells to EA3. In this way, it was hoped that clues could be obtained about whether EA3-induced inhibition of cell proliferation (as measured by the MTT assay) was due to cytotoxic or cytostatic effects.

As discussed in Chapter 4, apoptosis is generally a much quicker process than necrosis, occurring within just 1-3 h (Kerr *et al.*, 1972; Gavrieli *et al.*, 1992; Majno & Joris, 1995), so a relatively rapid cytotoxic response would be suggestive of the involvement of an apoptotic pathway. Additionally, by monitoring cell growth after the extract was removed from the culture, it was possible to ascertain if any EA3-induced cytostatic effects were reversible, or not.

6.1.4 Intracellular Ca^{2+} measurement

Another method employed to help distinguish between necrosis and apoptosis was the measurement of intracellular Ca^{2+} levels. A loss in cell membrane integrity can be demonstrated by measuring a rapid and prolonged influx of extracellular Ca^{2+} into the cytosol (Orrenius *et al.*, 1989; Endo, 2006). In contrast, as Ca^{2+} is a key second messenger, any rapid but transient change in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is indicative of a physiological response to a given stimulus (Wasilenko *et al.*, 1997; Endo, 2006). To assist the reader if necessary, the basic processes involved in Ca^{2+} signalling are provided in Appendix J.

Transient increases in $[\text{Ca}^{2+}]_i$ have been implicated in the regulation of a host of cellular processes in a wide variety of cell types (Campbell, 1990). Such Ca^{2+} signalling is essential for the growth, death, differentiation and function of cells (Dolmetsch *et al.*, 1997). Cell functions regulated by Ca^{2+} are too numerous to name individually, but include such basic processes as cell motility, contraction, secretion and division

(Clapham, 2007). Additional biochemical roles of Ca^{2+} include regulating enzyme activity (Nalamachu *et al.*, 1994), controlling gene expression (Giles & Hilmar, 1999) and, of particular significance here, initiating apoptosis (Tombal *et al.*, 1999; Sylvie *et al.*, 2000; Baumgartner *et al.*, 2009). Therefore, it was of considerable interest to investigate the dynamics of any changes to $[\text{Ca}^{2+}]_i$ in cancer cells exposed to EA3 and to compare any such changes to that of a known endogenous activator of Ca^{2+} signalling, such as histamine (Lee *et al.*, 2001; Akdis & Blaser, 2003; Montero *et al.*, 2003). PC3 cells were selected for this purpose as they express histamine receptors which are involved in Ca^{2+} signalling pathways (Lee *et al.*, 2001). They were chosen above other cancer cell lines studied as EA3 potently inhibited their proliferation (see Fig. 5.3) and because they grew well on glass. It was assumed that if necrosis was responsible for the observed cytotoxicity of EA3 in PC3 cells, any breach in membrane integrity would be revealed by an increase in $[\text{Ca}^{2+}]_i$ that increased suddenly and remained elevated. On the other hand, a Ca^{2+} transient would suggest some Ca^{2+} signalling taking place, which may be a normal signalling process or activation of cell death pathways. Increased intracellular Ca^{2+} is known to be an initial step in many pathways leading to apoptosis (Tombal *et al.*, 1999; Sylvie *et al.*, 2000; Olofsson *et al.*, 2008; Baumgartner *et al.*, 2009).

6.2 METHODOLOGY

6.2.1 Chemical composition

LC-MS for identification of non-volatiles was carried out on EA3 by a collaborator, Dr Shao Fang Wang (ChemCentre, WA). Briefly, LC-MS spectra were recorded on an Agilent Triple Quad 6400 LC-MS system. The column used in LC-MS was an Agilent Zorbax 80 Å, C18, 4.6 x 150 mm, 5 µm. An Electronic Spray Ionisation in negative ionisation mode was used. The typical source conditions were a gas temperature of 350°C, gas flow of 8 L/min, and the nebulizer set at 55 psi. The scan time was 500 s and

fragment voltage was 135 V. The software used to run LC-MS was Agilent Masshunter Workstation and software used for data analysis was Agilent Masshunter Qualitative Analysis (Santa Clara, CA, USA).

Due to the relative expense of LC-MS as well as the limited availability of the equipment, the absolute amounts of each of the main constituents present in EA3 were determined by HPLC-UV analysis. The HPLC-UV chromatograms obtained for EA3 after UV absorption at 254 nm and 350 nm were compared to those of a standard mixture containing known quantities of rutin, luteolin and apigenin. The equipment and conditions used have been described previously (Section 5.2.2.1).

6.2.2 Identification of active constituents

Three of the main components of EA3 identified by LC-MS, rutin, luteolin and quercetin, were tested for their antiproliferative effects on cancer cells compared to those of the whole extract. Briefly, these pure compounds were dissolved in DMSO at 10 mg/mL, diluted 1/20 in DMEM without phenol red and filter-sterilised. Further dilutions were made such that when 10 μ L was added to 100 μ L of cells in microtitre plates, the final concentrations of the compounds ranged from 200 μ g/mL down to 2.5 μ g/mL. EA3 was dissolved at 100 mg/mL, filtered and diluted as above such that cells were exposed to final concentrations of between 500 μ g/mL and 25 μ g/mL. The MTT assay in its final modified form (Section 2.6.1) was again employed to assess the cytotoxicity of these pure compounds compared to EA3 against a range of human cancer cell lines and one non-cancerous cell line.

6.2.3 Exposure and recovery

The CS system (see Sections 2.2.2.10 and 2.6.4) was used to evaluate the exposure kinetics of EA3 on different cancer cell lines. Briefly, cells in their appropriate culture medium were seeded at 10,000 cells per well and allowed to adhere and grow O/N at

37°C. Various dilutions of EA3 (initially 100 mg/mL in DMSO) were added in 10 µL aliquots to quadruplicate wells and the effects on cells tracked over time. Final concentrations of EA3 ranged from 25 to 500 µg/mL and cell growth was monitored over 48 h of exposure.

It was assumed that, in the presence of the extract, no change in cell confluency would indicate an overall cytostatic effect, whereby all cells had left the cell cycle or cell proliferation was in equilibrium with cell death. On the other hand, reduced confluency over time would indicate cells had lifted off the surface of the well, suggesting they had died or were at least very unhealthy. Thus, a flat line on the growth curve was taken to mean cytostaticity and any drop compared to initial levels indicated cytotoxicity.

In another experiment designed to establish if the EA3-induced inhibitory effects were reversible or not, after approximately 48 h exposure to various concentrations of EA3, cells were washed twice with warm balanced salts solution (BSS) and the culture medium replaced with cell line-appropriate fresh medium. The CS system was used to track changes in confluency levels for two days during exposure to EA3 and for a further three days after it was removed. In the absence of extract, increased confluency over time would indicate surviving cells were capable of proliferation, suggesting any EA3-induced cytostatic effects were reversible.

6.2.4 Intracellular Ca^{2+} measurement

In these experiments, PC3, MDA-MB-468 or A549 cells ($2-3 \times 10^6$ cells in 1 mL) were seeded onto round (25 mm in diameter) collagen-coated cover slips in 35 mm TC petri dishes. Cells were allowed to adhere and grow for 24 to 48 h under standard culture conditions. Just prior to use, medium was removed and the cells washed three times with Physiological Rodent Saline (PRS, see section 2.2.3.16) before being passively

loaded with 1 mL of the fluorescent Ca^{2+} -sensitive dye, Fura-2, bound to an acetoxymethyl ester group (Fura-2-AM) to make it cell-permeant (Huang & Jan, 2001; Jackisch *et al.*, 2000; Bruschi *et al.*, 1988). Fura-2-AM is readily converted into the calcium-sensitive form within the cell through the activity of endogenous esterases so the non-ionic detergent and dispersing agent, Pluronic F-127, was employed to prevent Fura-2-AM precipitation and promote dye dispersion (Poenie *et al.*, 1986; Drummond *et al.*, 1987). Hence, the loading solution consisted of 3 μM Fura-2-AM and 0.06% Pluronic F-127 in PRS ($=1.8 \text{ mM } \text{Ca}^{2+}$). Cells were incubated with this dye mixture for 25 min at RT in the dark.

Following Fura-2 loading, cells were rinsed three times with PRS before being left to bathe in PRS for a further 30 min. These steps removed the remaining extracellular Fura-2-AM and allowed time for the complete de-esterification of the Fura-2-AM within the cells into the cell-impermeant Ca^{2+} -sensitive Fura-2 free acid form (Moore *et al.*, 1990). The cover slip was then mounted in a chamber and placed on the stage of the microscope attached to the Ca^{2+} measurement apparatus (Section 2.2.2.11).

Under the 40X objective, a group of approximately 5-15 viable cells were selected and these were illuminated at alternating excitation wavelengths of 340 and 380 nm (bandwidth 10 nm). Fluorescence emission at 510 nm was captured and approximately three measurements were recorded every second. Current output from the photomultiplier tube of the spectrophotometer was converted to voltage and amplified for display using the supplied Optoscan control system. The spectrophotometer was connected to a personal computer for subsequent data analyses which were performed with Cairn software (see Section 2.6.4).

Raw fluorescence measurements depend on variable or poorly quantified factors such as the efficiency of the instrument, the effective thickness of the cells in the optical beam, and the number of cells and the concentration of dye inside them (Grynkiewicz *et al.*, 1985; Moore *et al.*, 1990). However, the ratio of the fluorescence intensities at 340 and 380 nm (F_{340}/F_{380}) takes into account such variations between experiments and is a very good, well-established indicator of $[Ca^{2+}]_i$ in groups of cells, and even single cells (Grynkiewicz *et al.*, 1985; Wasilenko *et al.*, 1997; Devipriya *et al.*, 2006). Hence, $[Ca^{2+}]_i$ was taken as the F_{340}/F_{380} ratio (sometimes referred to simply as the “ratio”).

As $[Ca^{2+}]_i$ increases as cells become sick or apoptotic (Orrenius *et al.*, 1989), cells were only considered healthy if resting ratios were below the arbitrarily determined value of 0.6. After a baseline ratio was established for at least 1 min, individual test agents were added via a syringe system (50-100 μ L aliquots) and the trace recorded for 5 to 10 min. EA3 was added at a final concentration of 500 μ g/mL (w/v), while the negative control was simply the vehicle, DMSO at 0.5% final concentration (v/v). The positive control employed was the endogenous agonist histamine, which was added at a final concentration of 100 μ M.

At the end of each experiment, the maximal ratio for that particular population of Fura-2-loaded cells was measured by saturating Fura-2 with Ca^{2+} . This was achieved by adding 10 μ M ionomycin, a calcium ionophore that increases the permeability of the plasma membrane to Ca^{2+} , consequently equalising the concentration of extracellular Ca^{2+} ($[Ca^{2+}]_o$) and $[Ca^{2+}]_i$ (Liu & Hermann, 1978; Himmel *et al.*, 1990). F_{340}/F_{380} ratios were translated into $[Ca^{2+}]_i$ values using the Grynkiewicz equation (Grynkiewicz *et al.*, 1985):

$$[\text{Ca}^{2+}]_i = K_d \frac{(R - R_{\min})}{(R_{\max} - R)} \beta$$

where

K_d is the dissociation constant for Ca^{2+}

R is the measured F_{340}/F_{380} ratio at a particular point

$R_{\min}$ is the F_{340}/F_{380} ratio in the absence of Ca^{2+}

$R_{\max}$ is the F_{340}/F_{380} ratio in the presence of 1 mM Ca^{2+}

β is the ratio at 380 nm excitation for zero (nominally) Ca^{2+} and saturating Ca^{2+} .

Constants previously established in this laboratory for another cell line (C_2C_{12}) were a K_d of 224nM, a β value of 7.63 and $R_{\min}$ and $R_{\max}$ values of 0.335 and 2.75, respectively.

6.3 RESULTS

6.3.1 Chemical composition

According to LC-MS analysis, the major compounds identified in EA3, in order of proportion, were rutin, luteolin, quercetin, apigenin and naringenin (Figure 6.1). A few other prominent peaks could not be identified by Dr Wang without a standard, although their molecular weights were known (given in red).

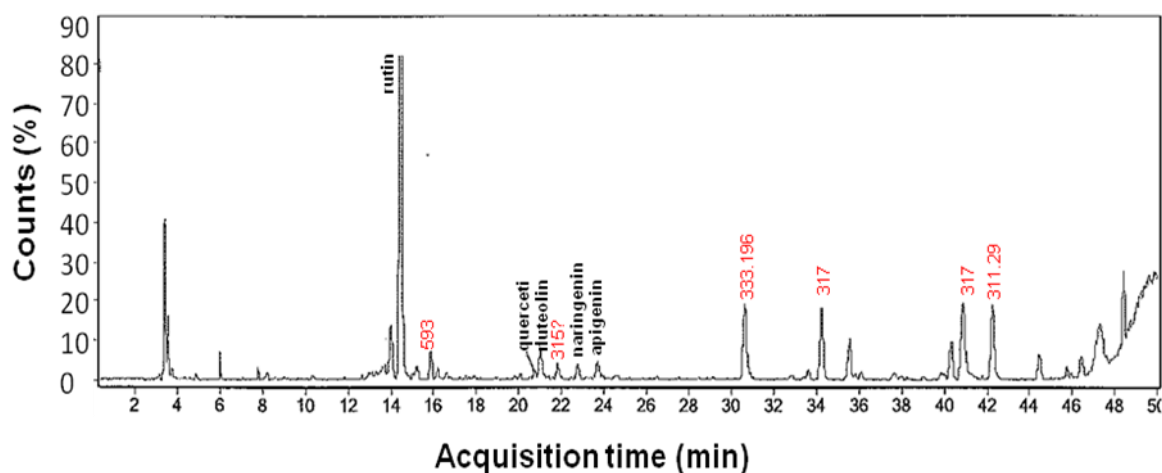


Figure 6.1 LC-MS chromatogram of EA3.

LC-MS was performed on EA3 using an Agilent Zorbax 80 Å, C18, 4.6 x 150 mm, 5 µm column and Electronic Spray Ionisation in negative mode. Source conditions were 350°C, 8 L/min gas flow with a scan time of 500 s and fragment voltage of 135V. The analyte was eluted by a gradient mobile phase system consisting of solvent A (acetonitrile) and solvent B (ammonia formate, adjusted to pH 3.0 with formic acid).

Based on the data derived from this LC-MS analysis, the absolute amounts of each of the main constituents present in EA3 were determined by HPLC-UV. The HPLC-UV chromatograms for EA3 compared to those of a standard mixture containing rutin, luteolin and apigenin are presented in Figure 6.2.

Retention times of the peaks in the sample corresponded to known compounds as presented in Table 6.1. The calculated percentage composition (w/w) is also shown.

Table 6.1 Identification of Peaks in Sample EA3

Retention time (min)	Compound name	Composition (g/100 g)
22.720	rutin	1.54
24.057	quercetin-3-glucoside	0.03
27.239	luteolin-7-glucoside	0.03
32.750	luteolin	0.03
34.250	apigenin	0.01

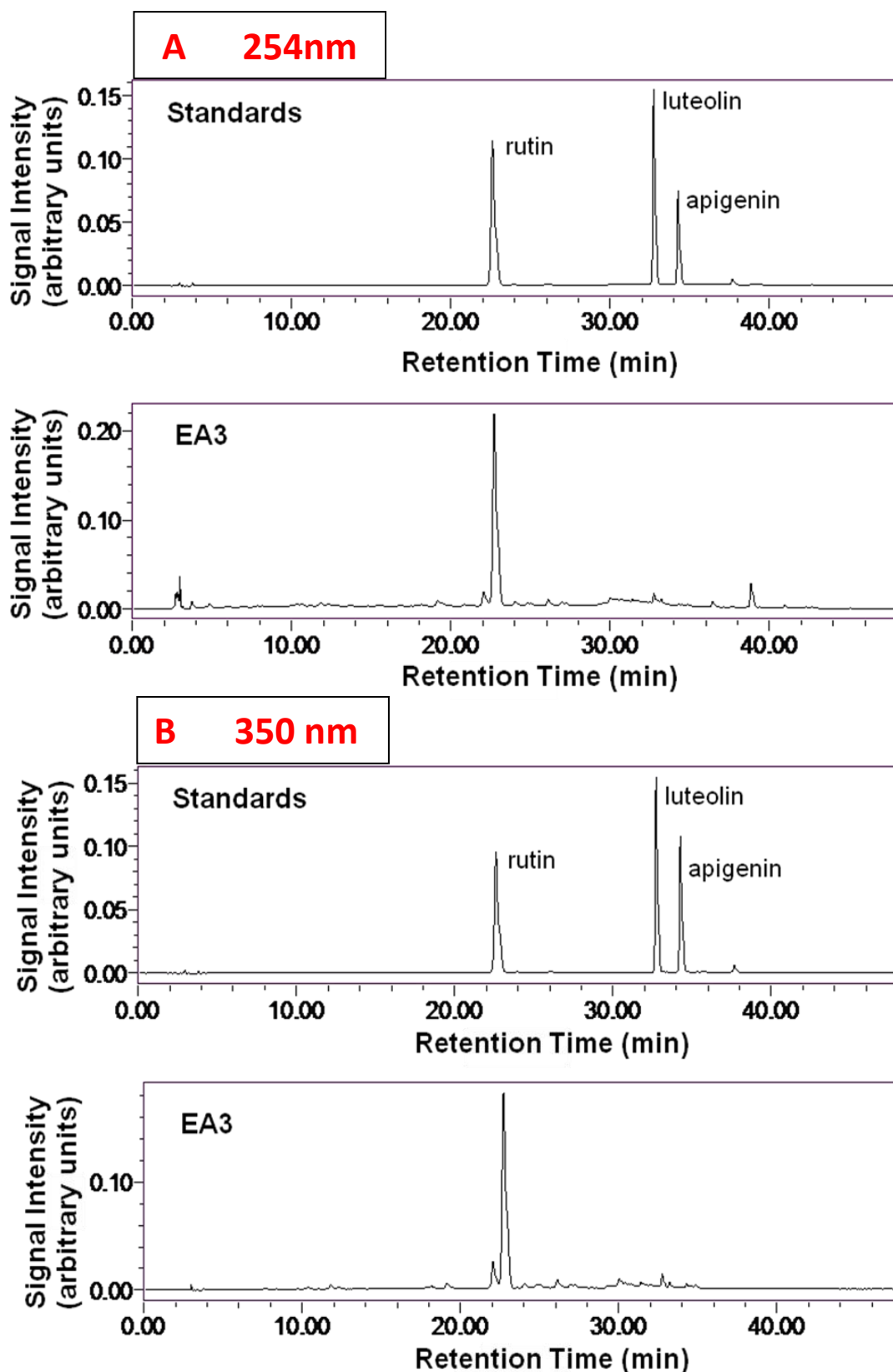


Figure 6.2 HPLC-UV chromatograms

HPLC chromatograms for EA3 compared to that of a standard mixture (containing rutin, luteolin and apigenin) after UV absorption at 254 (A) or 350 nm (B). HPLC-UV was performed on an Alliance Waters 2695 HPLC separation module pump with a Waters 2996 Photodiode Array Detector. Chromatography used an Apollo C18, 5 μ m 250 x 4.6 mm column (30°C). Solvent A was acetonitrile and solvent B was 0.6% H_3PO_4 in water.

6.3.2 Identification of active constituents

In order to determine if any of the main identifiable components of EA3 were likely responsible for its antiproliferative effects, human cancer cells were exposed to three purified compounds, rutin, luteolin and quercetin, for 48 h and their effects evaluated using the MTT assay and compared to those of the whole extract, EA3. The resultant dose-response curves are shown in Figures 6.3 to 6.6.

It is obvious from Figure 6.3 that, with the possible exception of MDA-MB-468 cells, rutin had no inhibitory effect on the proliferation of any cell line tested. If anything, it elicited a small stimulatory effect at lower doses (hormesis) against MCF7, Caco-2 and MRC5 cells. On the other hand, luteolin (Fig. 6.4) and quercetin (Fig. 6.5) both inhibited the proliferation of every cell line tested in a dose-dependent manner. For both these pure compounds, the greatest effects were seen against PC3 cells. Hormetic effects were observed for luteolin against MCF7 and Caco-2 cells, while quercetin caused hormesis against Caco-2, A549 and MRC5 cells.

However, given that the percentage of luteolin in the whole extract (EA3) is only 0.06% (Table 6.1), an EA3 concentration of 100 $\mu\text{g/mL}$ corresponds to only 0.06 $\mu\text{g/mL}$ luteolin. This value is over 40-fold less than 2.5 $\mu\text{g/mL}$, the lowest concentration of luteolin tested in dose-response studies and which caused no inhibition of proliferation of any cell line (Fig. 6.4). Quercetin was present in EA3 at half this level again, equating to 80-fold less than the minimum concentration tested (Fig. 6.5).

Looking at these data another way, the highest level of pure luteolin tested, 100 $\mu\text{g/mL}$, corresponded to an EA3 concentration of 167 mg/mL, 334-fold higher than the highest concentration tested, 500 $\mu\text{g/mL}$ (Fig. 6.6). Similarly, 100 $\mu\text{g/mL}$ quercetin

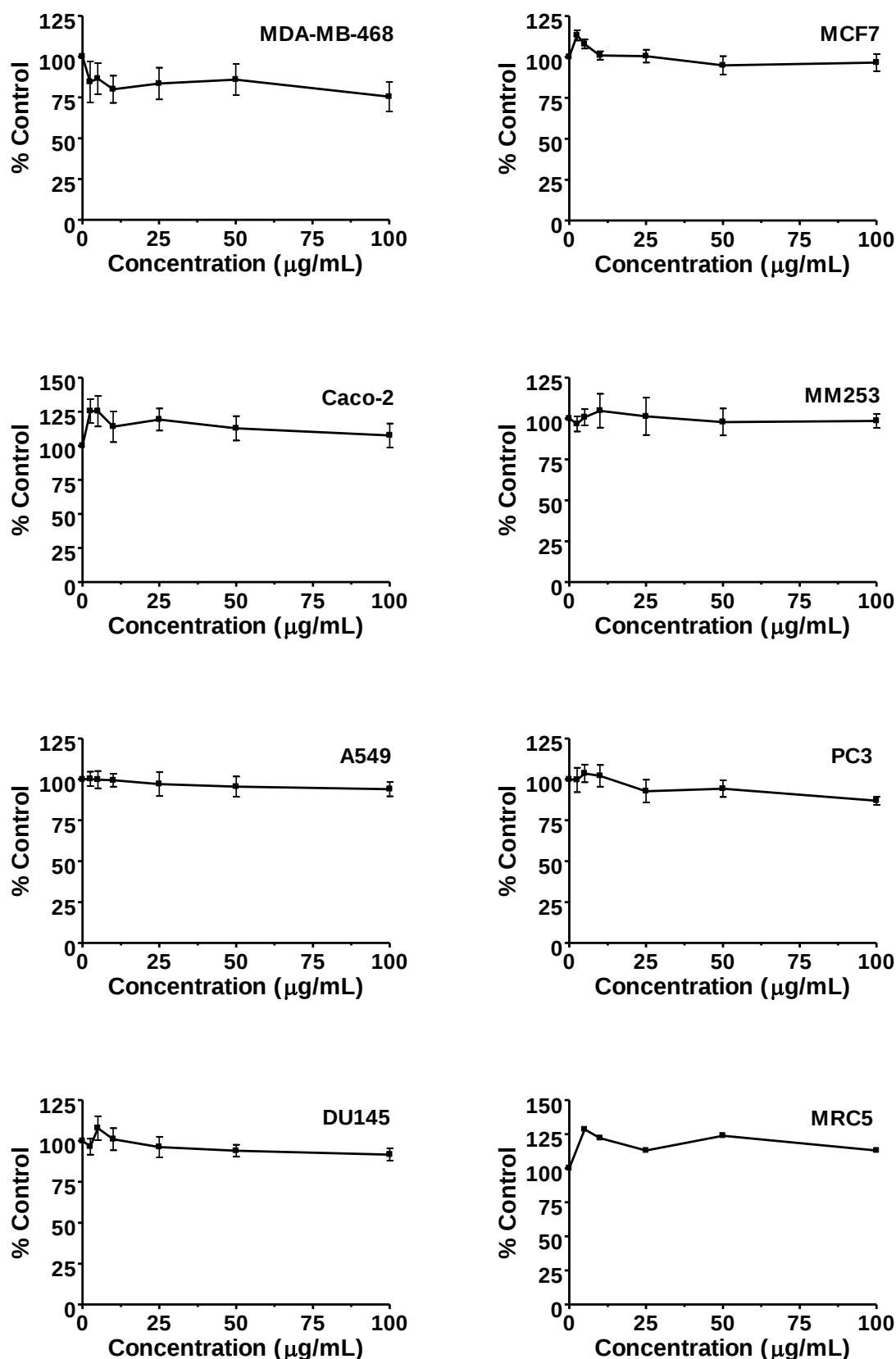


Figure 6.3 Effects of rutin on cell proliferation

Cells were exposed to various concentrations of rutin for 48 h before being subjected to the MTT assay. Results are means $\pm$ SE of at least 4 separate experiments, except for MRC5, which are the values obtained from a single experiment only.

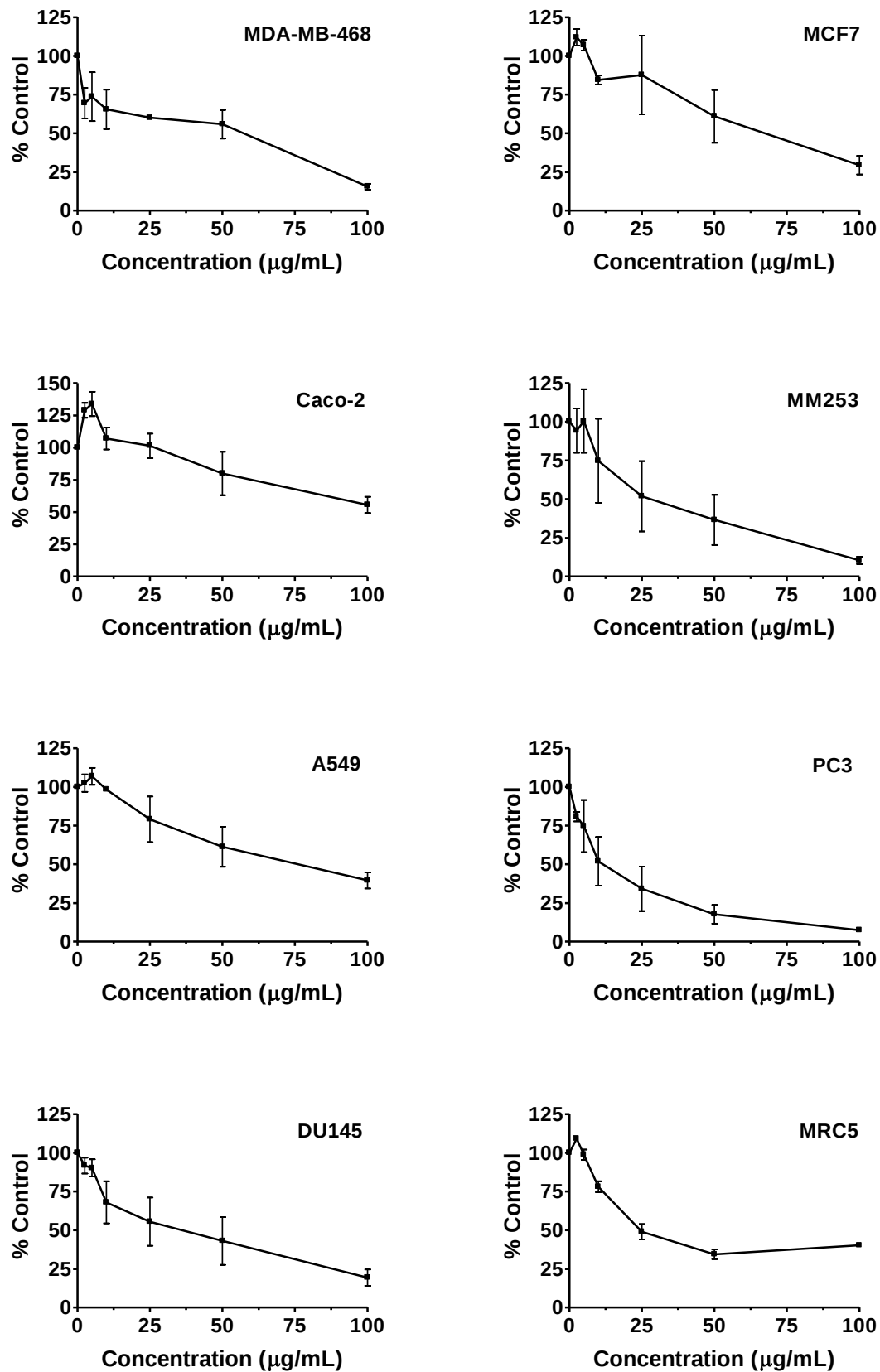


Figure 6.4 Effects of luteolin on cell proliferation

Cells were exposed to various concentrations of luteolin for 48 h before being subjected to the MTT assay. Results are means $\pm$ SE of at least 3 separate experiments.

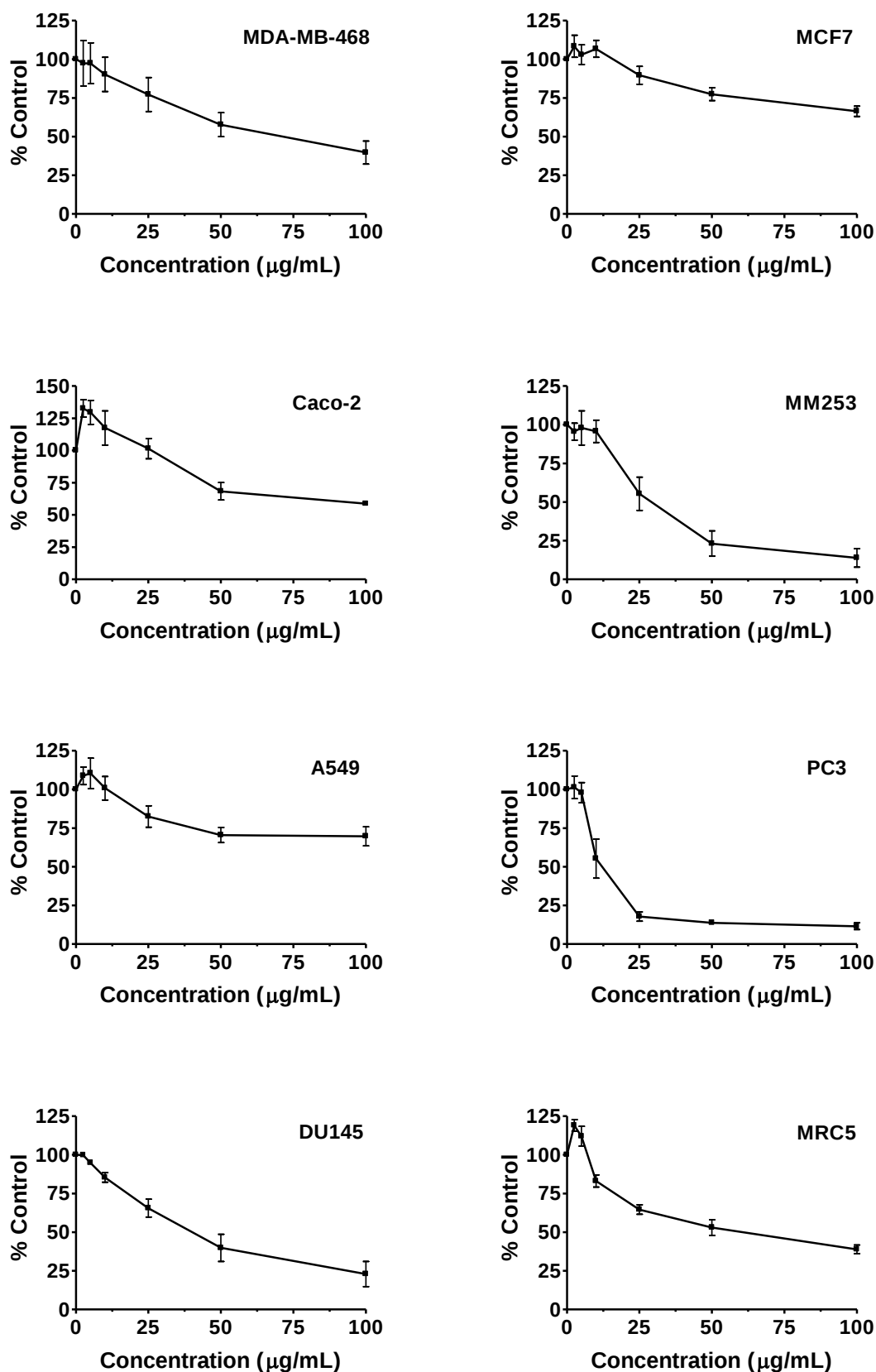


Figure 6.5 Effects of quercetin on cell proliferation

Cells were exposed to various concentrations of quercetin for 48 h before being subjected to the MTT assay. Results are means ± SE of at least 4 separate experiments.

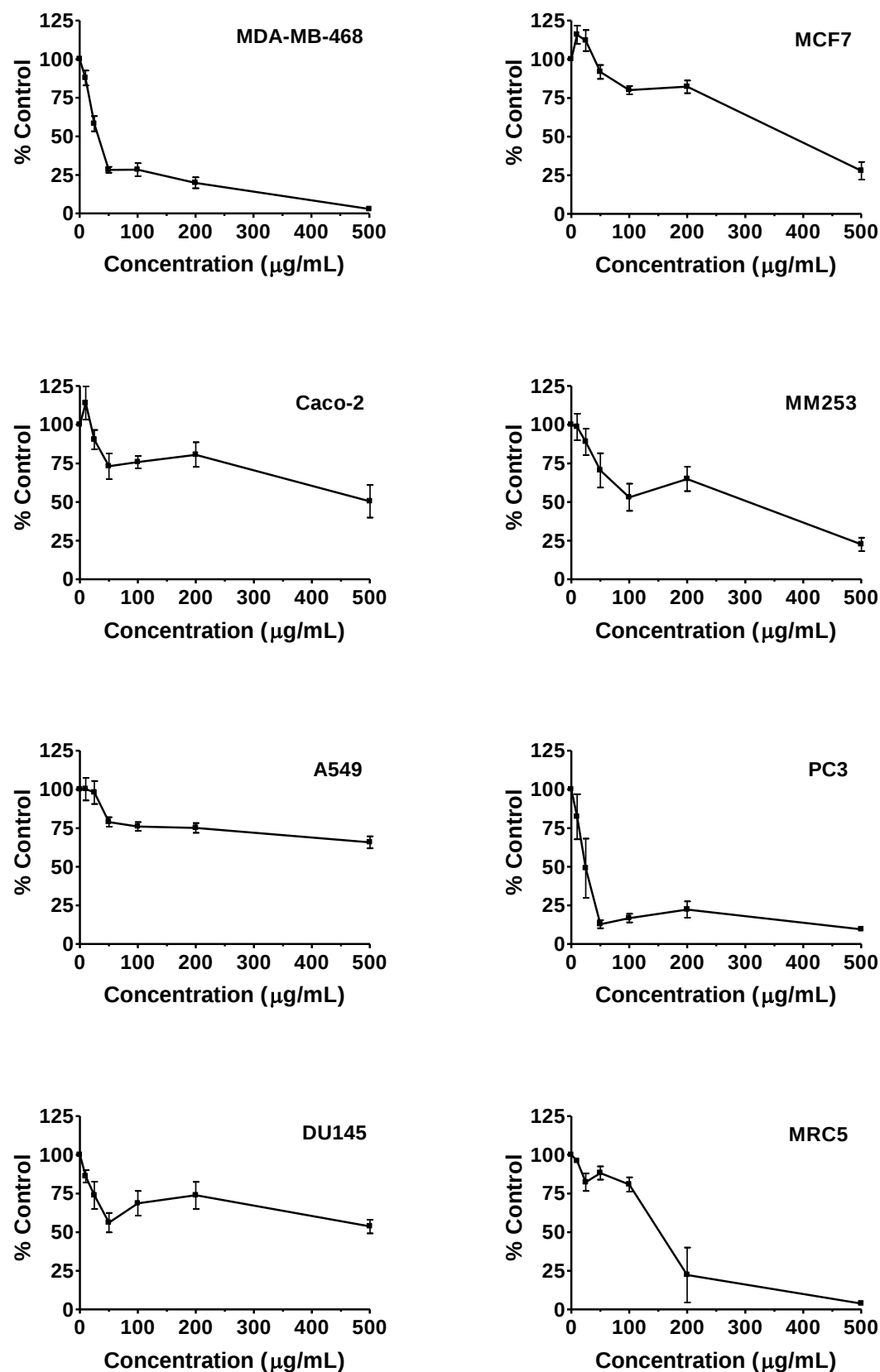


Figure 6.6 Effects of EA3 on cell proliferation

Cells were exposed to various concentrations of EA3 for 48 h before being subjected to the MTT assay. Results are means $\pm$ SE of at least 5 separate experiments.

corresponded to 333 mg/mL of EA3, over 666-fold more than was tested. In other words, although the dose-response curves of luteolin (Fig. 6.4) and quercetin (Fig. 6.5) appear similar in shape to that of the whole extract, EA3 (Fig. 6.6), they are not directly comparable.

Therefore, the concentrations of pure luteolin or quercetin required to induce a given level of inhibition were compared to the concentration of EA3 required to produce the same effect. For example, 50 µg/mL luteolin and 25 µg/mL quercetin produced similar degrees of inhibition of proliferation of PC3 cells as EA3 at 100 µg/mL (17% of control). Given the concentrations of luteolin and quercetin in EA3 are 0.06% and 0.03% (w/w), respectively, this means that these concentrations of pure luteolin and quercetin each correspond to approximately 83 mg/mL of EA3, 830-fold more than what was actually required to produce the same effect. Similar values were obtained for other selected inhibitory effects in PC3 cells and other cell lines. Hence, the magnitude of the observed cytotoxic effects of EA3 cannot be accounted for by luteolin or quercetin acting alone. Even if the individual effects of luteolin and quercetin were added together, this would only explain 1/415 of the observed cytotoxicity in these cells.

From these dose-response curves, IC_{50} values were determined, where possible, using GraphPad Prism software as described in Section 4.3.3. The fitted curves obtained by using the optimum model for each compound- (or the whole extract, EA3-) cell line combination are depicted in Figure 6.7. The IC_{50} values calculated in this way and related data are tabulated on the following page (Table 6.2). Of the five main constituents of EA3, luteolin and quercetin were the most active with IC_{50} values below 100 µM in Caco-2 cells after 48 h exposure.

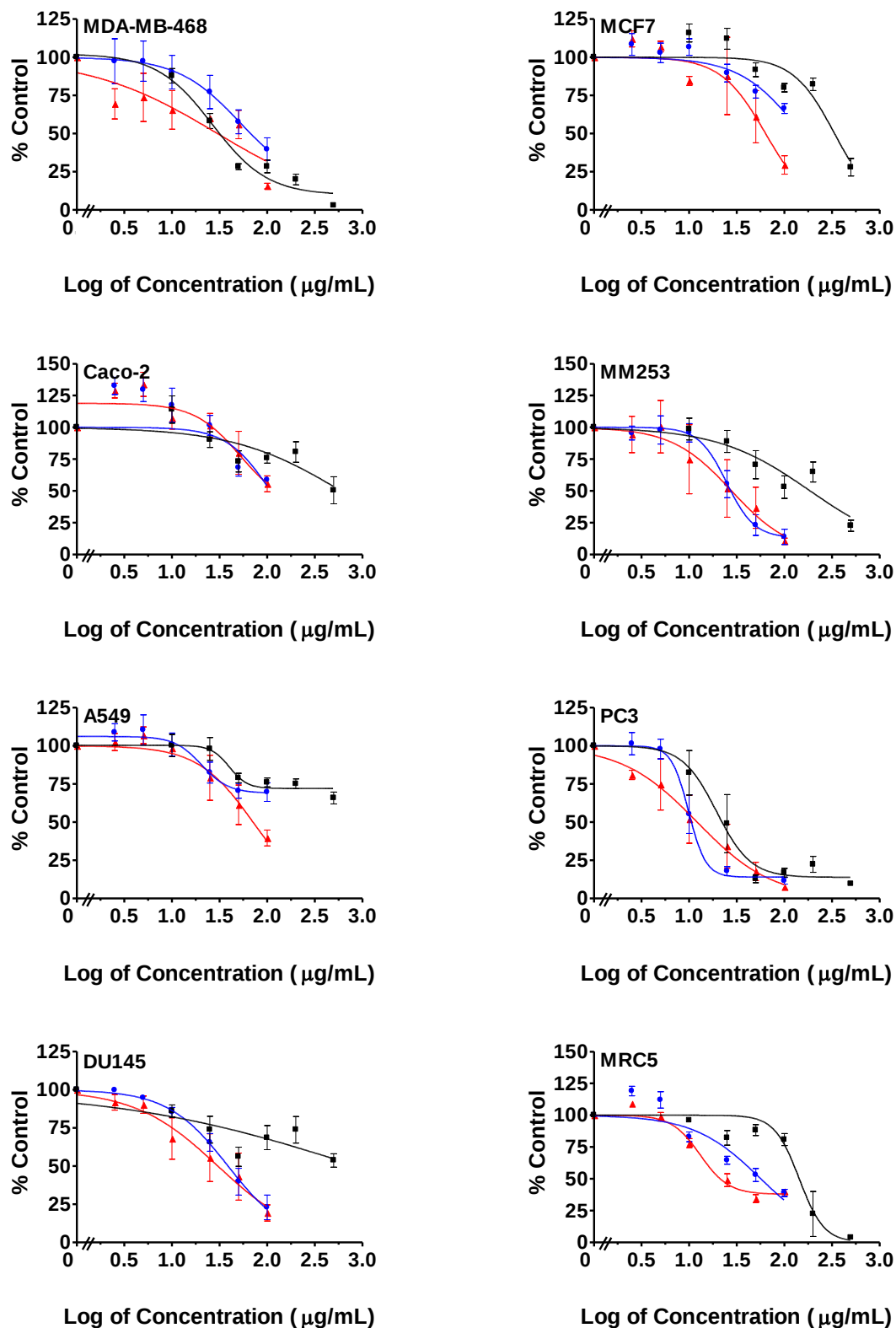


Figure 6.7 Nonlinear regression of pure compounds and Extract EA3 using the optimum model for each compound or extract-cell line pair.

Cells were exposed to various concentrations of the pure compounds, luteolin ($\blacktriangle$) or quercetin ($\bullet$), or the whole extract, EA3 ($\blacksquare$), as described above (Figs 6.3 -6.6). Dose values were transformed into $\log_{10}$ values and curves were fitted using Graph Pad Prism nonlinear regression dose-response (variable slope) according to the optimum method for each compound- or extract- cell line combination.

Table 6.2 IC₅₀ Values Calculated Using Optimum Model for Individual Cell Line and Pure Compound or Extract Combinations

MDA-MB-468	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Best Model	r ²	Hormesis present
Luteolin ▲	30.0	10.9 – 82.6	TB	0.791	
Quercetin ●	51.9	26.5 – 101.9	4P	0.998	
EA3 ■	26.6	11.4 – 62.0	4P	0.973	

MCF7	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Best Model	r ²	Hormesis present
Luteolin ▲	63.4	44.5 – 90.3	TB	0.927	*
Quercetin ●	151.7	68.6 – 335.9	TB	0.864	
EA3 ■	335.9	202.5 – 557.0	TB	0.871	*

Caco-2	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Best Model	r ²	Hormesis present
Luteolin ▲	51.8	0.421 - 6386	4P	0.823	**
Quercetin ●	31.8	9.14 – 110.7	4P	0.868	**
EA3 ■	595.3	130.9 - 2708	TB	0.735	*

MM253	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Best Model	r ²	Hormesis present
Luteolin ▲	27.9	21.1 – 36.8	TB	0.976	
Quercetin ●	24.7	21.9 – 27.9	T	0.997	
EA3 ■	186.0	95.3 – 362.8	TB	0.891	

A549	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Best Model	r ²	Hormesis present
Luteolin ▲	70.9	55.6 – 90.6	TB	0.969	
Quercetin ●	20.4	10.0 – 41.6	4P	0.961	*
EA3 ■	40.0	20.6 – 77.4	4P	0.949	

PC3	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Best Model	r ²	Hormesis present
Luteolin ▲	12.1	9.78 – 15.0	TB	0.989	
Quercetin ●	9.86	9.29 - 10.5	T	0.998	
EA3 ■	19.4	13.2 – 28.4	T	0.975	

DU145	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Best Model	r ²	Hormesis present
Luteolin ▲	30.3	22.7 – 40.4	TB	0.976	
Quercetin ●	38.8	36.0 – 41.7	TB	0.998	
EA3 ■	864.4	55.0 - 13592	TB	0.674	

MRC5	IC ₅₀ (μg/mL)	IC ₅₀ Range (95% CI) (μg/mL)	Best Model	r ²	Hormesis present
Luteolin ▲	13.0	8.15 – 20.8	T	0.976	
Quercetin ●	56.5	27.5 – 115.9	TB	0.843	*
EA3 ■	143.2	108.8 – 188.4	TB	0.951	

*at least one concentration > 110% of control; ** > 120% of control

6.3.3 Exposure and recovery

Using the CS system, the effects of EA3 on various cancer cell lines were monitored over time. Figure 6.8 is a representative graph depicting how different concentrations of EA3 affected the proliferation of cells over the course of 48 h culture at 37°C. From these graphs, the doubling times of the various cell lines were calculated (see Section 3.3.1) with and without EA3 (Fig. 6.9). For simplicity, this figure does not include the doubling times calculated for all concentrations of EA3.

From Figures 6.8 and 6.9 it is clear that EA3 did, indeed, inhibit the proliferation of all cell lines tested in a concentration and time-dependent manner. The inhibitory effects began within 90 min of exposure in the most susceptible cell lines, MDA-MB-468, MCF7, PC3, DU145 and MRC5. It was assumed that, following the addition of extract, a growth curve with a lower slope was indicative of a reduced rate of proliferation, while a flat growth curve implied complete inhibition of proliferation (or rate of cell proliferation = cell death). This assumption was supported by the very high, or incalculable, doubling times obtained from flat growth curves of some cell lines after EA3 exposure. Thus, the highest concentration used, 500 µg/mL, completely inhibited the proliferation of MDA-MB-468, MCF7, PC3, DU145 and MRC5 cells within 48 h. However, the same concentration only retarded the growth of the other three cell lines. MCF7 and PC3 cell proliferation was also totally inhibited by 200 and 100 µg/mL concentrations of EA3, although this effect required longer to manifest.

In a separate experiment, after 48 h exposure to various concentrations of EA3, the extracts were washed out, the medium replaced and the cells monitored for recovery over several more days. In this way, if the EA3-induced inhibition of cell proliferation was due to merely cytostatic effects, it could be ascertained if these effects were reversible or not. Data from a representative experiment are shown in Figure 6.10.

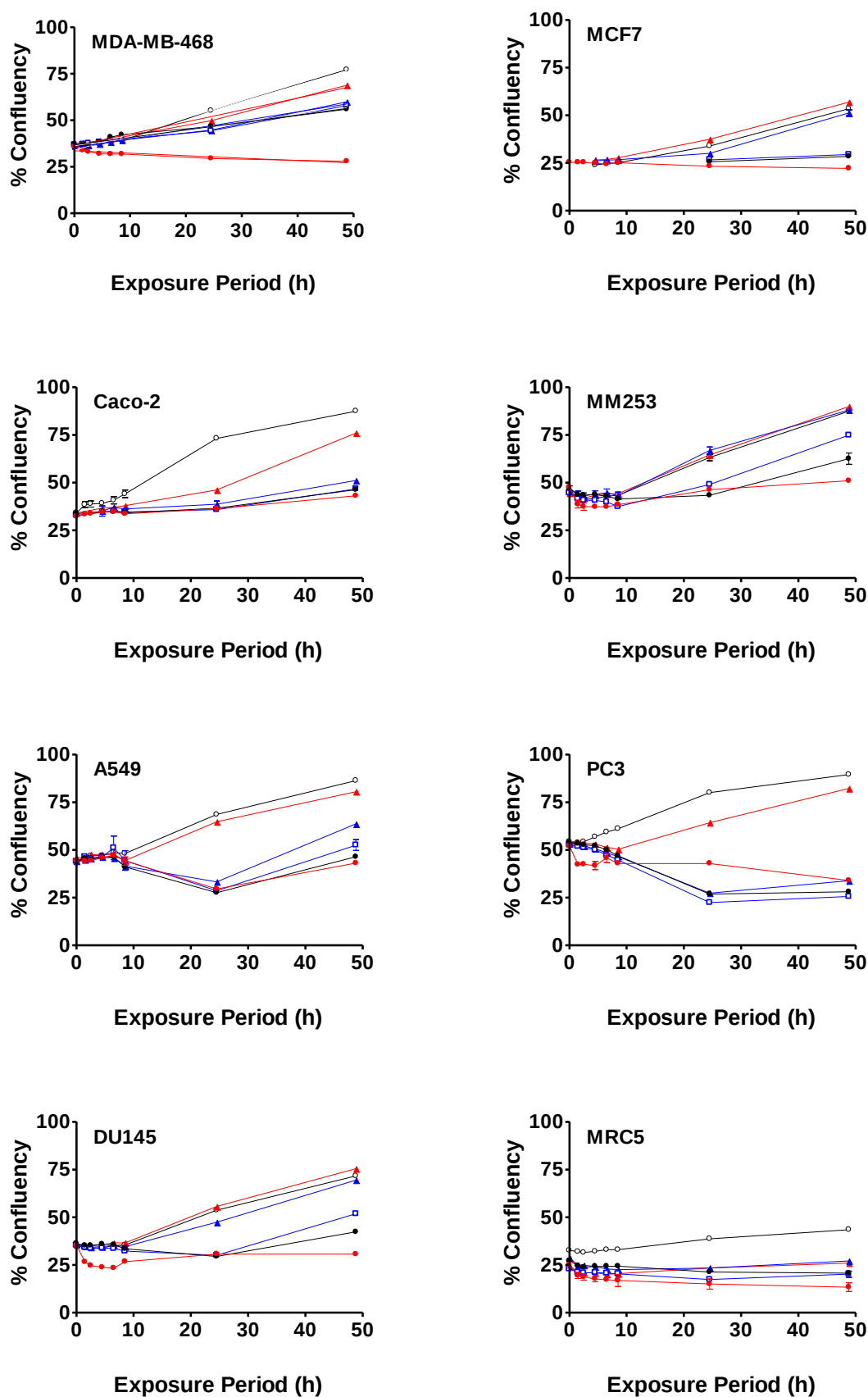


Figure 6.8 Effect of exposure time of EA3 on cell proliferation (CS system).

Cells were exposed to various concentrations of EA3, 0 (○), 25 (▲), 50 (▴), 100 (□), 200 (●) or 500 (●) µg/mL, and their confluency monitored by the Cellscreen system over time. Results are the means $\pm$ SE of quadruplicate wells of one representative experiment.

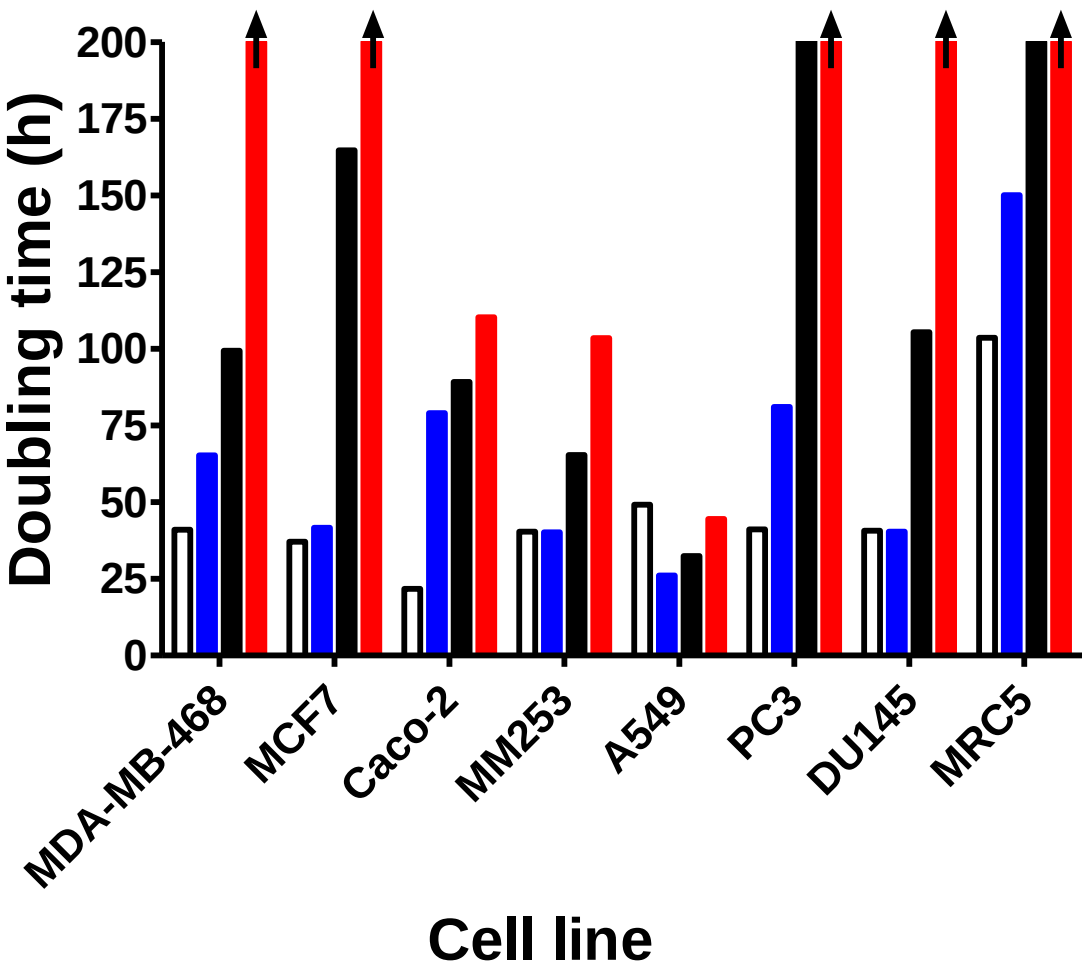


Figure 6.9 Effect of EA3 on cell doubling times.

Cells were exposed to various concentrations of EA3, 0 (□), 50 (■), 100 (■) or 500 (■) µg/mL, and their confluency monitored by the Cellscreen system over time (Fig. 6.8). Percent confluency values were transformed into logarithmic values and the doubling times calculated using the slope of the resultant linear regression lines fitted to data points over the final 40 h of exposure, as described in Section 3.3.1.

↑ denotes the calculated doubling time was above 200 µg/mL.

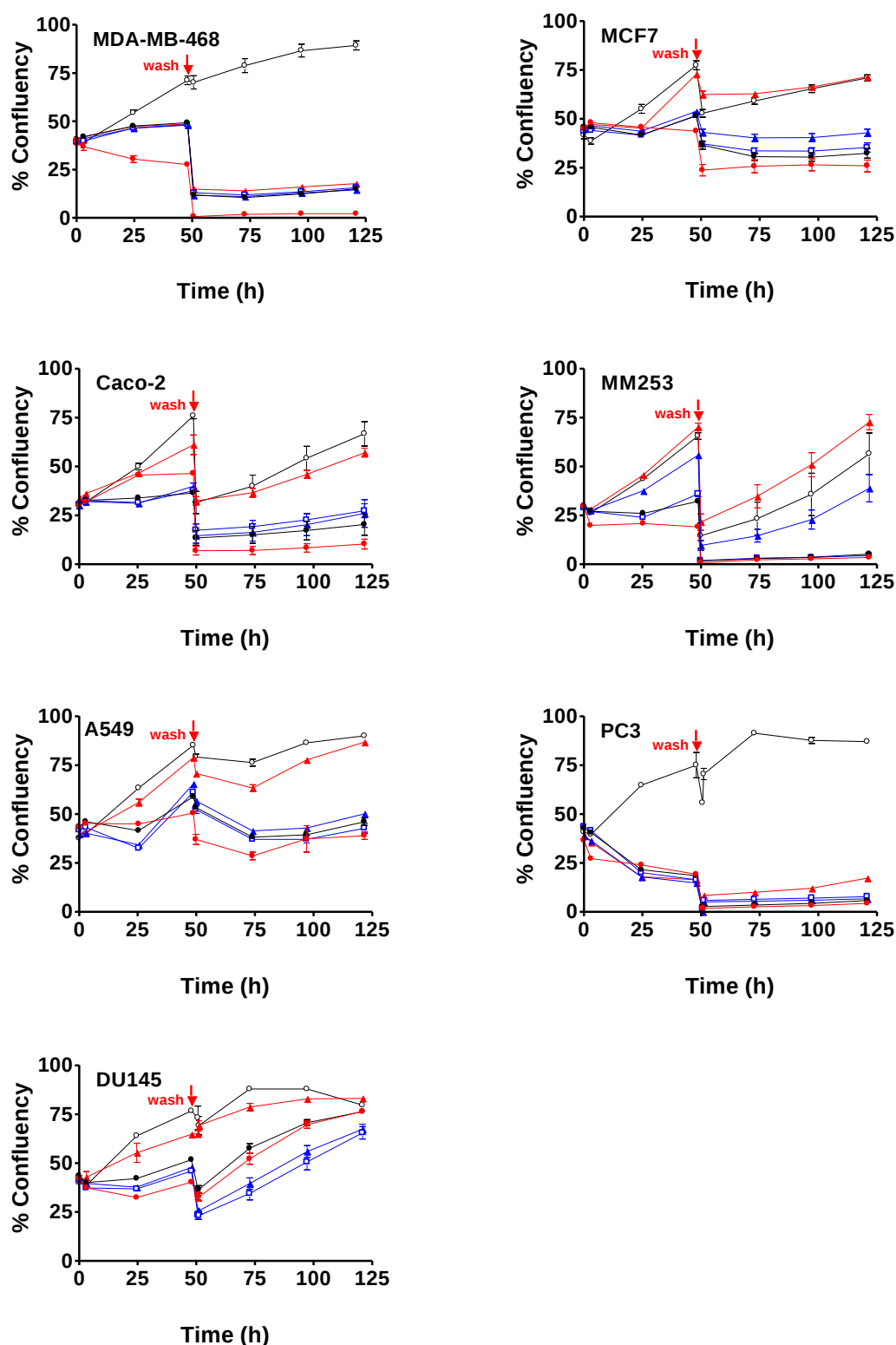


Figure 6.10 Reversibility of inhibitory effects of EA3 on cancer cell proliferation.

Cells were exposed to various concentrations of EA3, 0 ($\circ$), 25 ($\blacktriangle$), 50 ($\blacktriangle$), 100 ($\square$), 200 ($\bullet$) or 500 ($\bullet$) $\mu\text{g/mL}$, and their confluency monitored by the Cellscreen system over time. After 48 h exposure, EA3 was washed off ($\downarrow$), the medium replaced and the cells allowed to recover. Results are the means $\pm$ SE of quadruplicate wells of one representative experiment.

From Figure 6.10 it can be seen that the inhibitory effects of EA3 were sometimes, but not always, irreversible. After EA3 was removed, Caco-2, MM253, A549 and DU145 cells resumed proliferation within the 72 h period assessed. This resumption of growth was most evident when low concentrations of EA3 were used, but was also noticeable even when these cells were exposed to the highest concentration of EA3, 500 µg/mL. On the other hand, MDA-MB-468, MCF7 and PC3 cells did not recover from exposure to EA3 even at 50 µg/mL within 72 h of its removal.

It must be noted that the wash step itself removed many cells as evidenced by the drop in confluency between 48 and 50 h even when no EA3 was present. This effect was most noticeable in Caco-2 and MM253, but the remaining cells rapidly recovered, which was not the case for EA3-induced cells.

6.3.4 Intracellular Ca^{2+} measurement

The effect of EA3 on intracellular Ca^{2+} signalling was then examined in PC3 cells. When PC3 cells were exposed to 500 µg/mL EA3, $[\text{Ca}^{2+}]_i$ significantly increased and then gradually declined, plateauing between 5 min and 10 min post-peak (Fig. 6.11A). The mean data obtained from all the experiments on PC3 cells in which a response was recorded (n=5) are presented in Table 6.3. ANOVAs (Student Newman-Keuls, SNK) revealed significant differences between the mean resting ratio and the peak ratio after addition of EA3 ($p < 0.001$). Significant differences ($p < 0.001$) were also found between the mean resting ratio and the ratios at 5 min ($P < 0.01$) and 10 min ($P < 0.001$) post-peak, and between the mean peak ratio and the ratios 5 min and 10 min post-peak ($p < 0.001$), indicating that the ratio initially declined, but did not return to the resting level within the period tested. There was no significant difference between the ratios at 5 min and 10 min post-peak. This response was a consistent result, with a transient increase in the ratio observed in 13 of 14 tests using different cancer cell lines.

It is possible that the intracellular Ca^{2+} remained high for a considerable time, as in one experiment cells were monitored for 60 min after addition of EA3 and the ratio was still 0.269 ($= 231.5\text{nM } \text{Ca}^{2+}_i$) above the original baseline level observed before addition of EA3.

Histamine (100 μM) was employed as a positive control for a change in cytosolic Ca^{2+} in these experiments. Histamine at this concentration exhibited large transient change in intracellular Ca^{2+} in 5 out of 5 experiments in this study. A typical example is presented in Figure 6.11B. Mean data are presented in Table 6.3. Histamine induced a significant ($P < 0.01$) increase in $[\text{Ca}^{2+}]_i$, but the ratio then declined steadily towards the baseline level. While there was no difference between the mean peak ratio and the ratio 5 min post-peak, ten minutes after the peak the ratio was statistically similar to the resting ratio ($P < 0.05$). There was no difference between the ratios 5 and 10 min post-peak.

Statistical tests showed that, in PC3 cells, there was no significant difference ($P > 0.05$) between the mean values obtained for the Ca^{2+} responses elicited after exposure to EA3 and histamine for most parameters measured when individual two-tailed t-tests (with Welch correction) were performed. Importantly, neither the mean amplitude (peak normalised for baseline) nor the time taken to reach the peak (TTP) induced by histamine was significantly different from the values obtained for the equivalent EA3 measurements. The only parameter in which a significant difference was recorded was the ratios 5 min post-peak. Overall, the Ca^{2+} response of EA3 was very similar to the histamine-induced response, although the histamine-induced Ca^{2+} transient gradually returned to baseline within 10 min while the EA3 Ca^{2+} response was still significantly elevated compared to the original resting $[\text{Ca}^{2+}]_i$ level observed after 10 min.

Similar trends were observed when EA3 was added to MDA-MB-468 and A549 cells (data not shown). However, the resting ratios were higher in these cells and peak ratios were also correspondingly higher. Additionally, more time was required to reach peak values. The effects of histamine and ionomycin were not evaluated in these cell lines.

Table 6.3 Mean $\pm$ SE Data from F_{340}/F_{380} Traces of PC3 Cells

	EA3 (500 μg/mL)	Histamine (100 μM)
Resting Ratio	0.421 ± 0.013	0.503 ± 0.033
Peak Ratio	0.719 # ± 0.028	0.722 † ± 0.055
Peak + 5 min	0.528 # ^ ± 0.006	0.632 * ± 0.034
Peak + 10 min	0.563 # ^ ± 0.026	0.565 ‡ ± 0.041
Amplitude	0.297 ± 0.031	0.219 ± 0.033
TTP (s)	98.2 ± 10.8	107.8 ± 35.9
Peak $[Ca^{2+}]_i$ Increase (nM)	260 ± 30.0	198 ± 61.0
% of Maximal	36.3 ± 5.3	26.8 ± 4.9

The effects of EA3 and histamine exposure on $[Ca^{2+}]_i$ in Fura-2-loaded PC3 cells ($n=5$). The *Resting Ratio* is the basal F_{340}/F_{380} , the *Peak Ratio* is the maximum ratio attained, the *Amplitude* is the peak ratio minus the resting ratio, and *TTP* (“time to peak”) is the duration of time taken to reach the peak. *Peak + 5 min* and *Peak + 10 min* are the ratios at 5 min and 10 min post peak. *Peak $[Ca^{2+}]_i$ Increase* is an estimation of the maximal rise in $[Ca^{2+}]_i$, calculated using the Grynkiewicz equation. Finally, *% of Maximal* values are changes in $[Ca^{2+}]_i$ as a percentage of the fluorophore’s total capacity for Ca^{2+} , calculated using the mean ratios recorded upon addition of 10 μ M ionomycin. ANOVAs (SNK) were performed to assess changes in $[Ca^{2+}]_i$ for both EA3 and histamine. For EA3, # denotes a significant ($P<0.01$) difference between the value and the Resting Ratio, while ^ means the value is significantly ($P<0.001$) different from the Peak Ratio. For histamine, † denotes a significant ($P<0.01$) difference from the Resting Ratio, while ‡ means a significant ($P<0.05$) difference from the Peak Ratio. Significant ($P<0.05$) differences between histamine values and the corresponding EA3 value are denoted * (t-tests with Welch correction).

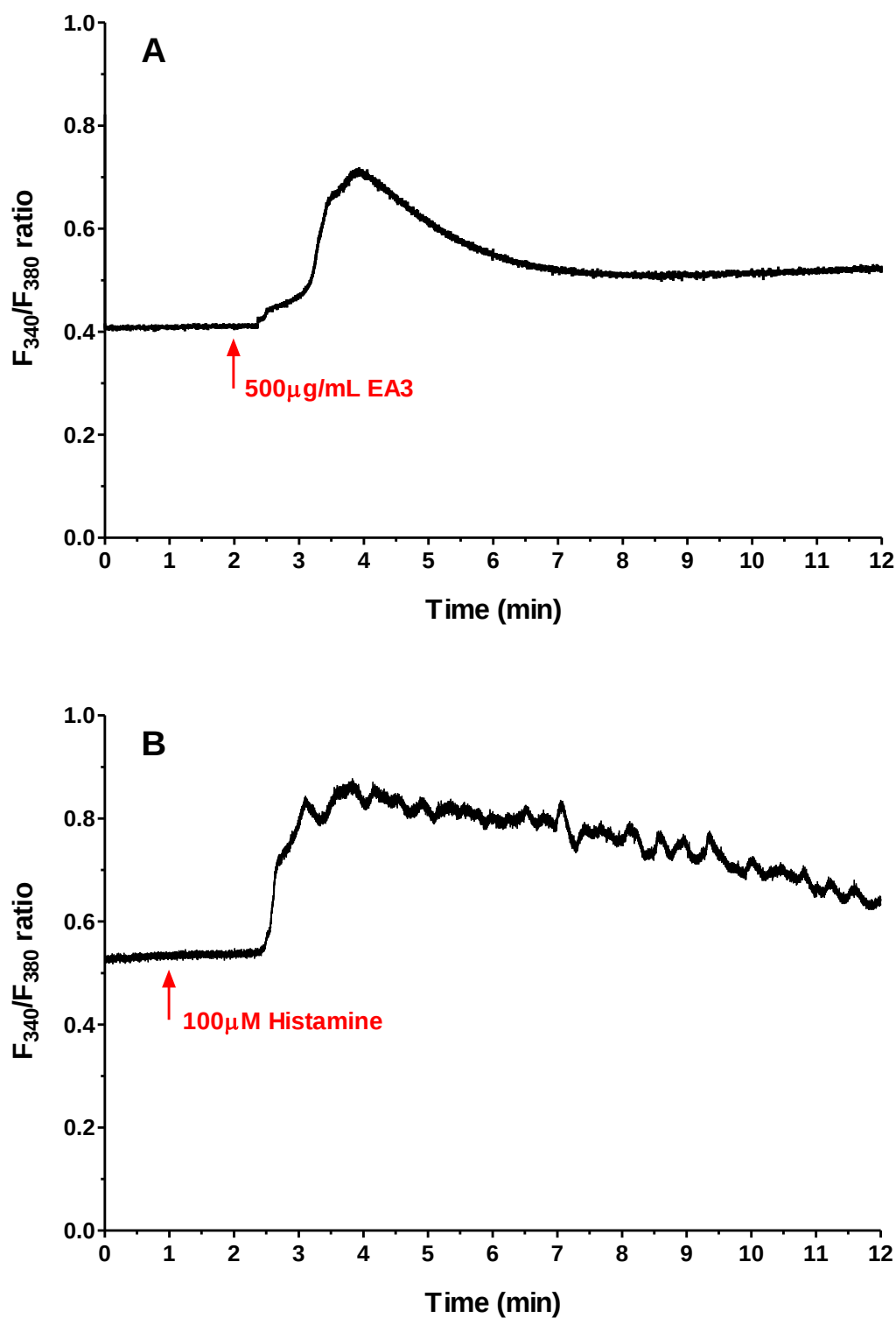


Figure 6.11 Representative traces showing effects of EA3 and histamine on intracellular Ca^{2+} levels.

500 $\mu\text{g/mL}$ EA3 (A) or 100 μM histamine (B) was added to a group of approximately 10 Fura-2-loaded PC3 cells and the fluorescence at 340 nm and 380 nm recorded for 10 min past the peak fluorescence value. The traces shown are the ratios of F_{340}/F_{380} and are indicative of $[\text{Ca}^{2+}]_i$.

The positive control for inhibition of cell proliferation used most often in this project (DFO) had a minimal effect on $[Ca^{2+}]_i$ when 1 mM was added to a group of 5-15 PC3 cells. In one experiment, a response was observed, but the peak ratio was only 0.085 above that of the resting ratio. This peak occurred 101s after addition of DFO and corresponded to a 20.6% increase in $[Ca^{2+}]_i$, representing just 6.5% of the total possible. The change in $[Ca^{2+}]_i$ was estimated to be only 67.0 nm.

To eliminate the prospect that the EA3- and histamine-induced Ca^{2+} responses were simply due to the vehicle, DMSO at 0.5% final concentration was added to PC3 cells. As shown in the representative trace, DMSO had no effect on the F_{340}/F_{380} ratio and, hence, $[Ca^{2+}]_i$ of these cells (Fig. 6.12). This lack of response was shown in 12 samples.

Also exemplified by Figure 6.12, 10 μ M ionomycin, a Ca^{2+} ionophore, consistently resulted in an immediate and dramatic increase in the F_{340}/F_{380} ratio to a level which was sustained for the remainder of the test period on 13 occasions. The amplitude of the peak ratio was significantly greater ($P < 0.001$) than the peak ratios obtained with both 500 μ g/mL EA3 and 100 μ M histamine.

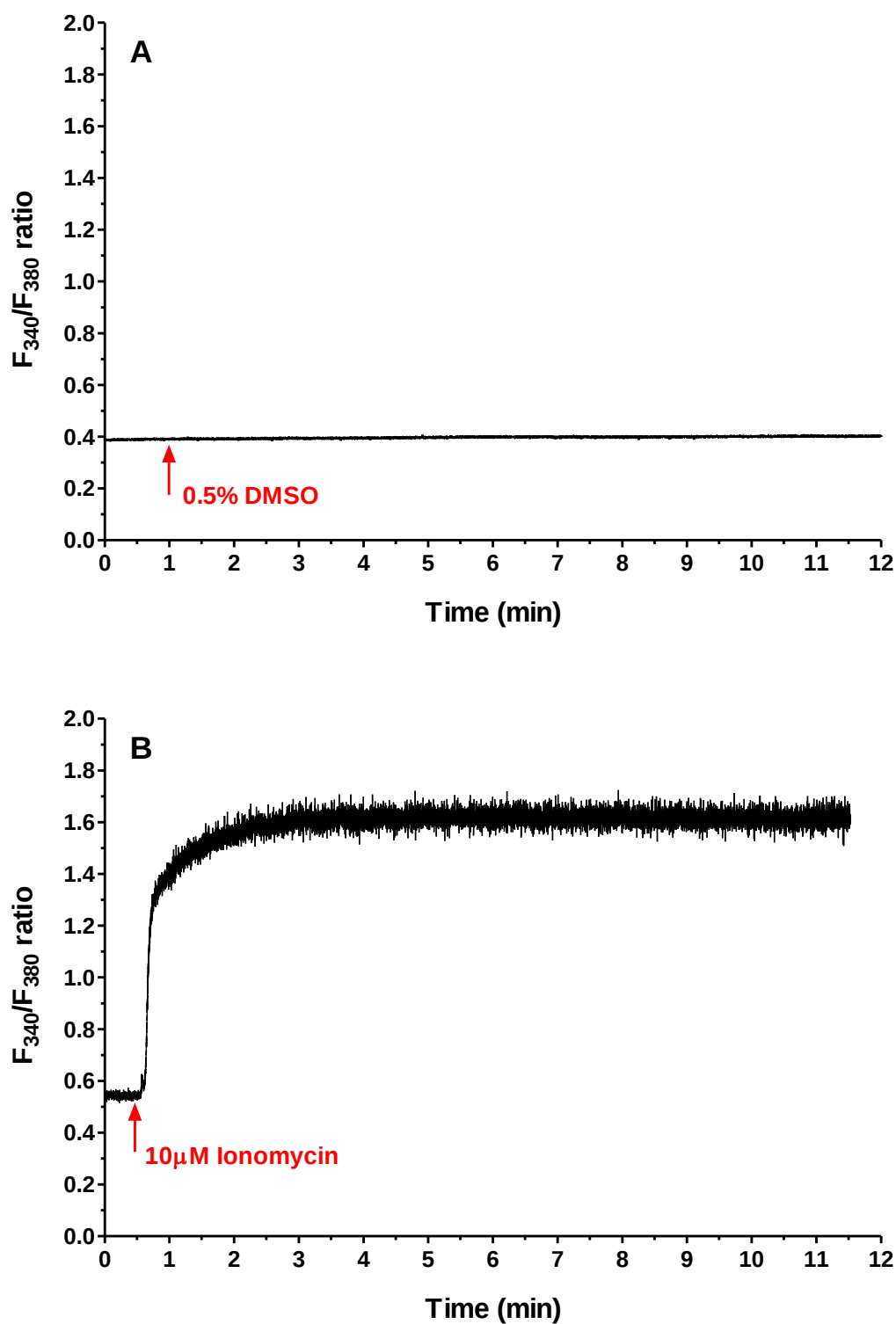


Figure 6.12 Representative traces showing effects of DMSO and ionomycin on intracellular Ca^{2+} levels.

0.5% DMSO (A) or 10 μ M ionomycin (B) was added to a group of approximately 10 Fura-2-loaded PC3 cells and the fluorescence at 340 nm and 380 nm recorded for 10 min past the peak fluorescence value. The traces shown are the ratios of F_{340}/F_{380} and are indicative of $[\text{Ca}^{2+}]_i$.

6.4 DISCUSSION

6.4.1 Chemical composition

The main constituents of EA3 identified by LC-MS were rutin, luteolin, quercetin, apigenin and naringenin. These compounds are all flavonoids, sometimes known as bioflavonoids, the largest and most important group of polyphenolic compounds that are universally present in flowering plants (Kuntz *et al.*, 1999; Brusselmans *et al.*, 2005). Flavonoids are one of the largest groups of plant secondary metabolites, with over 6,500 different compounds identified to date, although it has been speculated that thousands more are likely to exist (Ververidis *et al.*, 2007). They are primarily recognised as the molecules responsible for the red, yellow and orange pigments of many plant parts and are consumed regularly as part of the human diet (Middleton *et al.*, 2000). For example, flavonoids are prominent components of citrus fruits, but they are also found in other fruits, vegetables, seeds, herbs, spices as well as in tea and red wine (Middleton *et al.*, 2000). Flavonoid compounds also play important roles as plant defence mechanisms (Ververidis *et al.*, 2007). Flavonoids are low molecular weight compounds comprised of a basic 3-ring structure with various substitutions as seen in Figure 6.13 (Middleton *et al.*, 2000; Graf *et al.*, 2005).

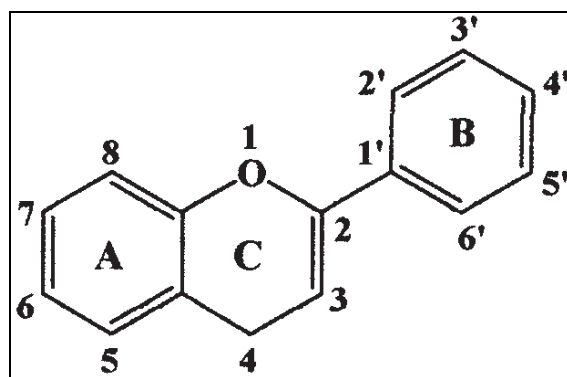


Figure 6.13 Basic structure and numbering system of flavonoids.

Flavonoids contain two aromatic rings (A and B) that are linked via an oxygenated heterocycle (ring C).

Note. From “Flavonols, flavones, flavanones, and human health: epidemiological evidence.” by B.A. Graf, P.E. Milbury and J.B. Blumberg, 2005, *Journal of Medicinal Food* 8(3), p. 282.

There are six commonly recognised subclasses of flavonoids and more information about the differences in their chemical structures can be found in Appendix K. For this study the important thing to note is that the structures of rutin, quercetin and luteolin are very closely related, differing only in the functional group (R) attached to carbon 3 (Fig. 6.14).

Quercetin mainly occurs in plants as a glycoside, of which rutin is an example (quercetin rutinoside) present in tea (Erlund *et al.*, 2000).

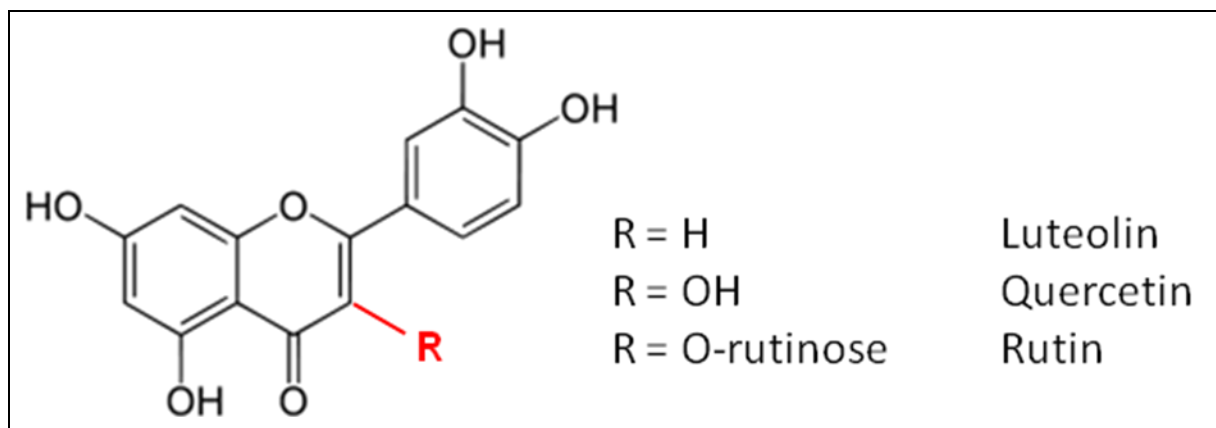


Figure 6.14 Chemical structures of luteolin, quercetin and rutin.

Note. From “Structure-activity relationships for antioxidant activities of a series of flavonoids in a liposomal system.” by A. Arora, M.G. Nair and G.M. Strasburg, 1998, *Free Radical Biology and Medicine* 24(9), p. 1356.

Besides the flavonoids, several other components of known molecular weight were also present in EA3. However, given the limited resources, unfortunately these could not be identified as part of the present study. Since the identified compounds are known to have antitumour activity (see Section 6.4.2), it was decided to focus the remaining experiments on comparing the actions of these pure compounds with those of the whole extract, EA3.

6.4.2 Identification of active constituents

The main components of EA3, as identified by LC-MS, were rutin, luteolin, quercetin, apigenin and naringenin. These compounds, like many flavonoids, have all been reported to possess antitumour properties (Kandaswami *et al.*, 2005; Plochmann *et al.*, 2007). For example, Kuntz *et al.* (1999) screened 36 flavonoids for their anticancer effects in three cancer cell lines and demonstrated all were growth-inhibitory to varying degrees. Quercetin has been shown to reduce cell proliferation, cause cell cycle arrest in the G₀/G₁ phase, the G₂/M-phase and the S-phase and induce caspase-3 activity and apoptosis in *in vitro* experiments with various cell lines (Mertens-Talcott & Percival, 2005). These properties of luteolin and quercetin are consistent with the cytostatic properties of extracts discussed in Chapter 4. Additionally, Han *et al.* (2001) showed that both luteolin and quercetin in nanomolar quantities were able to inhibit MCF7 cell proliferation. Evidence that these effects extend to the *in vivo* situation was provided by Devipriya *et al.* (2006), who showed quercetin reduced the tumour volume in rats induced for mammary carcinoma, and Selvendiran *et al.* (2006), who demonstrated luteolin significantly inhibited the growth of xenografted tumours in nude mice in a dose-dependent manner.

The results of the present study support those in the literature in that pure compounds of luteolin and quercetin both inhibited the proliferation of several cancer cell lines in a dose-dependent manner. After 48 h exposure, the calculated IC₅₀ values for luteolin were in accordance with several other studies (Saleem *et al.*, 2002; Chang *et al.*, 2005; Yoo *et al.*, 2009). Similarly, IC₅₀ values obtained for quercetin agreed with published data (Yoshida *et al.*, 1990; Wenzel *et al.*, 2004). Rutin did not inhibit the proliferation of any cell line tested, a finding which is also in agreement with those of other groups (Scambia *et al.*, 1994; Kawaii *et al.*, 1999; Chao *et al.*, 2007). It appears that rutin is not able to induce

antiproliferative effects until the glycosidic bond of the rutinose moiety is cleaved to yield quercetin (Campbell *et al.*, 2006). Indeed, as a group, flavonoid glycosides typically show little effect on the proliferation of cancer cells *in vitro*. It is likely that by making the molecules more polar and less planar, glycosidation blocks the entry of flavonoids into cells and may also sterically inhibit their binding to receptors involved in gene expression (Manthey & Guthrie, 2002). While several glycosidic flavonoids, including rutin, have shown antitumour activities *in vivo*, this is probably due to their antioxidant properties and their abilities to modulate the levels of detoxifying hepatic enzymes or inhibit angiogenesis (Deschner *et al.*, 1991; Shen *et al.*, 2002; Manthey & Guthrie, 2002; Guruvayoorappan & Kuttan, 2007).

However, while luteolin and quercetin are two of the main constituents of EA3, they are present at less than 0.1% (w/w). Calculations comparing the effects of pure compounds with the effects of the whole extract (see Section 6.3.2) indicated that the observed EA3-induced cytotoxicity/cytostaticity was not attributable to luteolin or quercetin acting alone. However, it is unusual for a single compound to be responsible for the observed activity. Indeed, very often several compounds are isolated from an extract which exert the same effect, although they may have different potencies (Houghton *et al.*, 2007). Therefore, the individual effects of pure luteolin and quercetin were added together to see if that would account for the observed bioactivity of EA3. These calculations implied that even if they had additive effects, the bioactivity of EA3 could not be explained by quercetin and luteolin.

However, according to Wagner², an international expert in the field of interactions between plant constituents, “it is not possible to compare extracts and pure constituents thereof, because interactions of constituents in extracts cannot be calculated because of the many possible complex and antagonistic interactions” (Wagner, H., personal communication, 7th June, 2010). Thus, it is quite possible that luteolin and quercetin could have acted synergistically, together with other flavonoids, other polyphenolics, and even other constituents, in the whole extract. This possibility is discussed in more depth in Section 8.2.6.2.

Unfortunately, due to time and budget constraints, only three of the secondary metabolites present in the extract could be examined as part of the current study. The choice of pure compounds tested was based on their relative presence in EA3 and previous studies implicating them as anticancer agents. However, it would also be worth examining the effects of other flavonoids, especially apigenin and possibly naringenin, which LC-MS revealed were also major components of EA3. Apigenin has been shown to inhibit proliferation and/or induce apoptosis in many cancer cells, including all those used in the present study except MM253, although inhibition of growth of other melanomas has been reported (Weiqun *et al.*, 2000; Yin *et al.*, 2001; Gupta *et al.*, 2001; Liu *et al.*, 2005). While there are reports of naringenin causing low-level inhibition of proliferation of some cell lines, these effects are markedly increased when it is used in combination with other flavonoids (So *et al.*, 1997; Knowles *et al.*, 2000; Campbell *et al.*, 2004; Brusselmans *et al.*, 2005; Campbell *et al.*, 2006).

² Emeritus Professor Hildebert Wagner is an internationally renowned pharmaceutical biologist. Based at the University of Munich's Institute of Pharmacy, he is the author of over 950 original papers and 30 review articles on various areas of pharmacognosy, including the pharmacological screening of plants and isolated constituents (<http://www.cup.uni-muenchen.de/pb/aks/wagner/>).

Indeed, this sort of synergistic interaction is quite common with flavonoids (Mertens-Talcott *et al.*, 2003; Campbell *et al.*, 2006; Kale *et al.*, 2008; Harasstani *et al.*, 2010).

Significantly, quercetin and luteolin have both been shown to interact synergistically with other compounds to inhibit proliferation of some cancer cells *in vitro* (Shen & Weber, 1997; Ackland *et al.*, 2005; Wu *et al.*, 2008a). Therefore, a future direction of this study is to investigate the effects of a range of cocktails containing quercetin and luteolin, as well as other flavonoids like apigenin, mixed together in various ratios to see if their individual inhibitory effects are potentiated when in combination (see Section 8.2.6.2).

Ideally, flavonoids would be removed from EA3 during the extraction process and the flavonoid-free extract tested for its ability to inhibit the proliferation of cancer cells. If the cytotoxic/cytostatic effects are still evident, then other constituents must be responsible. These would then need to be identified and individually tested for activity too. In this manner, eventually, it should be possible to clarify if the active principle/s is a known or novel compound.

While it seems likely that the observed effects of EA3 were due to luteolin and quercetin acting in synergy, future studies must be done in order to confirm this. There are many other constituents of EA3, including other flavonoids and other polyphenolics, that could be tested to definitively identify the compound/s responsible for its observed cytotoxicity/cytostaticity. While most of these substituents are known compounds, until these are proven to be responsible, the possibility exists that the antiproliferative effects may be due to a novel compound.

6.4.3 Exposure and recovery

EA3 inhibited the proliferation of all cell lines tested in a concentration and time-dependent manner (Figs. 6.8 and 6.9). The most susceptible cell lines were MDA-MB-468, MCF7, PC3, DU145 and MRC5 cells in which 500 $\mu\text{g/mL}$ EA3 caused total inhibition of proliferation within just 90 min. However, the same concentration only retarded the growth of the other three cell lines (Caco-2, MM253 and A549), which recovered to proliferate within 48 h. Even lower concentrations (200 and 100 $\mu\text{g/mL}$) of EA3 caused total inhibition of the proliferation of MCF7 and PC3 cells, but only reduced the rate of proliferation in other cells.

In PC3 and DU145 cells, % confluency actually decreased over time, suggesting some level of cytotoxicity at higher concentrations of extract. This cytotoxicity was rapid, occurring well within the time span of 1-3 h reported for cells to undergo apoptosis (Gavrieli *et al.*, 1992). Therefore, in these cells, it is feasible that EA3 induced cell death via an apoptotic mechanism. As the % confluency levels did not rise again, surviving cells had either left the cell cycle or were proliferating at the same rate as other cells were dying. Results of the next experiment in which extracts were washed out (see Fig. 6.10 and discussion below), indicate the former scenario in PC3 cells and the latter in DU145 cells.

Cytotoxicity was also observed in A549 cells, but these cytotoxic effects were not apparent until at least 8 h after addition of the extract, which may suggest cell death was via necrosis. However, in this case, surviving cells were able to proliferate.

Cytostatic effects were apparent when MDA-MB-468, MCF7 and MRC5 cells were exposed to 500 $\mu\text{g/mL}$ of EA3, as evidenced by the flat growth curves. In MRC5 cells, all

concentrations of EA3 were cytostatic, although it must be noted that even the control cells were not growing very fast.

From Figure 6.10 it can be seen that the inhibitory effects of EA3 were mostly irreversible at high concentrations. Except for A549 and DU145 cells, high concentrations of this extract generally caused cells to irreversibly leave the cell cycle, as even after they were washed out, cell proliferation did not resume within 72 h period assessed. This result suggests that, while EA3 induced cytotoxicity in both PC3 cells and DU145 cells, surviving DU145 cells were able to proliferate in the absence of extract, while all PC3 cells were irreversibly affected. In MDA-MB-468 and PC3 cells, concentrations as low as 50 µg/mL were enough to cause irreversible growth arrest.

Overall, these experiments revealed that EA3 causes cytotoxic effects in some cell lines and cytostatic effects in others, thus displaying selectivity. Additionally, the speed of the cytotoxic response in PC3 and DU145 cells was suggestive of apoptosis as necrosis is generally a slower process (Gavrieli *et al.*, 1992; Majno & Joris, 1995), although further studies are required to confirm this.

6.4.4 Intracellular Ca^{2+} measurement

Ca^{2+} is the most widely used second messenger, regulating a diverse array of cellular functions, including adhesion, motility, gene expression and proliferation (Campbell, 1990). Indeed, Ca^{2+} signals control almost every aspect of cellular life, from short-term cytoskeletal modifications to long-term alterations in gene expression (Clapham, 2007; Gallo *et al.*, 2006). However, too much intracellular Ca^{2+} is cytotoxic, so it is vital that a cell maintains a relatively low resting $[\text{Ca}^{2+}]_i$ and Ca^{2+} increases during signalling are

transient (Carafoli *et al.*, 2001; Clapham, 2007). Hence, to facilitate Ca^{2+} homeostasis and yet provide easily accessible Ca^{2+} when required for signalling purposes, excess Ca^{2+} is stored in intracellular organelles, such as the mitochondria, Golgi apparatus or, in particular, the endoplasmic reticulum (ER) (Campbell, 1990). A sophisticated system of membrane channels and pumps (see Appendix J) coordinates the entry of Ca^{2+} into the cytosol from the extracellular fluid or these intracellular storage organelles, and its subsequent extrusion from the cell (Clapham, 2007).

However, when a cell becomes injured, impairment of normal intracellular Ca^{2+} homeostasis usually results. Following injury, the integrity of the plasma membrane can become compromised and Ca^{2+} flows into the cytosol down its 20,000-fold concentration gradient from the extracellular fluid. Ca^{2+} extrusion systems are quickly overcome, leading to uncontrolled, sustained rises in $[\text{Ca}^{2+}]_i$ with cell death often ensuing. Ca^{2+} influx can also be enhanced due to changes in the opening of plasma membrane Ca^{2+} ion channels or release of Ca^{2+} into the cytosol from intracellular stores, constituting Ca^{2+} signalling (Campbell, 1990). Mobilisation of intracellular Ca^{2+} stores is the usual explanation behind rapid and transient increases in $[\text{Ca}^{2+}]_i$. Such a pattern is indicative of a physiological response and may or may not be associated with cell death (Orrenius *et al.*, 1989). There is evidence suggesting that very high $[\text{Ca}^{2+}]_i$ causes cell death through necrosis, whereas lower increases in $[\text{Ca}^{2+}]_i$ induced by milder insults promote cell death via apoptosis (Rizzuto *et al.*, 2003). A rise in $[\text{Ca}^{2+}]_i$ is associated with both early and late stages of some apoptotic pathways (Tombal *et al.*, 1999; Sylvie *et al.*, 2000; Baumgartner *et al.*, 2009). The aim of this series of experiments was to detect any changes in $[\text{Ca}^{2+}]_i$ when cells were exposed to EA3 in order to obtain clues to the mechanism by which EA3 induces cell death. The basic premise was that if cells were dying via necrosis, a rapid and sustained

increase in $[Ca^{2+}]_i$ of high magnitude would ensue, while a more transient, smaller response would indicate Ca^{2+} signalling, possibly suggesting a more regulated mechanism, such as apoptosis. On the other hand, a Ca^{2+} transient could also be activating a normal physiological change within the cells.

The compounds used elsewhere in this thesis as positive controls for proliferation studies were DFO and camptothecin (CPT). The effect of CPT on intracellular Ca^{2+} was precluded due to autofluorescence which interfered with fluorescence measurements, rendering the traces obtained nonsensical (data not shown). However, DFO was examined and was found to have very little effect on intracellular Ca^{2+} . This result was surprising as it contrasts with the findings of Wang and colleagues (2005) who, using a different fluorophore, demonstrated that DFO significantly ($P < 0.01$) increased $[Ca^{2+}]_i$ of Caco-2 cells in a dose-dependent manner. Nevertheless, the results of the current study indicate that DFO was not inhibiting proliferation of PC3 cells by acutely increasing Ca^{2+} . Considering that EA3 did acutely increase $[Ca^{2+}]_i$ in the PC3 cells, it is possible that EA3 and DFO act via different pathways. However, these experiments do not rule out the possibility that DFO may also increase cytosolic Ca^{2+} in the PC3 cells after an initial delay, which would not be detected in the 10 min monitoring window used in this study.

Histamine is a widely occurring chemical mediator that has been shown to mobilise Ca^{2+} in cells expressing the H_1 and H_4 type receptors (Tilly *et al.*, 1990; Thurmond *et al.*, 2008). Significantly, PC3 cells express the H_1 receptor and histamine has been shown to elevate $[Ca^{2+}]_i$ in a concentration-dependent manner in these cells, with an IC_{50} value of 1 μM (Wasilenko *et al.*, 1997; Lee *et al.*, 2001; Suzuki *et al.*, 2007). Wasilenko and colleagues

(1997) reported that 10 μM histamine caused an increase of $[\text{Ca}^{2+}]_i$ in PC3 cells which was 261nM above baseline levels. While this concentration of histamine was not enough to induce a detectable Ca^{2+} response in the same cell type under the experimental conditions used in this project, 100 μM histamine caused a mean increase in $[\text{Ca}^{2+}]_i$ 198nM above the basal level. This elevation in $[\text{Ca}^{2+}]_i$ was not significantly different to the increase published by Wasilenko *et al.* (1997, p169), who described histamine as one of the “most effective calcium agonists” of all the compounds they tested. Therefore, histamine was introduced as a suitable positive control for the experiments designed to evaluate changes in intracellular Ca^{2+} levels of PC3 cells.

Accordingly, the Ca^{2+} response of PC3 cells to histamine was compared to that of EA3. From Table 6.3, it appeared 500 $\mu\text{g/mL}$ EA3 caused a higher increase above basal intracellular Ca^{2+} levels than 100 μM histamine. However, there was no statistically significant difference between the amplitudes of the two responses. Similarly, the speeds of responses, as indicated by the times taken to reach peak (TTP) fluorescence values, were not significantly different for the two agents. Histamine is well known to be an effective mediator involved in many Ca^{2+} signalling processes (Lee *et al.*, 2001). For example, histamine receptors couple with and activate specific G-proteins which leads to enhanced Ca^{2+} mobilisation in the case of three of the four known receptor subtypes (Akdis & Blaser, 2003; Thurmond *et al.*, 2008). Therefore, the similarities between the EA3- and histamine-induced Ca^{2+} transients suggest that the action of EA3 could be, at least in part, via Ca^{2+} signalling. Further study needs to be done to resolve this suggestion.

The main difference between the effects of EA3 and histamine on $[Ca^{2+}]_i$ was that in the case of EA3, the $[Ca^{2+}]_i$ remained elevated above the initial resting $[Ca^{2+}]_i$ value for at least 10 min after activation whereas the $[Ca^{2+}]_i$ had returned to resting levels after 10 min after histamine application. This suggests that the histamine-induced Ca^{2+} response was transient, with the peak slowly decaying towards the baseline within the experimental time frame, while the EA3-induced Ca^{2+} response was biphasic, consisting of a peak then a plateau phase. For many cell types, it is well known that an initial rise in $[Ca^{2+}]_i$ is mainly due to the mobilisation of stored Ca^{2+} , while a secondary rise or plateau is due to sustained Ca^{2+} influx across the plasma membrane (Tilly *et al.*, 1990; Liou *et al.*, 2005; Chen *et al.*, 2010}. It is possible that the plateau phase recorded for EA3 was actually a slight (non-significant) rise in $[Ca^{2+}]_i$, but since this secondary rise was only gradual, it was probably not due to EA3-induced damage to the integrity of the plasma membrane. If a breach in membrane integrity had occurred, Ca^{2+} would flood down its considerable gradient into the cytosol and the trace would more closely resemble that of the ionomycin-induced Ca^{2+} response (Fig. 6.12). Previous experiments demonstrated that a crude methanol extract of the same source as EA3 reduced the proliferation of cancer cells within 30 min (section 5.3.5). Subsequent CS experiments described above revealed that inhibition of PC3 cell proliferation by EA3 was rapid and non-reversible, indicating a cytotoxic effect within this period. Given that a rapid influx of Ca^{2+} did not transpire within a similar time frame, it is therefore unlikely that EA3 at this concentration causes cell death via necrosis.

These results clearly demonstrate that some Ca^{2+} signalling is taking place upon exposure of PC3 cells to EA3. However, while the Ca^{2+} response to EA3 may be a signal for apoptosis, it could also be a part of normal physiological signalling processes. Further experiments, discussed in Chapter 8, are therefore required.

6.5 SUMMARY

The results of these experiments suggest that EA3 causes cytotoxicity in some cancer cell lines and yet has only cytostatic effects in others, illustrating a degree of selectivity. Both cytotoxicity and cytostaticity could be due to the synergistic actions of two or its major constituents, the flavonoids luteolin and quercetin. On the other hand, it is possible that the observed effects are attributable to other known or unknown compounds, acting alone or synergistically. EA3-induced cytotoxicity may involve Ca^{2+} signalling processes as EA3 clearly induced a transient rise in $[\text{Ca}^{2+}]_i$ in PC3 cells within minutes of their exposure to this extract. The Ca^{2+} response was biphasic which is consistent with release of Ca^{2+} from internal stores followed by extracellular Ca^{2+} influx. The pattern of the trace also indicates that the elevated $[\text{Ca}^{2+}]_i$ was unlikely to be due to a breach in the plasma membrane integrity within the experimental time frame. As cytotoxic effects were evident in susceptible cells within just 90 min of exposure to EA3, taken together, these results suggest that the observed EA3-induced could be due to apoptosis, as it is unlikely to be due to necrosis. However, further experiments to corroborate these findings are necessary. Accordingly, some possible future directions are considered in the General Discussion which follows the next and final experimental chapter.

Chapter 7: Two Different Biodiscovery Strategies

7.1 INTRODUCTION

As discussed in Chapter 1, the enviable record of successful hits from naturally derived compounds bears witness to the argument that bioprospecting is an excellent approach to drug discovery (Clardy & Walsh, 2004; Newman & Cragg, 2007). Indeed, despite an overall deviance towards HTS and CC in the 1980s and '90s, Big Pharma companies including Novartis, Merck, Pfizer, Wyeth and Bristol Myers Squibb maintained drug screening programmes based on bioprospecting during this period as the advantages of this traditional method of drug discovery over more modern techniques were recognised (CBD, 2008). Other pharmaceutical companies have renewed their strategies in favour of natural product (NP) drug development and discovery (Patwardhan *et al.*, 2005). Moreover, advances in instrumentation, robotics, and bioassay technology have improved the speed of purification and characterization processes, which have promoted renewed interest in bioprospecting amongst other researchers (Butler, 2004; Svarstad, 2005; Baker *et al.*, 2007; Laird *et al.*, 2008).

Approaches to selecting appropriate NPs for screening potential new bioactive compounds range from random selection to more guided selection strategies, such as the ethnopharmacological approach (Cordell *et al.*, 1991; Shoemaker *et al.*, 2005; McRae *et al.*, 2007). The random approach is basically a numbers game and is, therefore, more suited to large companies with lots of money and resources. In contrast, more time-consuming selection strategies guided by prior information about chemical make-up, traditional ethnomedical use or other clues have generally been the domain of academic institutions and small biotechnology operators. While the typical industrial drug discovery process

uses medium to high-throughput bioassay screening platforms to find promising compounds for a specific biological target, ethnopharmacology uses almost the opposite approach, whereby anecdotal efficacy of medicinal plants is tested in the laboratory (Gertsch, 2009).

As the title of this thesis asserts, the overall objective of this project was to use traditional Aboriginal medical knowledge as a guide to identifying plants that may be helpful in the treatment of cancer. To this end, extracts of plants identified by local Aboriginal people as having medicinal properties were evaluated for their anticancer potential. This screening programme and the subsequent analyses of bioactive compounds form the basis of Chapters 4 to 6. However, it was also of interest to compare this ethnopharmacological tactic to screening with a more random approach in order to provide evidence for the hypothesis that the ethnopharmacological method does, indeed, provide a greater chance of success. Hence, this chapter is concerned with screening a randomly selected NP sample for cytotoxic activity in cancer cells and comparing this to the effects of a sample with purported medicinal properties.

7.1.1 Random approach

The first samples to be examined in this study were extracts of a marine sponge with no known ethnomedical use. Besides plants, many other relatively available organisms have evolved biosynthetic pathways containing a vast array of NPs which enable them to adapt to a specific lifestyle. For example, marine invertebrates, such as sponges, are sessile organisms that rely on chemical defence systems for protection against predators. Certain sponges also produce other secondary metabolites including those that help prevent infections, facilitate reproduction and protect from ultraviolet radiation (Shimizu, 1985).

Thus, marine sponges are potentially a rich source of therapeutically useful bioactive compounds. Indeed, already a plethora of pharmacologically valuable novel NPs obtained from some sponges have been reported. Biological activities described so far include antifungal, antibacterial, anticancer, antiviral, antimalarial, antiinflammatory, anticoagulant, antituberculosis and immunosuppressive actions (Sipkema *et al.*, 2005).

7.1.2 Guided approach

The second samples screened were extracts of the plant, *Haemodorum spicatum*. This perennial herbaceous geophyte (bulb), is endemic to south-west Western Australia (Paczkowska, 1994; Woodall *et al.*, 2010). Commonly known as bloodroot due to the deep red colour of its bulbs (Clarke, 2007), Aboriginal names for *H. spicatum* include mardja, meen, born and bohn, depending on location (Paczkowska, 1994; Daw *et al.*, 1997; Woodall *et al.*, 2010). These bulbs were an important resource for local Aboriginal people. They were traditionally used as food, eaten either raw or roasted or ground up and used as a spice (Clarke, 2007; Flugge, 2009). Additionally, when cut, crimson red gelatin exuded from the bulbs and this could be used as a dye (Daw *et al.*, 1997; Flugge, 2009). There are also reports of extracts of the bulbs of various *Haemodorum* species being used as medicines (Dias *et al.*, 2009). For example, *H. spicatum* was used as a remedy for dysentery while *H. corymbosum* was used to treat snakebite (Bindon, 1996; Lassak & McCarthy, 2001). More significantly to this study, the compound responsible for the red pigmentation, haemocorin, has demonstrated antibacterial activity as well as purported antitumour activity (Cooke & Segal, 1955; Simpson, 1990; Daw *et al.*, 1997; Harborne *et al.*, 1999; Dias *et al.*, 2009). As bulbs from different geographical regions display varying degrees of colour (Woodall *et al.*, 2010) it was hypothesised that they would show corresponding variation in cytotoxicity against cancer cells.

7.2 METHODOLOGY

For both sets of samples, the MTT assay (Section 2.6.1) was employed to screen for bioactivity.

The negative control was the vehicle only, DMSO or methanol, at the same final concentration as in the sample. Methanol was used to dissolve powdered bulbs in later experiments as data (not shown) from a preliminary experiment in which fresh bulbs were steeped in methanol showed some bioactivity, albeit at very high concentrations (6 mg/mL).

Positive controls employed were deferoxamine mesylate (DFO) and deferriprone (L1) at 1 mM, camptothecin (CPT) and 5-fluorouracil (5FU) at 100 μ M, and paclitaxel (PAC), vinblastine sulfate (VIN) and etoposide (ETO) at 10 μ M final concentrations. To induce total cytotoxicity, 1 mg/mL HgCl₂ and 0.1% TX-100 were incubated with cells.

7.2.1 Random approach

A sample of a marine sponge of unknown taxonomy was collected randomly from Nelson Bay, NSW, Australia (32° 43' S, 152° 08' E) in February 2008 by Dr Ian van Altena (University of Newcastle). Frozen samples were stored at -8 °C prior to analysis. A voucher specimen was retained at the University of Newcastle.

Extraction of this sponge was performed by Ms Jessie Moniodis, an Honours candidate within the Discipline of Pharmacy (UWA). Details of the extraction procedure can be found in Appendix L. Briefly, the freeze dried sponge was crumbled manually and extracted with methanol. The crude methanolic extract was prefractionated and separated using various solvents. Subsequent fractions were dried under reduced vacuum and labeled 1-8. Sample 7 was the parent compound of samples 3, 4, 5, 6 and 8.

Dried extracts were dissolved in DDW at concentrations based on preliminary bioactivity data obtained from the brine shrimp assay (data not shown) and the quantities available. DMSO was added if necessary for complete dissolution and further dilutions were in DMEM. These were subsequently added in 10 μ L aliquots to 100 μ L cells in 96-well microplates before 48 h incubation under standard culture conditions. Concentrations used for each extract, including DMSO content, are summarised in Table 7.1.

Table 7.1 Concentrations of Sponge Extracts* Tested for Bioactive Effects

Extract Code	% DMSO added for dissolution	Neat concentration (mg/mL)	Range of final concentrations (μg/mL)	Highest final concentration of DMSO (%)
1	0.4	10	50-1,000	0.04
2	-	10	50-1,000	0
3	1.2	3	50-300	0.12
4	1.0	1	10-100	0.1
5	1.3	3	50-300	0.13
6	1.7	3	50-300	0.17
7	0.4	10	50-1,000	0.04
8	-	10	50-1,000	0

* Extracts were derived from a marine sponge as indicated in Appendix L.

7.2.2 Guided approach

Underground bulbs of *Haemodorum spicatum* plants from three distinct geographical regions, Albany, Esperance and Geraldton, were harvested in summer by Dr Geoff Woodall and collaborators (Centre for Excellence in Natural resource Management, UWA, Albany, WA). Soil was removed from field samples, whole plants were placed in an esky and transported to a facility in Albany. Bulbs were ground up and dried to a powder and transferred to UWA within a week. Here they were stored at room temperature in the dark until examined for bioactivity soon after. During autumn of the following year, the process was repeated on bulbs from Albany and Esperance only. Dried bulbs were dissolved in either DMSO (autumn bulbs) or methanol (summer bulbs) at 100 mg/mL on the day of use.

Samples were diluted in appropriate culture medium to give a range of concentrations from 6 mg/mL to 0.03 mg/mL (w/v). These were subsequently added in 10 μ L aliquots to 100 μ L cells in 96-well microplates and incubated for 48 h under standard conditions as used in previous chapters. In preliminary studies autumn bulbs were assessed for their ability to inhibit the proliferation of the two human breast cancer cell lines, MDA-MB-468 and MCF7, only. Summer bulbs were tested against these two cell lines as well as five other human cancer cell lines, Caco-2, MM253, A549, PC3 and DU145.

7.3 RESULTS

7.3.1 Random approach

The random sponge sample known as 080211NB09 had a tawny exterior and a dense packing which appeared dullish grey and was highly porous (Figure 7.1).



Figure 7.1 Marine sponge sample 080211NB09.

A photograph of the marine sponge sample collected from Nelson Bay, NSW, in February 2008. No sizing information was available.

The antiproliferative effects of extracts of the sponge were assessed against a panel of cancer cell lines and one non-tumourigenic line, MRC5 (Fig. 7.2). It must be noted that after the extraction procedures, only small quantities of each extract were obtained. As most of this was required for experiments pertaining to Ms Moniodis's Honours project, very little was available for these screening studies and optimal concentrations for each extract could not be established.

As can be seen from Fig 7.2, the most potent extract in terms of its antiproliferative ability was Extract 4, which produced the steepest curve. With the exception of Caco-2 cells, 100 µg/mL of this extract was enough to cause greater than 50% inhibition of proliferation in every cell line tested, including MRC5.

The greatest single level of inhibition of proliferation was obtained using 300 µg/mL of Extract 3. At this concentration, this extract resulted in >90% inhibition in all cancer cell lines and >80% inhibition of proliferation of MRC5 cells. At 100 µg/mL, >25% inhibition was achieved in four cell lines, A549, PC3, DU145 and MRC5.

Extract 6 showed a range of bioactivity across all cell lines. While still causing some inhibition of proliferation in other cell lines, 300 µg/mL of Extract 6 resulted in PC3 cell proliferation of only 12% of the control. The inhibitory effect of this concentration ranged upwards from this value for other cell lines to be 72% and 78% for MRC5 and Caco-2 cells, respectively. At 200 µg/mL, inhibition of proliferation was nearly 60% in MDA-MB-468, A549 and PC3 cells, while there was no effect in MRC5 cells. Lower concentrations generally resulted in higher % control values, indicating dose-dependence. Similar results were obtained with Extract 5, the most susceptible cell lines being MM253, A549 and PC3. Extract 7 caused some inhibition of proliferation of all cell lines at higher concentrations.

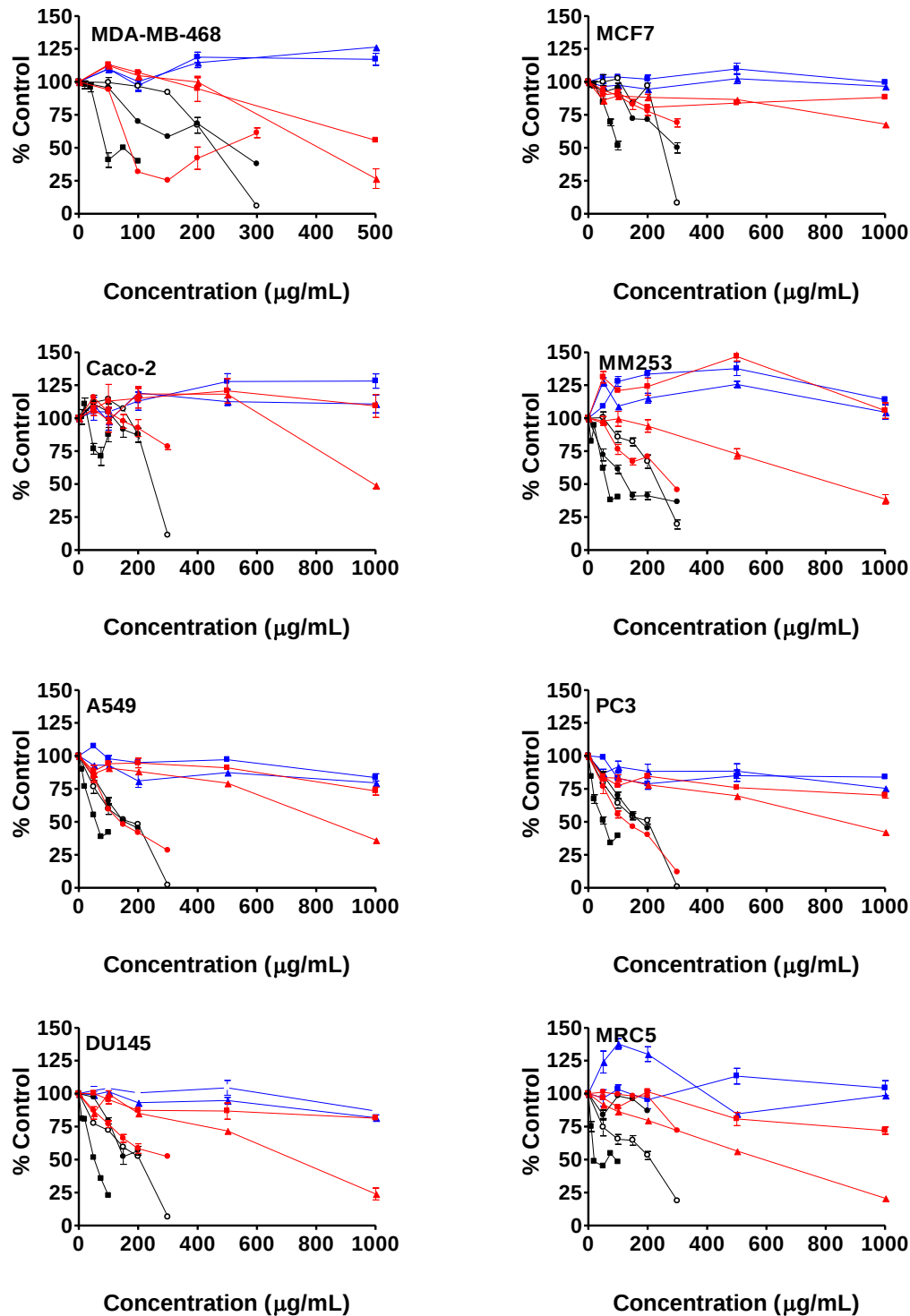


Figure 7.2 Effects of extracts of marine sponge on proliferation of cancer cell lines.

Cancer cells were exposed to a range of concentrations of various extracts, 1 (▲), 2 (■), 3 (○), 4 (■), 5 (●), 6 (●), 7 (▲) or 8 (■), of a marine sponge for 48 h. The MTT assay was used to assess changes in cell proliferation. Results are expressed as % of control values and are the means $\pm$ SE of quadruplicate wells from two separate experiments.

Where possible, IC_{50} values were calculated from fitted nonlinear regression dose-response (variable slope) curves of log-transformed concentrations (Fig 7.3). These are presented along with their corresponding 95% confidence intervals and r^2 values in Table 7.2. The Top- and Bottom-fixed model described in section 4.3.3 was the optimum model in all cases. Numbers in red represent IC_{50} values >15% above the highest concentration of that extract tested. As this means the programme performed extrapolation beyond the tested range, these values are not necessarily reliably accurate.

Extracts 1, 2 and 8 caused little or no inhibition of proliferation in any cell line over the concentration ranges tested, thus IC_{50} values were not calculated for these extracts.

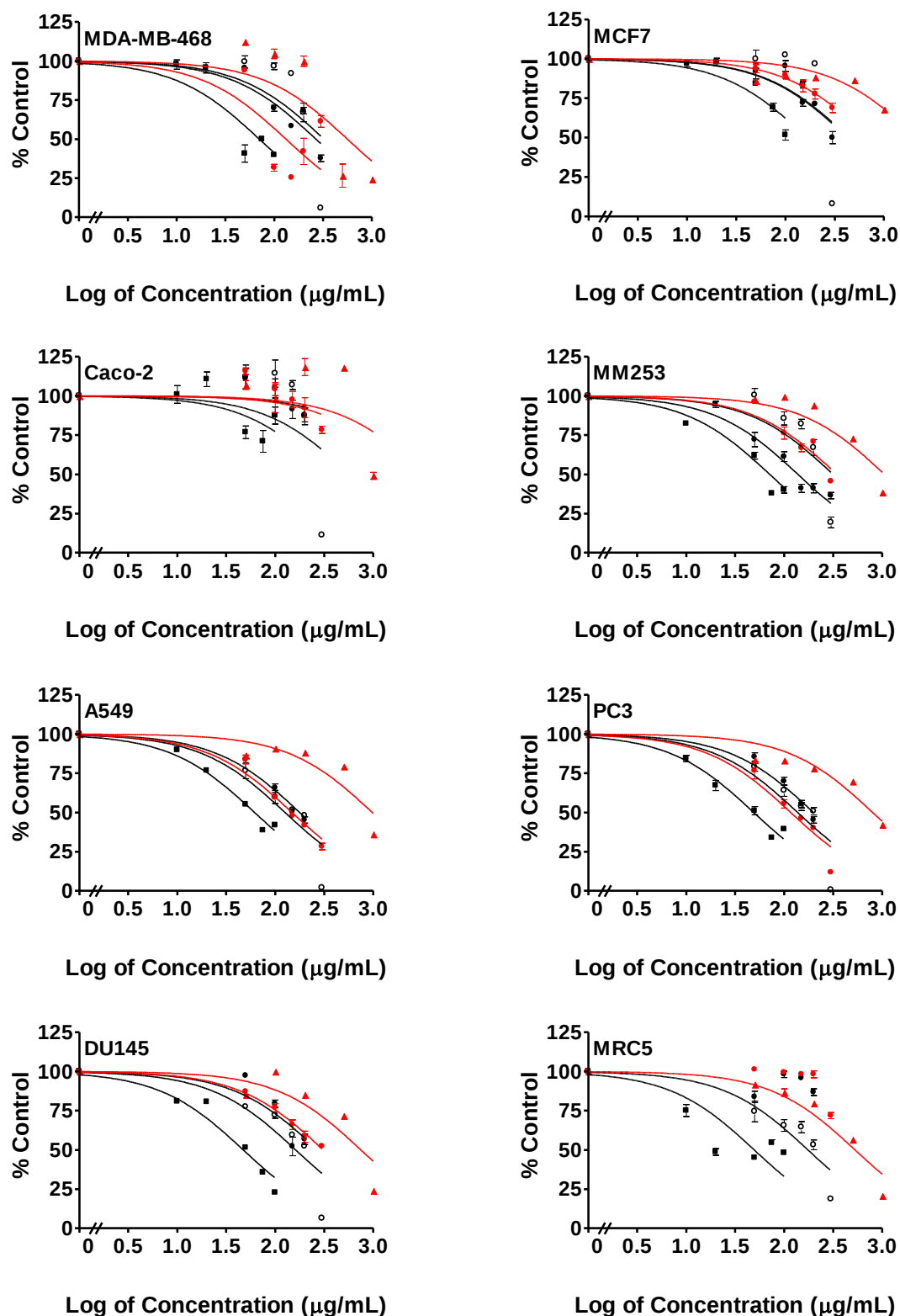


Figure 7.3 Nonlinear regressions of sponge extracts.

Dose-responses for extracts 3 (○), 4 (■), 5 (●), 6 (●) and 7 (▲) for each cell line from Fig. 7.2. Concentrations were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope with the top parameter set at 100% and the bottom parameter fixed at 0%).

Table 7.2 IC_{50} Values Calculated Using Top- and Bottom-Fixed Model for Individual Cell Line and Extract Combinations

MDA-MB-468				MCF7		
Extract	IC_{50} ($\mu\text{g/mL}$)	IC_{50} Range (95% CI)	r^2	IC_{50} ($\mu\text{g/mL}$)	IC_{50} Range (95% CI)	r^2
3	323.4	76.9 – 1359	0.517	426.8	74.4 – 2450	0.400
4	70.2	36.1 – 136.7	0.835	164.4	101.9 – 265.3	0.865
5	266.7	167.6 – 424.5	0.861	442.6	272.5 – 718.8	0.834
6	131.3	42.8 – 403.0	0.505	703.8	646.6 – 766.1	0.991
7	559.4	148.7 – 2105	0.723	2145	1176 – 3915	0.600

Caco-2				MM253		
Extract	IC_{50} ($\mu\text{g/mL}$)	IC_{50} Range (95% CI)	r^2	IC_{50} ($\mu\text{g/mL}$)	IC_{50} Range (95% CI)	r^2
3	574.4	61.1 – 5404	0.326	315.3	118.4 – 839.5	0.662
4	339.3	115.3 – 999.1	0.483	71.0	42.8 – 118.0	0.882
5	2591	215.0 – 31221	0.256	137.8	112.2 – 169.3	0.972
6	2233	391.9 – 12725	0.344	344.2	238.4 – 497.0	0.898
7	3354	255.4 – 44033	0.272	1056	583.3 – 1910	0.878

A549				PC3		
Extract	IC_{50} ($\mu\text{g/mL}$)	IC_{50} Range (95% CI)	r^2	IC_{50} ($\mu\text{g/mL}$)	IC_{50} Range (95% CI)	r^2
3	125.8	63.6 – 248.7	0.828	137.1	64.0 – 293.8	0.793
4	61.6	50.0 – 75.8	0.977	48.9	38.6 – 61.8	0.972
5	177.7	142.1 – 222.2	0.975	199.4	154.7 – 257.0	0.966
6	147.9	117.0 – 187.0	0.970	116.3	77.4 – 174.9	0.930
7	977.1	509.8 – 1873	0.822	798.7	519.8 – 1227	0.884

DU145				MRC5		
Extract	IC_{50} ($\mu\text{g/mL}$)	IC_{50} Range (95% CI)	r^2	IC_{50} ($\mu\text{g/mL}$)	IC_{50} Range (95% CI)	r^2
3	161.2	78.2 – 332.5	0.790	174.4	105.4 – 288.7	0.860
4	47.1	33.0 – 67.1	0.951	49.4	22.7 – 107.2	0.860
5	270.4	143.0 – 511.1	0.820			
6	307.9	282.1 – 336.1	0.992			
7	747.5	341.0 – 1639	0.816	526.8	334.7 – 829.2	0.936

IC_{50} values were calculated for five extracts of marine sponge against various cell lines using the top- and bottom- fixed parameter model in GraphPad Prism.

Numbers in red represent IC_{50} values >15% above the highest concentration of extract tested and therefore are considered possibly inaccurate.

7.3.2 Guided approach

Figure 7.4 shows *H. spicatum* bulbs from Albany and Esperance both whole and in cross-section.

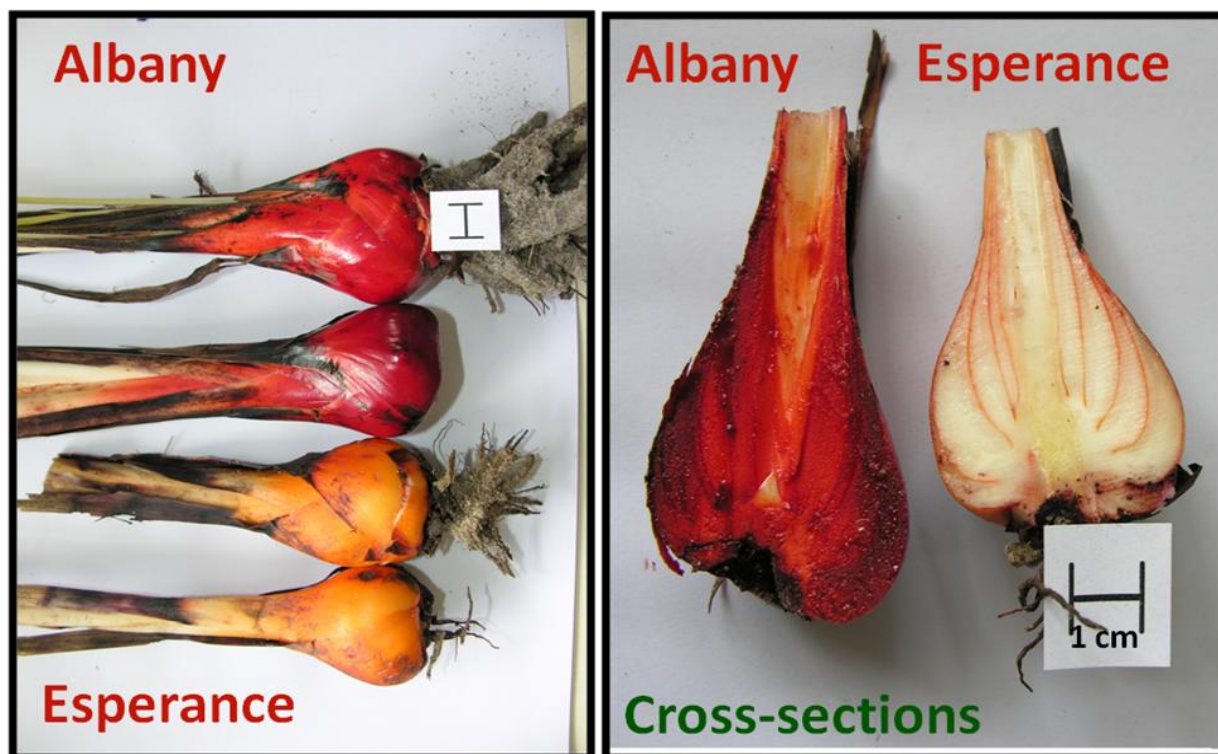


Figure 7.4 *Haemodorum spicatum* bulbs from different regions.

Underground *H. spicatum* bulbs were collected from Geraldton or Esperance. Soil was removed and photographs taken of whole bulbs (left) or after longitudinal cross-sectioning (right). The sizing bars on white cards indicate 1 cm lengths.

Clearly, there was a distinct difference in pigmentation of bulbs from Albany compared to those from Esperance. Samples from Albany display a deeper red hue compared to their counterparts from Esperance, which are more orange. The depth of colour did not appear to change according to season (Woodall, G., personal communication, 3rd March 2010).

The effects of *H. spicatum* extracts obtained from three different geographical regions, Albany, Esperance or Geraldton, on the proliferation of two breast cancer cell lines, MDA-MB-468 and MCF7 are depicted in Figure 7.5.

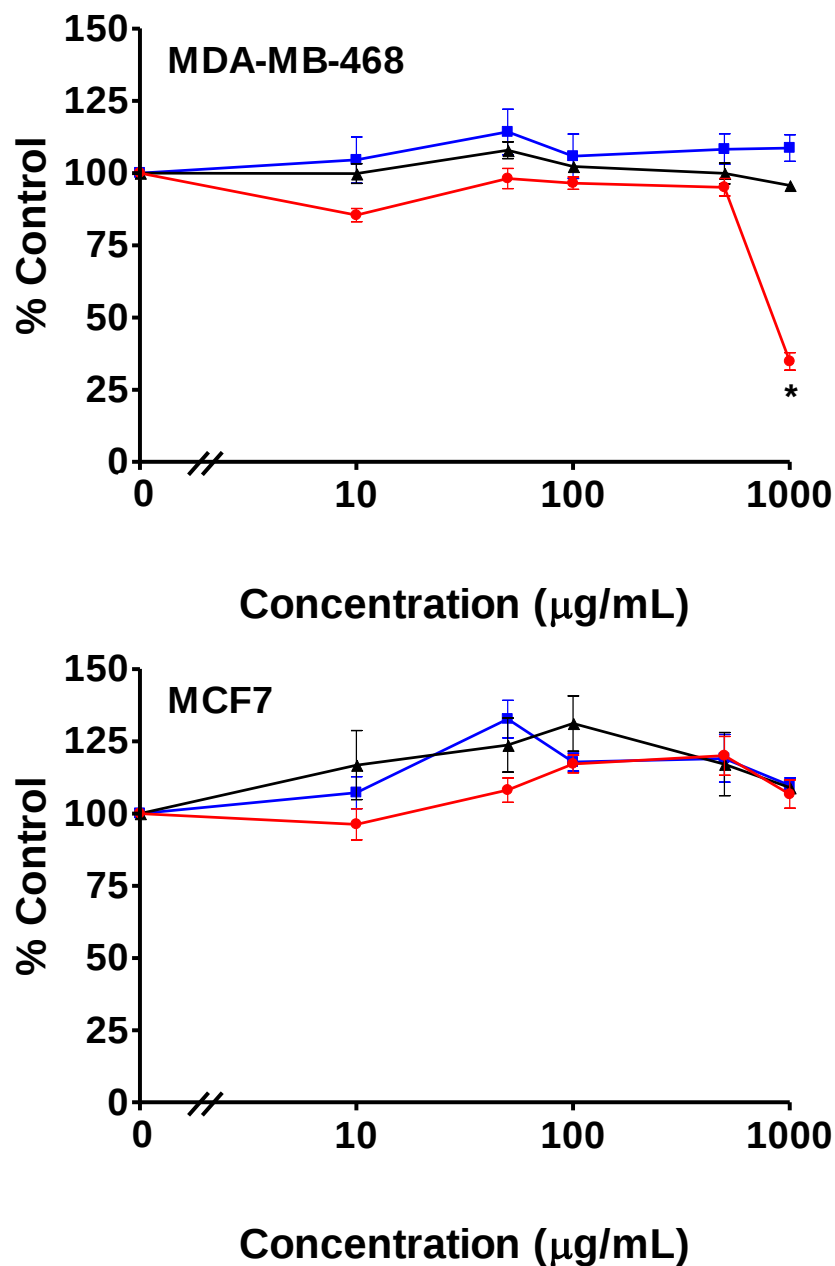


Figure 7.5 Effects of *H. spicatum* extracts from different regions on proliferation of two breast cancer cell lines.

MDA-MB-468 and MCF7 cells were exposed for 48 h to a range of concentrations of dried autumn bulbs from Albany (●), Esperance (▲) or Geraldton (■) dissolved in DMSO and diluted accordingly in medium. The MTT assay was used to assess changes in cell proliferation. Results are expressed as % of control values and are the means $\pm$ SE of quadruplicate wells from three different samples collected from the same region (Esperance and Geraldton) or the means $\pm$ SE of quadruplicate wells of a single sample (Albany).

* denotes significantly different from DMSO control ($P < 0.01$).

It is clear from Figure 7.5 that only the sample from Albany had any effect on either cell line over the concentration range tested. At 1,000 $\mu\text{g/mL}$, the highest concentration used, the proliferation of MDA-MB-468 cells was inhibited by more than 65%. Using a one-way ANOVA and Dunnet's Multiple Comparisons Test, this was the only significantly different value from the DMSO control ($P < 0.01$).

Samples collected from Albany and Esperance in summer of the following year were also tested for bioactivity, this time against a larger panel of human cancer cell lines. These bulbs were dissolved in methanol on the day of use and diluted accordingly before being subjected to the MTT assay for evaluation of their effects on cancer cell proliferation. The results of these dose-response experiments are shown in Figure 7.6.

From Figure 7.6 it is evident that neither the *H. spicatum* samples from Albany or Esperance caused any significant effect. While the highest concentration of the Albany sample, 6 mg/mL, caused some reduction in proliferation of MCF7 and PC3 cells, neither of these values was statistically significant from the negative control ($P > 0.05$).

This general lack of bioactivity is perhaps more obvious from Figure 7.7, which shows the effects of just the highest concentration of extracts, 6 mg/mL, on all the cell lines in the panel compared to the positive controls DFO, L1 and CPT.

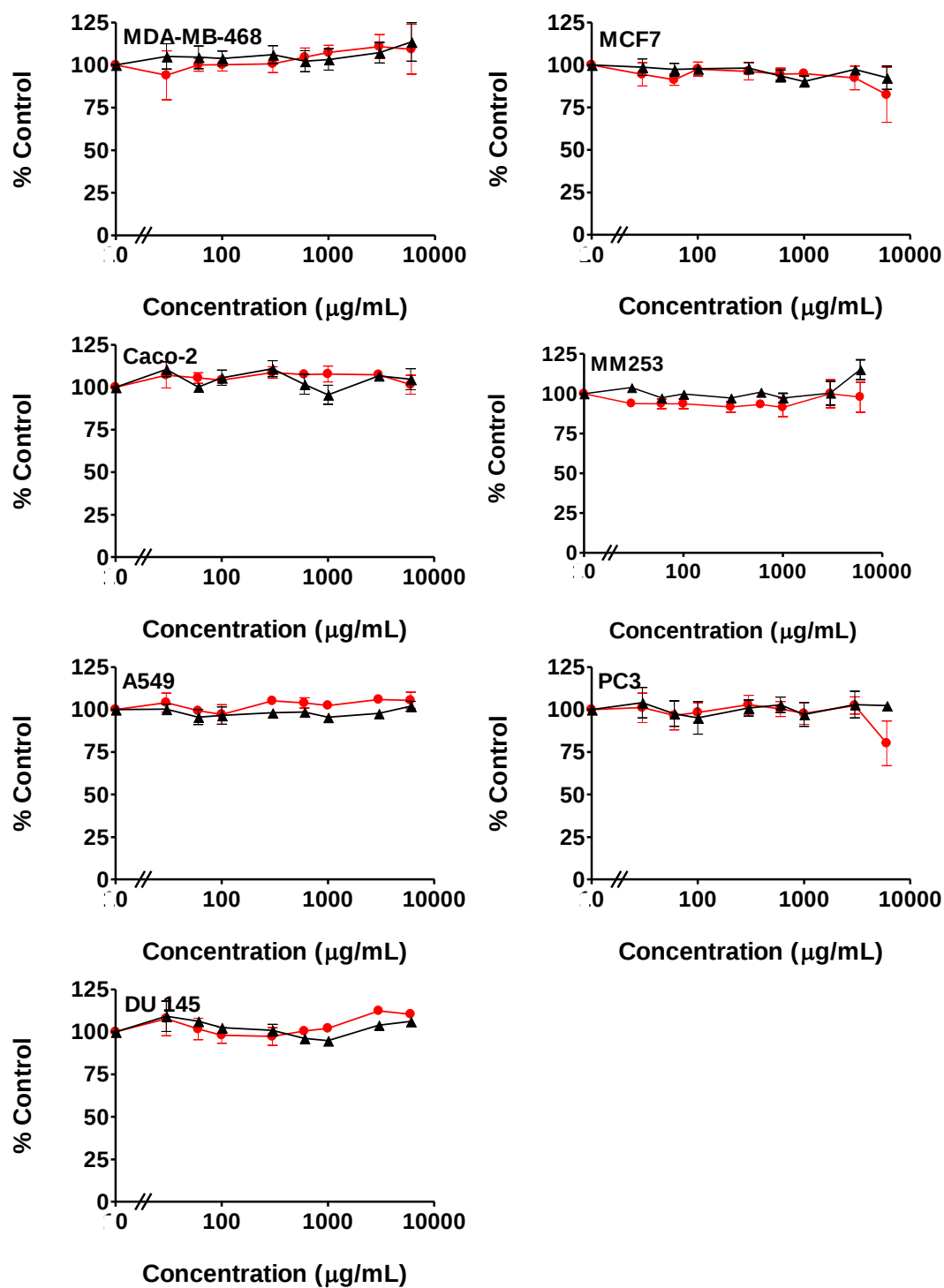


Figure 7.6 Effect of *H. spicatum* extracts on different cancer cell lines.

Seven different cancer cell lines were exposed for 48 h to a range of concentrations of dried *H. spicatum* summer bulbs collected from Albany (●) or Esperance (▲) dissolved in methanol and diluted in medium on the day of use. The MTT assay was used to assess changes in cell proliferation. Results are expressed as % of control values and are the means $\pm$ SE of quadruplicate wells from four separate experiments.

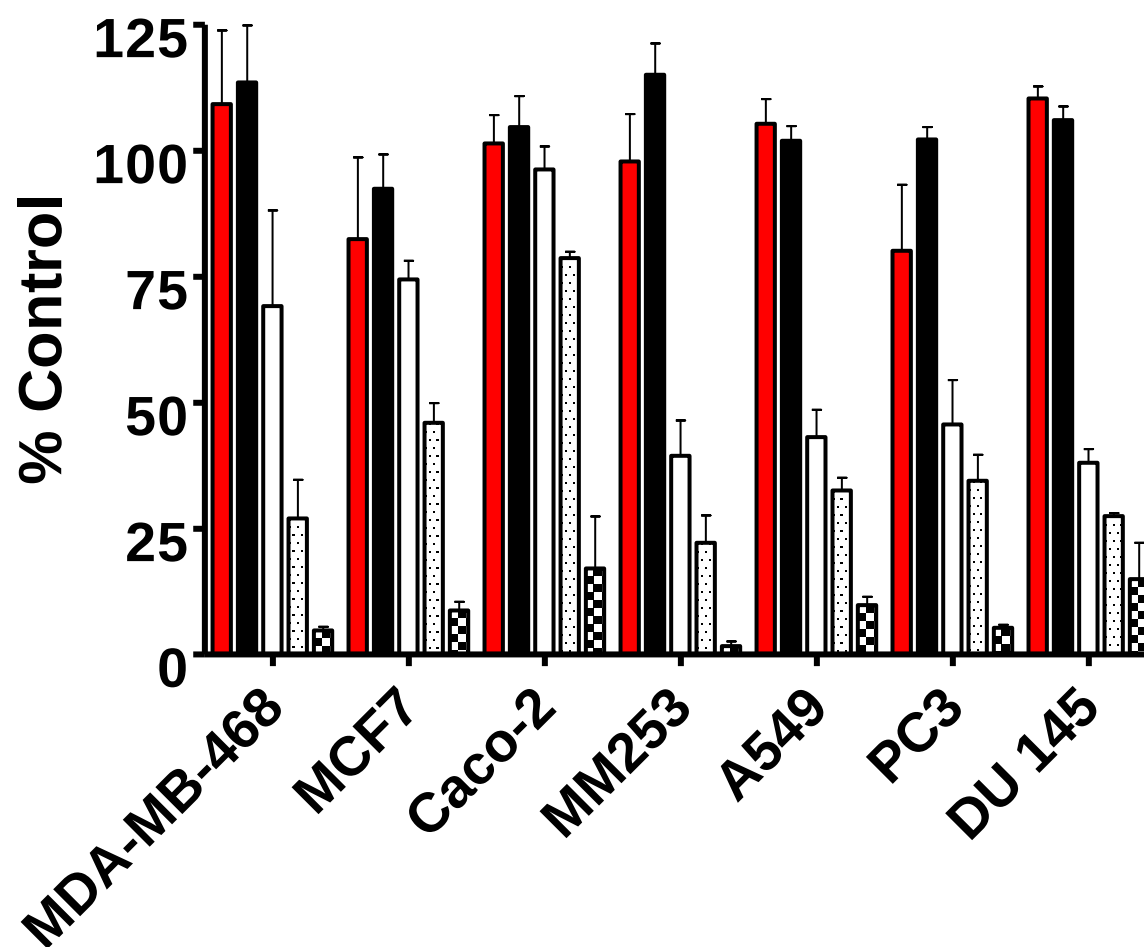


Figure 7.7 Effect of *H. spicatum* extracts on different cancer cell lines.

Cancer cell lines were exposed to *H. spicatum* summer bulbs from Albany ($\blacksquare$) or Esperance ($\blacksquare$) at 6 mg/mL (w/v) in methanol or 1 mM of DFO ($\square$), L1($\boxplus$) or 100 μ M CPT ($\boxtimes$) for 48 h before changes in proliferation was assessed by the MTT assay. Results are expressed as % of control values and are the means $\pm$ SE of quadruplicate wells from at least three separate experiments.

Other positive controls used, namely 100 μ M 5FU and 10 μ M PAC, VIN and ETO, also inhibited proliferation of all cancer cell lines tested to a greater degree than the *H. spicatum* sample from Albany. These data are not included in the figure for the sake of brevity. HgCl₂ and TX-100 caused 100% cell death.

7.4 DISCUSSION

7.4.1 Random approach

It must be emphasised that the collection and subsequent extraction of sponge material was performed by others, who generously provided me with a limited supply of material for testing in these cytotoxicity screening studies. Unfortunately, this meant, once depleted, no further samples were available for assessment.

The extracts of marine sponge assessed for bioactivity in these experiments were chosen based on preliminary data and availability by a collaborator, Ms Jessie Moniodis (Pharmacy, UWA). Ms Moniodis showed that some of these extracts were cytotoxic to brine shrimp (see section 2.7.2.1) and the concentrations chosen were based on her data. Some promising data were gathered despite having only enough sample for two experiments. For example, Extract 6 caused strong inhibitory effects in PC3 cells while having little effect against MRC5 cells, suggesting selectivity. A similar trend, albeit not as strong activity, was also recorded for this extract in some other cancer cell lines. Similarly, Extract 5 was also somewhat selective, but its activity was generally not as potent.

Extract 3 was also very potent at inhibiting the proliferation of every cell line tested. Indeed, at 300 μ g/mL, Extract 3 caused almost total inhibition of A549 and PC3 cells, while inhibiting the others by at least 20%. Unfortunately, this was also the case for the non-tumourigenic cell line, MRC5 cells, suggesting the effect was likely due to a non-

specific mechanism rather than any particular anticancer properties. Similarly, Extract 4 showed very high, but non-cancer cell specific, bioactivity.

Extracts 2 and 8 were not effective at inhibiting the proliferation of cancer cells, in agreement with preliminary data which showed no bioactivity of these extracts in a BSLA (Moniodis, J., personal communication, 13th May 2010). Despite being judged bioactive in the BSLA, Extract 1 was not active against the panel of cancer cell lines tested, at least at <1 mg/mL. However, the concentrations used in the BSLA were much higher and >69 mg/mL was required to induce >50% brine shrimp death (Moniodis, J., personal communication, 26th May 2010).

It was disappointing that the lack of more sample precluded these interesting extracts from further study. While it may have been possible to obtain more sponge material and perform additional extractions, since the objective was simply to compare screening data between two different approaches to biodiscovery, further experiments in this vein were beyond the scope of the current project.

7.4.2 Guided approach

It is known that *H. spicatum* bulbs of different regions have different degrees of red pigmentation (Woodall *et al.*, 2010). Clearly, the bulbs collected from Albany were a deeper red than those from Esperance (Fig. 7.2). According to Woodall (Woodall, G., personal communication, 17th April 2007), bulbs from Geraldton are similarly lighter red in colour compared to those from Albany. This red pigmentation is due to the amount of the phenylphenalenone, haemocorin, within the bulbs (Cooke & Segal, 1955). Therefore, differences in colour reflect differences in haemocorin levels. Thus, bulbs from Albany have more haemocorin than those from either Esperance, which lies

390 km NE, or Geraldton, approximately 760 km NNW of Albany. Variations could be due to local soil conditions, microclimate and/or genetic differences.

In a preliminary experiment, dried *H. spicatum* bulbs collected from Albany, Esperance or Geraldton and dissolved in DMSO had little effect on the proliferation of the two human breast cancer cell lines, MDA-MB-468 and MCF7 (Fig. 7.4). Only the sample from Albany showed any significant bioactivity, and this was just at the highest concentration tested (1,000 µg/mL) and in MDA-MB-468 cells only. Additionally, no dose-dependency was apparent. Hence, the *H. spicatum* experiments were put on hold until fresh samples became available the following year.

Figures 7.5 and 7.6 depict the effects of *H. spicatum* bulb samples collected from Albany and Esperance. Clearly, besides a moderate (but not significant) effect in MCF7 and PC3 cells by the sample from Albany, no bioactivity was evident against the panel of cell lines over the range of concentrations tested. Given the positive controls were capable of inhibiting proliferation of every cell line, it must be assumed that the system was working and the findings were credible.

Therefore, there was no evidence that bulbs from different regions had differential cytotoxic effects, despite their variability in colour. This would seem to undermine the claim that haemocorin has antitumour activity (Daw *et al.*, 1997). However, it may simply be that the levels of haemocorin were not high enough in the bulbs, even the reddest ones from Albany, to cause a detectable effect, or that the extraction process failed to isolate or enrich for the antitumour compound. In order to test this, it would be necessary to develop an assay to quantitate the levels of haemocorin in samples instead of simply relying on a colour judgment. The cytotoxic activity of known amounts of pure haemocorin could then be measured and compared to the amount of haemocorin in

bulbs from different regions and seasons. However, this was beyond the scope of the present research.

Another possibility is that by the time the samples arrived from Albany (5 days), the haemocorin had oxidised, resulting in a loss of bioactivity. This notion was strengthened by an experiment in which a methanol sample of bulbs from Albany was tested a short time after collection and then again one year later. Moderate bioactivity present in the fresh sample was lost upon storage (data not shown). This effect was thought to have been due to the oxidation of haemocorin during that period. However, there was no obvious loss in colour of the sample to support this theory.

Alternatively, perhaps the haemocorin in *H. spicatum* is in the wrong chemical form to cause any anticancer effects. According to Harborne *et al.* (1999), the aglycone of haemocorin displays antitumour effects, but it is the cellobioside that is present in a close relative of *H. spicatum*, *Haemodorum corymbosum*. If this is also the case in *H. spicatum*, that may explain the lack of bioactivity shown.

Then again, perhaps another compound in the crude extract masked, or even antagonised, the cytotoxic activity of the haemocorin. Such interactions are common when crude extracts are screened and is one of the arguments against bioprospecting (Farnsworth & Bingel, 1977). On the other hand, sometimes the ingredients of mixtures act synergistically and bioactivity is lost upon isolation of the active ingredient (Houghton *et al.*, 2007). These interactions and their possible significance to the samples evaluated in this thesis, as well as to bioprospecting in general, will be discussed further in the next chapter.

7.4 SUMMARY

In Chapter 4 of this thesis it was demonstrated that of the plant species identified by local Aboriginal people as having a traditional medical use, over half displayed bioactivity in initial screening studies. This chapter aimed to compare the confirmed high success rate of the ethnopharmacological approach to drug screening with a less guided approach. To this end, various extracts of a randomly harvested marine sponge of unknown taxonomy were assessed for their effects on a panel of human cancer cell lines. Out of eight extracts tested, two displayed some moderate, selective cytotoxic activity for cancer cells. In contrast, bulbs of the native plant, *H. spicatum*, were also tested and found to have no discernible bioactivity, despite its traditional medicinal use and published claims that a major constituent has possible antitumour effects. Overall, then, these results do not support the hypothesis that guided approaches to biodiscovery, such as choosing samples based on ethnomedical knowledge, are superior to random sampling as measured by their hit rate in screening studies. However, it must be remembered that the final concentrations of *H. spicatum* and sponge extracts were not equal. Moreover, since the sample size was just two, these results provide only anecdotal evidence at best.

Chapter 8: General Discussion

This chapter seeks to integrate the elements of the foregoing chapters and to discuss the significance of the study. Additionally, issues and limitations raised in the main body will be discussed in more depth. Some possible future research directions will also be suggested. However, to do that, it is first necessary to summarise the major findings of each of the experimental chapters. While these summaries are basically duplications of those appearing at the end of each results chapter, they are reproduced again here in one place amongst outlines of every chapter to highlight how each chapter links to the next, making clear the framework for the entire study.

8.1 SUMMARIES

8.1.1 Chapter 1: General Introduction

In Chapter 1, a broad overview of the published literature surrounding the three broad themes of cancer, drug discovery and ethnopharmacology was provided. In reviewing the relevant literature, a rationale for the current project was developed.

8.1.2 Chapter 2: Materials and Methods

A basic materials and methods section, this chapter provided a portal for common procedures used in the experiments described in later chapters.

8.1.3 Chapter 3: Optimisation of Experimental Techniques

This chapter described the validation and optimisation of the experimental design. Firstly, the panel of cell lines chosen was justified and their growth characteristics defined in order to optimise the culture conditions required for experiments. Secondly, the rationale for choosing the MTT assay to monitor cytotoxic effects of test agents was provided, and the refinement of the routine procedure hitherto employed in this laboratory was recounted. Next, the suitability of DMSO as a vehicle was examined and shown to be appropriate. Finally, a fitting positive control, DFO, was chosen from a

range of potentially suitable compounds. From these studies, a standard protocol was devised which was used in subsequent experiments to evaluate the cytotoxic activity of extracts of plants traditionally used as medicines by Aboriginal people.

8.1.4 Chapter 4: Preliminary Screening of Plant Extracts

A total of 25 plants from two distinct areas were chosen for evaluation of their anticancer potential based on traditional Indigenous medical knowledge of those species. Relevant plant parts were harvested and crude methanolic extracts derived. These plant extracts were assessed for their bioactivity against four different human cancer cell lines using the MTT assay to measure changes in cell proliferation. Preliminary screening narrowed the field to a pool of 16 promising extracts which were assessed for their dose-responses against the same four cell lines. Based on these data, 6 extracts were chosen for more thorough assessment of their effects, including morphological changes, the speed of their actions and their dose-response relationships against a panel of several more cancer cell lines and one non-cancerous cell line. These six extracts exhibited differences between each other and between cell lines, indicating a degree of selectivity. The most promising four of these methanolic extracts, 5, 6, 21 and 28 were chosen for further evaluation, the results of which constitute Chapter 5.

8.1.5 Chapter 5: Evaluation of Most Promising Plant Extracts

More plant samples of those with the most bioactive methanolic extracts in the initial screen were collected. These were extracted to give aqueous, methanol or ethyl acetate fractions which were subjected to various chemical analyses and assessed for their specificity and selectivity against cancer cells. On the basis of these cytotoxicity studies, the extract with the greatest potential as an anticancer agent, the ethyl acetate extract of *Eremophila duttonii* (EA3), was chosen for further study, which was the focus of the following chapter.

8.1.6 Chapter 6: Further Evaluation of Plant Extract, EA3

The results of experiments undertaken in Chapter 6 suggested that EA3 causes cytotoxicity in some cancer cell lines and yet has only cytostatic effects in others, illustrating a degree of selectivity. Both cytotoxicity and cytostaticity are possibly attributable to the actions of two or its major constituents, the flavonoids luteolin and quercetin, acting synergistically. However, it is also possible that the observed effects of EA3 are due to other known or novel compounds. EA3-induced cytotoxicity appears to be mediated via Ca^{2+} signalling processes as EA3 clearly induced a transient rise in $[\text{Ca}^{2+}]_i$ in PC3 cells within minutes of their exposure to this extract. The Ca^{2+} response was biphasic which is consistent with release of Ca^{2+} from internal stores followed by extracellular Ca^{2+} influx. The pattern of the trace also indicates that the elevated $[\text{Ca}^{2+}]_i$ was unlikely to be due to sudden chemical damage to the cells as there was no evidence of a breach in the plasma membrane integrity within the experimental time frame. As cytotoxic effects were evident in susceptible cells within just 90 min of exposure to EA3, taken together, these results suggest that the observed EA3-induced cytotoxicity is likely due to apoptosis, not necrosis.

8.1.7 Chapter 7: Two Different Biodiscovery Strategies

This chapter aimed to compare the confirmed high success rate of the ethnopharmacological approach to drug screening with a less guided approach. To this end, various extracts of a randomly harvested marine sponge of unknown taxonomy were assessed for their effects on a panel of human cancer cell lines. Out of eight extracts tested, two displayed some moderate, selective cytotoxic activity against cancer cells. In contrast, bulbs of the native plant, *H. spicatum*, were also tested and found to have no discernible bioactivity over the concentration range studied, despite its traditional medicinal use and published claims that a major constituent has possible antitumour effects. Overall, then, these results do not support the hypothesis that guided

approaches to biodiscovery, such as choosing samples based on ethnomedical knowledge, are superior to random sampling as measured by their hit rate in screening studies.

8.2 DISCUSSION

8.2.1 Significance

Aboriginal people living in traditional communities had an encyclopaedic knowledge of Australian plants and animals and of the seasonal changes of their environment. They knew, for example, that when the bat-wing coral trees flowered, it was time to go and dig mangrove crabs out of the mud. The Aboriginal awareness of life cycles of animals and plants was gathered over thousands of years and was understood by everyone in the community. Despite not being written down, it was shared from generation to generation through example, song and dance so that every child learnt the significance of the natural signs. This TK was specific for each local area and covered not only food resources, but extended to the medical uses of various plants. At one stage, all members of the family knew the names, location, structure, value and application of medicinal plants (Isaacs, 1987; ACNTA, 1988).

Aboriginal people have historically shared their TK generously. Indeed, it was their open revelation of details of edible seeds, fruits and roots, water sources, and how best to catch animals that convinced sceptical outback settlers of the authenticity of Aboriginal claims to tribal lands (Isaacs, 1987). However, as a consequence of having no written language and young Aboriginals moving away from traditional lands and showing a declining interest in their heritage, this well of information is in serious danger of drying up. For this reason, a project aimed at determining the therapeutic effectiveness of Aboriginal traditional medicines was undertaken in the mid 1980s. The importance of this endeavour was recognised by both the Northern Territory and

Federal Governments who jointly supported the project as part of a Bicentennial Commemorative Programme (Stack, 1989). It resulted in the publication of an Aboriginal Pharmacopoeia which linked traditional Aboriginal medicine with modern science (ACNTA, 1988). Over 40 Aboriginal communities shared their traditional ethnomedical knowledge about specific bush medicines which is detailed in the internationally recognised document, along with a botanical description for each species and the chemical composition and therapeutic activity known to that time. More recently, the web-based *Customary Medicinal Knowledgebase* has been developed as an integrated multidisciplinary resource to document, conserve and disseminate the valuable Aboriginal TK that is scattered throughout the published literature and amongst various Aboriginal communities (Gaikwad *et al.*, 2008).

Isaacs (1987) suggested that the greatest repository of centuries of botanical knowledge and experience lies with Aboriginal women rather than men. Theoretically, everyone in a community knew the names and locations of all useful plants, but sex roles were well defined in traditional communities. It was the women who were the gatherers of plant foods and herbal medicines while the men were preoccupied with preparing for the hunt. While men knew all the plants, it was the women who knew the finer details of their respective uses (Lassak & McCarthy, 2001). For this reason, the plants collected for this study were those identified by mostly Aboriginal women guides.

The Australian continent covers a vast range of botanical environments: from tropical coast to rainforest, from open scrub to wet sclerophyll forest, from woodland to desert and from temperate riverine environments to snowy alpine mountains. Aboriginal people once lived in all these areas and continue to live in most, utilising the natural foods and medicines unique to Australia (Isaacs, 1987). This project has looked at

plants traditionally used as medicines by Aboriginal people of just two desert communities. Screening for cytotoxic activity verified the bioactivity of several of these medicinal plants, scientifically validating what the Aboriginal people have known for thousands of years. Several flavonoids identified in one particular species, *Eremophila duttonii*, accounted for some of its observed bioactivity, which agreed with published data. However, the compound likely responsible for the main cytotoxic/cytostatic actions of this species could not be definitively identified as part of this study, leaving open the possibility that the inhibitory effects were due to a new chemical entity (NCE).

Given the success of this study, it would be interesting to further evaluate the anticancer potential of other bioactive plants identified in the initial screen. This approach could also be used to evaluate plants for use against other diseases besides cancer. While several extracts displayed selective antibacterial properties and one showed modest antiviral activity in independent screening tests performed (at another university) on the same plants examined in the current study (Evans *et al.*, 2007), many other disease targets could also be assessed (see Section 8.2.7.2). Provided that local guides can be persuaded to help, the entire procedure could then be repeated for other Aboriginal communities living in other regions of Australia. In this way, not only will Aboriginal TK be recorded for preservation, but it will also hopefully be scientifically validated too. By using ethnopharmacological knowledge from other Australian landscapes, the chances of discovering a novel therapeutically useful agent will be multiplied.

As described in the General Introduction to this thesis, one of the main goals of this particular study was to identify a potential anticancer agent for chemotherapeutic use outside of the Indigenous community who provided the lead, hopefully resulting in a direct economic benefit to that community. While a NCE was not identified, it is still

possible that further tests may reveal that the observed bioactivity of plant extracts were due to a novel compound/s.

However, a second objective of the study was the validation of Traditional Aboriginal medical knowledge. Therefore, even though a novel compound could not be identified, the fact that extracts of the medicinal plant were bioactive is still significant. In the end, if bioactivity is not due to a NCE that can be exploited, then maybe it does not really matter what the active principle/s are. Perhaps the real point is that, regardless of what compound/s are responsible, *something* in the plants, acting alone or in combination, is bioactive. It seems likely that the various active constituents of extracts have to work as a whole, not in isolation, to be most effective. This would support the idea of the usefulness of traditional herbal medicines.

Therefore, this study may be considered a positive step towards reconciling traditional Aboriginal knowledge with Western medicinal ideals. While many more experiments are required before any anticancer drug can be developed, these preliminary results may pique the interest of a pharmaceutical company willing to invest in such a project. In such a case, the Aboriginal communities would be considered as major stakeholders of the enterprise. If not, marketable products based on some plants tested in the present study may still be feasible. As outlined in a previous section (1.2.3), there is a growing demand for medicines based on T/CAM, so the communities may choose to develop extracts of plants as alternative remedies for treatment of cancers and supply an industry partner with the required raw materials.

An excellent example of a successful international company founded on Aboriginal ethnomedical knowledge is Mount Romance, a manufacturer of a range of beauty

products based on the sandalwood tree, *Santalum spicatum*, and located in Albany, WA ("Mount Romance," 2010). A groundbreaking partnership between Mt Romance, Aveda (a US-based multinational cosmetics corporation) and the Kutkabubba Aboriginal community (represented by the Songman Circle of Wisdom) was launched in 2004 at Murdoch University in Perth (WA), representing the world's first case of indigenous intellectual accreditation. According to this protocol, Aveda and Mt Romance each donate \$50,000 to the Kutkabubba community for using the land and knowledge of the Aboriginal people. Ongoing funds are used by the community for any purpose they desire. Under the accreditation protocol, the partnership provides a new approach to protecting indigenous knowledge, which is very different from the patenting law. The Indigenous accreditation is a voluntary undertaking under a sustainability framework, allowing a holistic approach to indigenous knowledge (Marinova & Raven, 2006). As such, it represents a good model for partnerships between Indigenous communities and other groups who wish to apply their TK or employ their skills for commercial gain.

Another option is for Aboriginal communities themselves to set up a cottage industry based on skin creams or lotions to be sold directly to the public. Various examples of such cottage-style industry products traditionally used by Aboriginals exist. Many, such as the herbal remedy Gumbi(y) Gumbi(y) (*Pittosporum phylliraeoides*) and Emu Oil are sold at local markets or via various internet sites and can be found simply by doing an online search (e.g. "Gumby Gumby," 2010; "Essentially Bliss," 2009). Both Aboriginal communities involved in the current project already run viable businesses directly from their lands. The Titjikala community in the Northern Territory operates a tourist resort and sells authentic Aboriginal artwork directly to travellers who visit their purpose built gallery ("Remote communities," 2009; "Tapatjatjaka," n.d). Similarly, the Scotdesco Aboriginal Community operates several enterprising schemes, including running Aboriginal cultural

awareness presentations and Wirangu language and cultural experience camps. In addition, they sell books on the Wirangu language, various arts and crafts, and jars of Gujaru, a natural bush remedy to reduce pain, relieve breathing difficulties and dry itchy skin or rashes ("Scotdesco," 2010). Provided that further tests indicate no human toxicity, other herbal remedies based on any of the plants that showed bioactivity in the current studies could be similarly developed and marketed as traditional bush medicines with the additional selling point that the effects have been scientifically validated. However, even if it proves to be unviable to profit financially from this knowledge, it is hoped that Aboriginal people who shared their TK of medicinal plants will still feel empowered by the realisation that it has been scientifically verified and therefore seen to be valuable by the Western world.

8.2.2 Limitations

Even though the research described in this thesis has demonstrated clear cytotoxic effects of a plant extract (EA3) against cancer cells, it must be recognised that cytotoxic activity *in vitro* does not necessarily translate to clinical significance or even antitumour activity *in vivo*. For example, it may not be practicable (or cost-effective) to extrapolate the dose from that giving activity *in vitro*, to that which would be required for the size of a person. More importantly, physiological factors including absorption and metabolism may cause discrepancies between *in vitro* and *in vivo* activity (Houghton *et al.*, 2007). However, while *in vitro* cytotoxicity is not always the most effective or reliable means of predicting *in vivo* antitumour activity, *in vitro* cytotoxicity assays are generally rapid and inexpensive, and are therefore the most popular methods for initial tests (Dewick, 2002), hence the use of the MTT assay in the current study.

Nevertheless, it is vital that these results are not over-interpreted, which, according to Gertsch (2009), is often the case in many ethnopharmacologically-based articles in the literature. This means recognising the dangers inherent in focussing on just a part of

something that, as a whole, is much more complex, just as in the old tale of the blind men and the elephant. In this context, it must be stressed that the findings of this study are preliminary only, and many more experiments are required before they can be accurately correlated with human use.

There were many other issues arising from the current study that require commentary. For simplicity, this will be broken down such that each experimental chapter is discussed in turn. Conclusions, limitations or implications of each topic will be addressed, as well as some possible future directions.

8.2.3 Chapter 3: Optimisation of Experimental Techniques

8.2.3.1 *Cell lines*

According to Lord (1987), any successful therapy should ideally eliminate the abnormal cells while leaving all normal cells functionally undisturbed. In practice, this objective is usually never realised, particularly in the case of cancer chemotherapy. Even the most successful anticancer drugs have often been chosen because of their ability to preferentially inhibit rapidly dividing cells. Their therapeutic use represents a predictable compromise which seeks to achieve elimination of neoplastic cells while causing minimal damage to normal cells. Even in the best situations, undesirable side effects are inevitable.

This is because some normal cells (e.g. cells of the epithelial lining of the gut, bone marrow and hair follicles) grow at a faster rate than most tumour cells, and chemotherapeutic agents may kill these cells more efficiently than cancer cells, resulting in devastating side effects or even dose-limiting toxicities which can allow tumour cells to escape treatment and develop drug resistance (Keyomarsi & Pardee, 2003). Nevertheless, it may be possible to markedly reduce any cytotoxic effects on normal cells by exploiting other differences in their cell cycle

regulation (Keyomarsi & Pardee, 2003) or by employing special drug delivery systems that specifically target cancer cells (Dharap *et al.*, 2005; Lord, 1987).

Indeed, cancerous cell lines provide a very good model for studying the effects of various potential anticancer agents at the cellular level. While host factors such as drug metabolism, neurohormonal mechanisms, immune response and other physiological interactions often modify the effects and toxicity of a drug within the body, *in vitro* results generally give a good indication of cytotoxicity (Friedman *et al.*, 1984; Hofs *et al.*, 1992; Korting & Schafer-Korting, 1999). Indeed, Shrivastava *et al.* (1992) showed that an accurate *in vivo* LD₅₀ dose could be predicted from *in vitro* data for at least 75% of the compounds tested. There is usually a good correlation between lack of potency *in vitro* and lack of antitumour activity *in vivo*. However, high cytotoxic potency *in vitro* does not necessarily predict activity *in vivo* (Mirabell *et al.*, 1986; Cushion *et al.*, 2006). Thus, while not able to give definitive answers regarding anticancer activity, cell lines are very useful tools for initial screening programmes. As this project was, in essence, a screening study, the use of cell lines was considered an appropriate methodology. The significance of the cell lines chosen was that they represented the five most commonly occurring cancers in Australia.

According to the third “law of toxicology”, humans are animals and therefore the study of animals can provide useful insight into effects on humans (Goldstein & Gallo, 2001). Thus, the next step in this work will be to further evaluate promising extracts or compounds in *in vivo* studies (after obtaining appropriate Animal Ethics approval). This will mean inoculating cancer cells subcutaneously in nude mice to induce tumours and then administering the active compound/s to determine if a significant reduction in the

size of the tumours occurs over time. The physiological indicators of normal metabolism will also be monitored.

8.2.3.2 *MTT assay*

Much effort was expended in optimizing the MTT assay as it was the primary method of assessing cytotoxicity in these studies. In particular, time was spent selecting an appropriate solvent for solubilising the MTT formazan crystals as this process is crucial to the accuracy and reproducibility of the MTT colourimetric method (Li & Song, 2007). Initially, it was determined that the best extraction buffer consisted of 10% SDS, 50% isobutanol, 0.1M HCl (see Appendix C). This was based on this buffer producing the highest absorbance readings and so it was inferred that this would translate to the greatest sensitivity. However, background absorbance values were unacceptably high using this buffer, which interfered with the interpretation of data. The high background levels were probably due to undissolved formazan crystals which were apparent in some wells containing comparatively many viable cells even after mixing. The same phenomenon was reported by Li and Song (2007) who used isopropanol. As in the current study, these authors switched to DMSO for extraction because, in contrast, formazan was completely dissolved within 2 min using this solvent. Significantly, after assessment of many formazan solvent systems, DMSO was also the solubilising agent adopted by the NCI anticancer drug screening programme (Alley *et al.*, 1988).

The main modification to the original assay procedure was discarding the contents of the wells before the addition of MTT. This change reduced interference effects associated with the mixture of substances in wells, resulting in lower background absorbances.

Overall, the MTT assay was shown to be a reliable indirect method of measuring cell viability and hence appropriate for assessing cytotoxicity in the current study. However, it should be mentioned that there can be limitations with the MTT assay as metabolic activity may be altered by various conditions or chemical treatments, or there can be chemical interactions with MTT itself (Plumb, 1999; Ulukaya *et al.*, 2004; Ganapathy-Kanniappan *et al.*, 2010). While some compounds inhibit the reduction of MTT, others can augment it, or at least appear to due to chemical interference (Schubert, 1997; Ulukaya *et al.*, 2004; Ahmad *et al.*, 2006). When certain compounds are tested, the use of MTT as an indicator of metabolically active mitochondria has been shown to overestimate the number of viable cells by comparison with the ATP, DNA, or trypan blue determination (Ulukaya *et al.*, 2004; Wang *et al.*, 2010).

Increased absorbances due to MTT-formazan production via upregulation of mitochondrial dehydrogenases or chemical interference results in an underestimation of the antiproliferative effects of the test compound. It is possible that if this occurred in the initial screening studies of the current study, potential useful agents may have been lost in the elimination process as they were falsely classed as inactive. Inhibition of MTT reduction or chemical interference causing lower absorbances would lead to a bias that produced false-positives. This would not have been such a problem as false-positives were likely to have been removed at a later stage, after the modifications to the MTT assay were introduced. The removal of all compounds that would potentially interfere with the MTT assay prior to the addition of MTT went some way towards alleviating such problems, but it should be acknowledged that residual amounts may have had some influence on results obtained. In later experiments, the CS system was employed to back up the data obtained from cytotoxicity studies using the MTT assay.

While the direct effects of extracts on MTT reduction were not measured, their effects on absorbance were taken into account by measurement in cell-free media.

8.2.3.3 **Vehicle**

DMSO is commonly used as a carrier solvent and is therefore often included in experiments as a vehicle control (Damm *et al.*, 2001; Chang *et al.*, 1996; Dent *et al.*, 1999). For example, Xiao and Singh (2002) used DMSO to dissolve the chemopreventative agent they were testing against PC3 cells and so used an equal volume of DMSO (<1%) in control wells and Yamamoto *et al.* (1998) used 1% DMSO as the vehicle control in their studies.

At low levels DMSO does not generally affect cell viability or proliferation. For example, Song *et al.* (2002) and Anderson *et al.* (1998) demonstrated that 0.1% DMSO had no effect on cell number, cell viability or ³H- DNA synthesis when incubated with PC3 cells for 48 h and DMSO added at concentrations between 0.6-1% did not appear to change the cell cycle parameters or appearance of HT-29 colon cancer cells (Shiff *et al.*, 1996; Siavoshian *et al.*, 2000). DMSO at 0.1% was shown to be irrelevant to morphology, viability and proliferation of MDA-MB-468 cells (Prassas *et al.*, 2008). Kuntz *et al.* (1999) used DMSO as a vehicle control at concentrations up to 2% in studies on cell proliferation, cytotoxicity and apoptosis in four cell lines, including MCF7 and Caco-2. However, Blom *et al.* (1998) found that DMSO had a stimulatory effect on MCF7 cell proliferation at low concentrations, but decreased cell proliferation at concentrations of 0.8 and 1%.

Vesey, *et al.* (1991) showed that while only slight morphological differences between control Hep-G2 cells and those treated with 2% DMSO could be observed under a phase-contrast microscope, scanning electron microscopy showed that DMSO-treated

cells were flatter and spread out more than their untreated counterparts. More relevant to this thesis, DMSO reduced cell proliferation in a dose-dependent manner, such that significant differences were observed even at final concentrations of only 1%. However, these results were described for a cell line in which DMSO induces differentiation.

While DMSO can induce the differentiation of some cell lines, including the murine erythropoietic line FLC (Friend *et al.*, 1971) and the human hepatoblastoma-derived Hep-G2 (Vesey *et al.*, 1991), the only cell line of those employed in this study that can undergo differentiation is Caco-2 and it does so spontaneously anyway upon attaining confluence and with regular changes of culture medium (Mariadason *et al.*, 1997). Besides, other researchers (Wolter & Stein, 2002) have used DMSO as the carrier vehicle in differentiation studies using Caco-2 cells, so it was a considered decision to use it here.

8.2.3.4 Controls

As the carrier vehicle, DMSO was therefore employed as the negative control in cytotoxicity assays. Initial studies used a fixed concentration of 1% DMSO (v/v), as this was the maximum amount that cells incubated with plant extracts were exposed to. However, it was subsequently shown that DMSO itself did inhibit proliferation of some cell lines in the panel in a dose-dependent manner (Fig. 3.12). Therefore, it was necessary to include extra control wells consisting of DMSO at concentrations corresponding to the amount in wells containing different dilutions of plant extract (or positive controls). While this step complicated the procedure, it was deemed crucial to the accuracy of the data.

Based on effectiveness against all cell lines in the panel and reproducibility the primary positive control chosen was DFO (Fig. 3.13 and 3.14). This control acted as a universal

internal standard to ensure the system was reproducible and that experiments performed on separate days could be compared. L1 and CPT were sometimes also used as additional controls. Other compounds tested were not suitable due to ineffectiveness in one or more cell lines. Upon reflection, this ineffectiveness may have been due to an overestimation of cell viability by the MTT assay as reported for some cytotoxic agents, including vinblastine and paclitaxel (Sobottka & Berger, 1992; Ulukaya *et al.*, 2004). To confirm this, the direct effects of these compounds on MTT reduction could be evaluated by showing dose-dependent changes in absorbance readings in cell-free media. Regardless, these other drugs were inappropriate using the system employed in the present study and were not investigated further.

It is worth mentioning here that tamoxifen (TAM) was trialled against all cell lines in the panel. This might seem a curious tactic at first, given that TAM is widely recognised as the standard therapy for hormonally-sensitive breast cancers (Zarubin *et al.*, 2005; Valachis *et al.*, 2010). This is because its mechanism of action is primarily via estrogen receptor-dependent modulation of gene expression (Zheng *et al.*, 2007). However, TAM at higher doses ($>10\ \mu\text{M}$) has also been shown to have (cytostatic and cytotoxic) antiproliferative and pro-apoptotic activity against many estrogen receptor-negative cells, possibly due to its ability to generate oxidative stress and activate signal transduction pathways (Perry *et al.*, 1995; Cai & Lee, 1996; Robinson *et al.*, 1998; Ferlini *et al.*, 1998).

Of the cancer cells used in the current study, only MCF7 expresses estrogen receptors. However, TAM at low concentrations ($<10\ \mu\text{M}$) did not affect the proliferation of these cells under the conditions employed. The reason for this was not investigated, but is probably not related to TAM interfering with the MTT assay as it is not known to do so

and is frequently used to evaluate antiproliferative activity (Al-Joudi *et al.*, 2005; Bopp & Lettieri, 2008; Rossi *et al.*, 2009). However, 50 μ M TAM dramatically reduced the proliferation of both breast cancer cell lines, MDA-MB-468 and MCF7 cells, suggesting an estrogen receptor-independent mechanism as MDA-MB-468 cells are estrogen receptor-negative (Wang *et al.*, 1997). Nevertheless, it did not inhibit the proliferation of any other cell line tested and so was not suitable as a universal positive control.

8.2.4 Chapter 4: Preliminary Screening of Plant Extracts

8.2.4.1 Physical properties

The physical properties of plant extracts that were assessed in these studies were chosen in order to eliminate the possibility that any cytotoxic effects were due to simple physical assault. As the pH and osmolality were shown to be similar to controls, it was concluded that cells were not damaged due to excessive acidity or alkalinity or hyper- or hypo-tonicity. As the principal method used to assess cytotoxicity, the MTT assay, was spectrophotometrically-based, the colour of the extracts caused initial problems via colour interference with MTT formazan. However, this issue was resolved by adapting the method by removal of the extracts before addition of MTT.

8.2.4.2 Initial screening

According to Boyd and Paull (1995), the least interesting or useful (but most common) response to a random selection of chemical structures is none at all. A similarly unremarkable profile is one in which one or more concentrations of the tested compound produce/s growth inhibition and/or cytotoxicity of basically the same magnitude across all the cell lines of a panel. Of course, most screening studies, including the NCI programme, are capable of identifying highly potent, indiscriminate direct cell poisons, but that is not a particularly useful attribute of a screen. What is important to ascertain is evidence of a differential response between cell lines (Boyd & Paull, 1995). It is especially desirable to show selective activity for cancer cells

compared to non-cancerous cells. Therefore, as part of the current study, both potency *and* selectivity were key when selecting plant extracts with promising cytotoxic or cytostatic effects.

In that light, the most promising plant extract from the initial screen was Extract 3. However, unfortunately, the limited amount of this extract precluded it from selection. While it was feasible that more material could have been obtained for further study, it must be remembered that a major objective of this research was to discover a cytotoxic agent that could potentially be developed so as to economically benefit the Aboriginal community that provided the TK that originally identified the plant of interest. Extract 3 was derived from the roots of *Thysanotus exiliflorus*, a plant that, according to the Aboriginal women guiding the collections, is only found after “a good rain” and, indeed, was not found on three subsequent expeditions after the original collection took place. Moreover, the roots of *T. exiliflorus* are only very small, so harvesting enough of them to produce an adequate supply for a drug company to derive the active constituent would likely not be economically viable. A cottage-style industry based on the crude extract would likely be, similarly, unviable. However, many important current chemotherapeutics, including Taxol[®], are chemically synthesised or semi-synthesised after having been developed from natural product (NP) leads (Cragg & Newman, 2005). This means that there is still potential for Extract 3 to be of use as an anticancer agent and generate income for the providers of the TK via royalties paid by the drug company that develops it. Obviously, this enterprise would require the expertise of a chemist and a sophisticated chemistry laboratory as well as many more thousands of investment dollars, none of which were available for this PhD project.

Another option could be to investigate other, larger species of the *Thysanotus* genus for similar levels of bioactivity in the hope that harvesting a greater biomass will mean greater yields of bioactive compounds. However, while over 40 species of *Thysanotus* have been identified, they are very alike in that they are all perennial herbs characterised by distinctive fringed lily-like purple flowers (Brittan, 1981; Gathe & Watson, 2008). As monocotyledons, they do not typically display any secondary thickening, the usual means for plants to increase their girth. Only *T. spiniger* has been reported to exhibit secondary growth, but this is in the form of a woody underground stem (Rudall, 1995) and is unlikely to mean much greater yields of plant matter. While being herbaceous definitely does not preclude a plant from being cultivated for harvest (e.g. wheat and barley) and a lack of true secondary thickening does not necessarily correspond to small plant size (e.g. palm trees), *Thysanotus* species have generally proved difficult to maintain in cultivation despite being relatively easily propagated from seed (ANPSA, 2007).

Perhaps there could be some merit in procuring a botanist to research the potential cultivation of *Thysanotus*. The goal would be to produce large quantities of the bioactive constituents present in *T. exiflorus* by ongoing harvest. However, given the growth restrictions of *Thysanotus* species, a more realistic idea might be to harvest just enough plant matter for identification of the active compound/s so that subsequent laboratory synthesis may be possible. This synthesised compound could then be further studied in terms of its potency and specificity for cancer cells. This scenario is very similar to what happened in the case of the major cancer chemotherapeutic, paclitaxel, the discovery and development of which is discussed further in Section 1.4.4.1.3 and Appendix M. However, this synthesis would also require the collaboration of other scientists, in this case organic chemists, and both options would require a great deal of

capital and many years of research. Unless government funding could be obtained, the challenge would be to find an investment company willing to inject the millions of dollars necessary. However, without further evidence of the potency and specificity of the crude extract, this would seem to be a catch-22 situation. If funding could be secured to follow either avenue of further research, the process would be coordinated by members of the Titjikala community whose TK provided the lead in the first place and any profits generated would naturally flow directly to the community.

8.2.4.3 *Dose-response*

Cytotoxicity is one of the most common biological parameters measured following experimental manipulation, largely because it is easily measured and is dose-dependent (Cho *et al.*, 2008). For this reason, cytotoxicity, as measured by the MTT assay, was used in these studies to assess the effects of various plant extracts on cancer cell lines. However, during the course of this research it became apparent that lower concentrations of an extract did not always correspond to lower levels of inhibition of cell proliferation. Indeed, in some cases, low doses of an extract caused an increase in cell proliferation. In other words, the data were inconsistent with the generally accepted monotonic sigmoidal, threshold model of dose-response (Calabrese *et al.*, 2006a).

Given the dose-response relationship is so fundamental to toxicology, a search for an explanation in the published literature uncovered the phenomenon of *hormesis*, an alternative model of dose-response. Hormesis is a biphasic dose-response concept in which opposite effects are displayed at high and low doses, although typically it is characterised by a low-dose stimulation and a high-dose inhibition (Calabrese, 2008b). It is seen with a wide variety of toxic agents and NPs in multiple organ and cell systems, regardless of endpoint measured, leading to the proposal that hormesis is broadly generalizable (Calabrese, 2005a; Calabrese, 2008a). Generally, hormetic

responses are modest, with values typically about 30-60% above control levels (Calabrese & Baldwin, 2003a; Calabrese, 2008a). The width of the low-dose stimulatory range is generally limited to about one order of magnitude (Calabrese & Baldwin, 1998; Calabrese, 2008a). For interest, more information on the hormetic model is provided in Appendix N. As the stimulation of cancer cell proliferation observed after exposure to some plant extracts occurred only at low doses and was less than 150% of control values (e.g. see Fig 5.3), it was concluded that hormetic effects were present in these cases.

As explained further in Appendix N, because hormesis has been observed for so many different species, substances and endpoints, it is thought that it is a generalised mechanistic strategy, but no single molecular mechanism has yet been proposed to explain its existence. Nevertheless, this high generalizability suggests that the hormetic dose-response strategy has been selected for and is adaptive in nature (Stebbing, 1998). It has been suggested that the hormetic process may be a common tactic for cells to allocate resources when a response to low-level metabolic perturbations is required (Calabrese & Baldwin, 2003). Thus, it represents an overcompensation to an alteration in homeostasis. When the dose progressively increases, the system's capacity to compensate becomes overwhelmed, the toxicity threshold is exceeded, and toxic effects are seen (Calabrese *et al.*, 1999).

The actions of agonists and opposing receptor subtypes may help account for numerous cases of hormetic-like biphasic dose-responses. In this model, a single agonist may bind to two receptor subtypes, one activating a stimulatory pathway and the other an inhibitory one. The receptor subtype with greatest agonist affinity would typically have fewer receptors, hence a lower capacity, and its pathway activation effects would

dominate at lower doses. Conversely, the other receptor subtype would have lower agonist affinity, more receptors and greater capacity, becoming dominant at higher concentrations (Calabrese, 2008). A variation on this theme may explain the hormesis seen in the extract-cell interactions of the current study. As the plant extracts contained many different compounds (ligands), cell receptors could have had specificity for activating cell growth with certain ligands at low concentrations, but binding to other, less specific, ligands at higher levels which resulted in cell death.

In the experiments of this study, hormesis accounted for some of the inaccuracies in calculating valid IC_{50} values. This is because the conventional nonlinear regression procedure (used by GraphPad Prism) is based on the simple log-logistic model, which is not appropriate for biphasic relationships (Beckon *et al.*, 2008). While formulae have been developed to calculate IC_{50} values when hormesis is present (Brain & Cousens, 1989; Vanewijk & Hoekstra, 1993; Schabenberger *et al.*, 1999), it was not possible to adopt these models as part of the current study due to unavailable expertise.

Nonetheless, it is acknowledged that fitting a log-logistic model and simply ignoring the presence of hormetic effects can lead to serious bias and erroneous inferences (Schabenberger *et al.*, 1999). Therefore, the IC_{50} values obtained in this study when hormesis was present were taken with some reservation.

However, there are more serious concerns with hormesis than merely making fitted dose-response curves look untidy and complicating IC_{50} value determinations.

Assuming hormesis is a real effect and not an artefact, there are grave implications in not getting the dose right. Consider the likely scenario of, for example, an anticancer chemotherapeutic with a relatively long biological half life that displayed hormetic low dose stimulation. In this case, as the drug is metabolised within the body, its effective

dose is reduced to subtoxic levels, which would result in an increase proliferation of the very cancer cells the medicine (at higher doses) is targeting. Additionally, as the hormetic stimulatory zone is close to the pharmacologic threshold, there is a distinct possibility that a desired therapeutic dose could be toxic to some individuals due to inter-individual variability (Calabrese, 2008a). Obviously, either of these cases would be harmful to a patient and is the main reason why plant extracts displaying high levels of hormesis were excluded from the pool for further study as suitable drug candidates.

8.2.4.4 *Morphological changes*

Morphological changes to cancer cells upon addition of plant extracts were evaluated qualitatively under phase contrast light microscopy at 400X total magnification. This technique was suitable to establish cell death and to distinguish the relative effects of different extracts on different cell lines for screening studies. However, it was not possible to definitively say via which mechanism, apoptosis or necrosis, cells were likely to have died. In retrospect, it would have been better to have stained the cells with hematoxylin-eosin or one of the Romanowski group of stains (based on methylene blue and eosin) as this allows the classical hallmarks of apoptosis such as cell shrinkage, membrane blebbing and formation of apoptotic bodies to be viewed more easily (Hengartner, 2000; Doonan & Cotter, 2008; Krysko *et al.*, 2008). Sometimes it is also possible to see other features characteristic of apoptosis, like nuclear pyknosis (chromatin condensation) and karyorrhexis (DNA fragmentation) under light microscopy (Canalejo *et al.*, 2000). However, fluorescence microscopy using fluorescent dyes (e.g. Dapi and Hoechst stains 33258 and 33342) that stain the nuclei of the cells is more widely used as it enhances differentiation of smaller apoptotic nuclei and visualisation condensed chromatin at the nuclear membrane and even nuclear fragmentation (Doonan & Cotter, 2008), and perhaps this technique could be employed in future studies that focus on one or two extracts or compounds.

While it is possible to perceive many of the characteristic features of late apoptosis using light and fluorescence microscopy, to improve resolution electron microscopy could be employed. Scanning electron microscopy (SEM) provides detailed information about the cell surface, and the membrane in particular, enabling visualization of membrane blebs. For example, using SEM, Canalejo *et al.* (2000) visualised an intact cell membrane with nuclear changes that included chromatin condensation and fragmentation as well as the occasional apoptotic body surrounded by a cell membrane. On the other hand, transmission electron microscopy (TEM) facilitates the analysis of sectioned specimens providing internal images of the cell. Where detection of morphological features of apoptosis by regular microscopy is difficult, TEM can assist. Moreover, the shape adopted by the condensed chromatin can be visualised by TEM, providing information about the biochemical nature of the pathway. For example, chromatin adopts a different morphology depending on the type of death programme initiated. Caspase-dependent apoptosis mostly induces strong chromatin compaction in crescent shaped masses at the nuclear periphery, whereas caspase-independent apoptosis often results in lumpy, incomplete chromatin condensation (Doonan & Cotter, 2008). However, both SEM and TEM are considerably more expensive than light microscopy, more time consuming and require specialist training and equipment (Huerta *et al.*, 2007) and so would only be used to confirm apoptosis after other techniques had been used.

Necrosis is mostly defined in negative terms, by the absence of features characteristic of apoptosis (Krysko *et al.*, 2008). However, Rello *et al.*, (2005) described several morphological features of necrotic cells. In early necrosis there was typically immediate formation of membrane bubbles followed by coalescence and rupture, while disorganization of the cytoplasm, homogeneous nuclear condensation and final

chromatin disintegration within flattened polygonal cell remnants still attached to the substrate characterised late necrosis.

Another improvement to the experimental design and possible future direction would be to monitor changes in morphology over time. This is because all microscopic techniques can only detect apoptosis at a single point in time. It is known that apoptosis is a relatively rapid procedure, with cell elimination occurring within 1-3 h of initiation (Kerr *et al.*, 1972; Gavrieli *et al.*, 1992). Thus, using microscopy, it is possible to miss the characteristic markers of apoptosis (Huerta *et al.*, 2007). Additionally, *in vitro*, in the absence of phagocytic cells, apoptotic cells can undergo secondary necrosis, which shares many morphological features with primary necrosis (Saraste & Pulkki, 2000; Krysko *et al.*, 2008). By examining cell morphology at regular intervals over a long time course, it may be possible to “catch” apoptosis as it happens.

Another way to discriminate between primary and secondary necrosis is to stain the nucleus with propidium iodide. As secondary necrotic cells have already passed through an apoptotic stage, their nuclei are fragmented and/or condensed and chromatin structure is lost so nuclei stain homogeneously with propidium iodide. On the other hand, necrotic cells have uncondensed nuclei with prominent nucleoli. Secondary necrosis can also be distinguished from primary necrosis by detection of caspase-3 and -7 activity in the supernatant (Krysko *et al.*, 2008) (see Section 8.2.6.3).

However, even if these experiments were performed, it is still not certain that apoptosis could be unambiguously distinguished from necrosis. Apoptosis and necrosis represent two extremes of cell death, and there is often a continuum of apoptosis and necrosis in response to high and low doses of the same stimulus (Yeung *et al.*, 1999; Kemény-Beke *et al.*, 2006; Hsuuw & Chan, 2007; Shang *et al.*, 2009). Features of both apoptosis and

necrosis may even coexist in the same cell (Doonan & Cotter, 2008; Shang *et al.*, 2009).

Therefore, the long-standing view of two distinct death programmes is an oversimplification of a much more complex process and this should be considered when performing morphological examination of cell death (Doonan & Cotter, 2008).

8.2.4.5 *Exposure and recovery*

The MTT assay is often described as a cytotoxicity assay (Sgouras & Duncan, 1990; Fotakis & Timbrell, 2006; Bopp & Lettieri, 2008), but it does not actually allow for the discrimination between cytostatic (growth arrest) and cytotoxic (cell death) effects (Plumb, 1999). All it can reveal is whether there are fewer or more viable cells present than there would have been without the treatment. Therefore, in order to distinguish between cytostaticity and cytotoxicity, cells were exposed to extracts for various periods and then the extracts were withdrawn to give the cells an opportunity to recover and resume proliferation. This approach has been widely used to discern the antiproliferative effects of numerous test agents in a range of cell types under diverse conditions (Pollack *et al.*, 1996; Pedro *et al.*, 2005; Marzano *et al.*, 2007; Pascutti *et al.*, 2009; Fang *et al.*, 2009).

Using this method, the inhibitory effects of several extracts were shown to be cytotoxic or irreversibly cytostatic. However, in retrospect, for each exposure period, it would have been good to have measured cell numbers at various endpoints, not just at a single point in time, so that proliferation could have been assessed more readily. In this way, % inhibition could have been viewed on a graph against recovery time on the x-axis.

Interpretation of data could also have been improved by the inclusion of another control, a time zero point, marking the moment of addition of the test agent (and control). MTT-formazan absorbance (optical density, OD) at time zero (OD_{zero}) could then be subtracted from the absorbance reading after various recovery periods (OD_{treated}) and the

vehicle control (OD_{control}) and plotted on a graph against recovery time. As absorbance reflects cell numbers on the day of assay, if $OD_{\text{treated}} > OD_{\text{zero}}$, a cytostatic effect can be inferred, whereas an $OD_{\text{treated}} < OD_{\text{zero}}$ indicates cell death has occurred.

Using the formula, $[(OD_{\text{treated}} - OD_{\text{zero}})/(OD_{\text{control}} - OD_{\text{zero}})] \times 100\%$, the result will be
= 100, if an agent does not affect cell growth or viability,
= 0 to 100, if it induces a cytostatic effect, or
= -100 to 0, if it induces cell death (cytotoxicity) (Monks *et al.*, 1991; Fabbri *et al.*, 2006).

8.2.5 Chapter 5: Evaluation of Most Promising Plant Extracts

8.2.5.1 Sample collection

It is well established that the chemical make-up of an organism is dependent on the locality of collection (Cragg & Newman, 2002). In addition, seasonal variation may play a part in the biochemistry of plant cellular products (e.g. germination). Indeed, in most plants, the synthesis and accumulation of secondary metabolites is regulated by both space and time. For example, in the case of compounds involved in defence mechanisms, more vulnerable tissues like seeds, seedlings, buds and young tissues are, generally, defended more than older, senescing tissues, sequestering larger amounts of defence chemicals or even sometimes actively synthesising them. Organs important for survival and multiplication (e.g. flowers, fruits and seeds) are almost invariably a rich source of defence compounds (Wink, 1999). Liu *et al.* (1998) showed that the concentration of the secondary metabolite, camptothecin, in leaves declined with aging and as the growing season progressed. In addition, it is known that soil composition and other geographical features can influence the potency of plant secondary metabolites (Seigler, 1998; Cragg & Newman, 2002). For example, increased light intensity tends to increase the amount of terpenoids and phenolic compounds present in a plant and water

stress leads to increased terpenoid, alkaloid and condensed tannins, among others (Seigler, 1998).

Therefore, the original plan of this study included experiments to determine whether the time of year or the geographical location from which the plant was harvested had any effect on the potency of the final product. Bearing in mind that one of the initial concepts of this project was to validate traditional Indigenous knowledge and thereby empower Aboriginal people, any variation in activity could have enormous consequences on the credibility of any cottage industry products generated.

Unfortunately, time and financial constraints meant that it was not possible to address these issues fully in this study. It was not possible to say which particular environmental differences, if any, accounted for the variation observed between extracts of Samples 3 and 4 and between 5 and 6 in terms of their secondary metabolite profiles or their bioactivities. While the differences in chemical composition were subtle, they were, nevertheless, present. However, future directions of this research would include properly evaluating the extent of any variation in chemical composition or bioactivity between extracts of plants spatially or temporally removed. This information could provide useful when setting up any cottage industry to harvest a local product.

8.2.5.2 *Extract preparation and analyses*

The extraction process and chemical analyses of the current study were performed by specialist chemists. Obviously, it would take a person untrained in such procedures much longer to accomplish the work described in this thesis, if it ever was completed at all. This highlights the importance of collaborative efforts, not only in ethnopharmacological research, but in all areas of science that require diverse areas of expertise.

A good example of a successful biodiscovery collaboration is Queensland's Natural Product Discovery Unit (NPD), a formal partnership between the international pharmaceutical company Astra Zeneca and the Eskitis Institute for Cell and Molecular Therapies at Griffith University, as well as the Queensland Museum (collection of marine samples) and the Queensland Herbarium (terrestrial collections) (Laird *et al.*, 2008). This exclusive relationship lasted 17 years from 1993, but collaboration on specific projects continues and the know-how and infrastructure allowing further development remains. Each partner contributed funding, specialised expertise and/or facilities in return for monetary (potential and real) and non-monetary benefits (e.g. technological experience and biodiversity information). For example, Griffith University is now able to identify, separate and convert a NP into a medicinal chemistry product, which removes much of the complexity and cost traditionally associated with NPs. To 2007, the collaborative efforts of the NPD resulted in the discovery over 700 new bioactive compounds from its approximately 45,000 specimens, many of which are new to science. While no new drugs have resulted from the partnership, this is not unusual given the long timelines for drug-discovery and development, especially for NPs, and the low odds of developing a commercial product in this sector (Laird *et al.*, 2008).

Several other Australian companies involved in drug discovery from NPs have collaborations with academic or government institutions and other commercial concerns. The Queensland-based life science company, EcoBiotics Ltd, which has Access and Benefits Sharing Agreements (ABAs) with the Drug Discovery Group at the Queensland Institute of Medical Research (QIMR) and private landholders, recently announced plans to fast-track human clinical trials of EBC-46, a small molecule isolated from the fruit of a north-Queensland rainforest tree, after it showed very promising

results against a range of inoperable tumours in horses and dogs ("First cancer drug," 2009). To further develop and market its products, EcoBiotics actively builds partnerships with companies with complementary expertise and technologies ("EcoBiotics," 2010). To this end they have also signed research and development agreements with industry partners in the USA, Europe, Japan and Australia. These partners include Antisoma, a British biotechnology company specialising in novel cancer chemotherapeutics, and Jurox, an Australian veterinary pharmaceuticals company (Laird *et al.*, 2008; "Antisoma," 2006).

Another collaborative partnership is between the Australian Institute of Marine Science (AIMS), which has an ABA with the State of Queensland, and the agricultural company Nufarm Pty Ltd. Other examples of Australian biodiscovery companies that collaborate with government institutes and/or other corporations include Entocism Pty Ltd (ACT), BioProspect Ltd (WA) and Xenome Ltd (Qld) ("New drug," 2002; Laird *et al.*, 2008; "Xenome," 2009; "BioProspect," 2010).

Clearly, these relationships must be advantageous or they would not subsist. What is implicit by the existence of these collaborations is that it is unrealistic for one company to be responsible for the entire drug discovery and development process. It follows, then, that one person cannot be expected to be competent in all the various aspects of biodiscovery and at some point must join forces with others. In the case of the current study, a single operator worked alone in a laboratory with limited funds and equipment, so it made sense to entrust the extraction and subsequent chemical analyses of bioactive fractions to chemists with the appropriate expertise. In this way, time that would otherwise be spent on learning and performing the techniques was dedicated to more cell biological experiments.

In retrospect, however, the collaboration with the chemists should have been closer. Ideally, after the extractions were performed, further fractionations directed by the results of cytotoxicity screening (bioassay-guided fractionation) could have led to the isolation of the active principle/s. The widespread presence of known active “nuisance” compounds or “frequent hitters”, like tannins and polyphenols, including quercetin and luteolin, could have been detected and removed (dereplication) to enhance the chance of finding more interesting compounds (Butler, 2004; Lang *et al.*, 2008; Gertsch, 2009). Unfortunately, funding was an obstacle to this happening as part of the current study.

8.2.5.3 *Extract cytotoxicity*

The aim of this study was to find extracts of plants that could be of use in the treatment of cancer. To this end, the effects of various plant extracts were assessed for their cytotoxic effects on a panel of human cancer cell lines. To differentiate between specific anticancerous bioactivity and non-specific, general cytotoxicity, extracts were also tested against a non-cancerous cell line, MRC5. Selective anticancer activity was presumed when extracts displayed more bioactivity against the cancer cells compared to MRC5 cells. Additionally, general toxicity was also assessed by employing the brine shrimp lethality assay (BSLA). The theory was that if brine shrimp (which are normal organisms) were killed by a particular plant extract, then that extract would be toxic to all normal cells, including noncancerous human cells, grounds for elimination from the pool of extracts displaying promising anticancer activity. From these studies, the extract that showed the most potential as an anticancer agent, EA3, was subjected to a more intensive test to assess if the observed cytotoxicity was simply due to general toxicity or a more physiologically controlled, selective mechanism. Ca^{2+} entry into the cell was measured (see Chapter 6) and shown to be transient, indicating that no breach in plasma membrane integrity had occurred and, hence, cytotoxicity was a physiologically controlled event.

Of course, it should have been possible to determine any losses in membrane integrity by measuring the release of enzymes from cells in culture into the incubation media (see Appendix O). However, due to various reasons, discussed further in Appendix O, none of these assays were suitable for the current study. Mainly, it was because the extracts were coloured and absorbed at the same wavelengths used in these commonly used assays. Another alternative, assessing the cells' abilities to take up or release a radioisotope tracer was also not possible due to the lack of facilities and funding.

Thus, the unavailability of a suitable scalable assay for measuring general cytotoxicity meant it was beyond the scope of this project to examine the mode of action of every extract. Hence, the quest for a single extract to study further was continued by successively eliminating extracts displaying qualities suggestive of inferior potential as an anticancer agent. Nonetheless, determining the modes of action of the rejected extracts is a possible future direction of this study.

8.2.6 Chapter 6: Further Evaluation of Plant Extract, EA3

8.2.6.1 *Chemical composition*

While UV and MS detection are very powerful tools for identifying specific structural features and providing information about the molecular weight and nature of the individual constituents of an extract, only tentative structures can be assigned from these data. Complete elucidation of the configuration of the substituents on the skeletal structure usually requires complementary information from Nuclear magnetic resonance (NMR) experiments (Koehn & Carter, 2005; Cheng *et al.*, 2008). Although beyond the scope of this study, NMR spectroscopy to determine the molecular structures of interesting constituents is something that can be done in the future.

8.2.6.2 **Identification of active constituents**

Theoretically, the activity shown by a mixture, such as an extract, is due to the activities of the individual constituents (Houghton *et al.*, 2007). Therefore, fractionation, resulting in the isolation of individual compounds, should mean aliquots have a higher activity than the original extract. This is the foundation of lead compound discovery from NPs. This approach has led to the introduction of many important drugs (e.g. vincristine) and also provides a basis for standardising extracts for predictable activity (Houghton *et al.*, 2007).

However, several factors undermine the assumptions underlying this approach, making the process of biodiscovery not that simple. For one, it is unusual for a single compound to be responsible for the activity observed, and frequently several constituents are isolated which exert the same effect, although they may differ in their potency. Additionally, the most active compound is not necessarily responsible for the major part of the effect (Houghton *et al.*, 2007). Therefore, it is critical to determine the concentration of each compound in an extract and correlate it with the dose-response characteristics being tested. It is important that the most active compound is present in sufficiently high quantities in the extract to produce an activity. Until the amounts of active compounds in an extract are quantified, it is not possible to conclude the identity of the compounds responsible for any observed effects (Houghton *et al.*, 2007). In the current study, while pure compounds of luteolin and quercetin induced cytotoxic/cytostatic effects in several cancer cell lines, they did not display activity at the concentrations present in the whole extract, EA3.

However, as many ingredients of plants can work together to produce their effects, fractionation of an active extract will not necessarily produce active principles (Dufault

et al., 2001). Sometimes, the activity of the whole extract may be less than that predicted from the activities of the major constituents (Ren *et al.*, 2004; Aspollah Sukari *et al.*, 2010). This is due to antagonistic interactions of the individual constituents, often because of competition between two or more compounds for the enzyme active site (Houghton *et al.*, 2007). On the other hand, it is quite common for a whole extract to have an activity greater than the sum of any one of its individual fractions (Williamson, 2001; Houghton *et al.*, 2007; Prakash *et al.*, 2009). This is what occurred in the current study where the sum of the cytostatic/cytotoxic actions of luteolin and quercetin was not as high as that of the whole extract, EA3.

One possible reason why a loss of activity may occur following fractionation is decomposition of the active compounds during the process. Decomposition, or transformation of constituents to less active substances, may arise due to reactions with the solvents used. Oxidation is another common occurrence due to the methods employed in fractionation. Additionally, many plant extracts contain antioxidants, which might protect labile substances in a crude extract. However, fractionation may separate these protective substances from the vulnerable compounds, which become quickly oxidised (Houghton *et al.*, 2007). As commercially purchased pure compounds of quercetin and luteolin were used in the current study, and not compounds isolated from the whole extract, decomposition did not account for the lower activity of these compounds compared to the whole extract. Instead, it was postulated that these compounds have synergistic effects within the whole extract. This synergy, which has been claimed for a long time by herbalists, is actually a very commonly observed phenomenon in phytotherapy research (Williamson, 2001; Gilbert & Alves, 2003; Houghton *et al.*, 2007; Prakash *et al.*, 2009). Synergistic effects can be produced if the constituents of an extract affect different targets (Wagner & Ulrich-Merzenich, 2009).

In the current study, the individual cytotoxic effects of pure samples of luteolin and quercetin implied that the cytotoxicity/cytostaticity of the whole extract, EA3, was not attributable to these major constituents, even if added together (see Section 6.3.2). However, the calculations were based on the use of pure compounds, and were therefore not directly comparable to the effects of the whole extract because of the many possible complex interactions (synergistic and antagonistic) between all the constituents of the mixture (Wagner, H., personal communication, 7th June, 2010). Therefore, it is possible that, in the combination, luteolin and quercetin did act synergistically to produce the observed bioactivity. “It is impossible to give typical ranges for synergistic effects” (Wagner, H., personal communication, 8th June 2010), so it is not known if other constituents were also acting synergistically with quercetin and luteolin, or if the observed bioactivity of EA3 was entirely due to the interactive effects of these two flavonoids.

While there are methods and statistical models available to ascertain the degree of interaction between two or more individual compounds (see Appendix P), time constraints precluded experiments to be performed that would allow this determination. Indeed, very few studies have reported the systematic identification of the exact components in a crude NP extract that act together synergistically as there are serious logistical barriers to conducting bioassay-guided isolation of synergistic components (Jones *et al.*, 2006). Nevertheless, a future study would be to use the isobol method of assessing additive and synergistic interactions by measuring the concentration of individual components required to exert a particular effect compared to their actions in various fractional combinations (Stergiopoulou *et al.*, 2008; Wagner & Ulrich-Merzenich, 2009). For example, cocktails containing luteolin and quercetin in different

fixed ratios (e.g. 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10) would be tested for their antiproliferative effects and the IC₅₀ values plotted on an isobologram. From this, an interaction index (γ) could be derived. When $\gamma = 1$, no interaction is assumed, while $\gamma < 1$ signifies synergy and $\gamma > 1$ antagonism (Wagner & Ulrich-Merzenich, 2009). Additionally, combinations involving other flavonoids found in EA3 (e.g. apigenin), and even other constituents, could be tested and the effects compared to those of the whole extract in order to obtain interaction indices.

As there were many other compounds present in EA3 besides flavonoids, a future study could be to elucidate the structure of the unidentified compounds and evaluate them for anticancer activity too. In this way, a NCE may be identified and if it does happen to have anticancer activity, a potential novel anticancer drug may have been discovered.

8.2.6.3 *Exposure and recovery (Cellscreen)*

While the Cellscreen (CS) system provided an excellent method of monitoring cell growth, the software unfortunately became corrupted, resulting in a total system breakdown. This meant several planned experiments could not be completed. The problem has since been resolved and future directions would involve studying the effects of other promising extracts on both cancer cell and non-tumourigenic cell growth, as well as the actions of individual constituents, as identified by HPLC.

Nevertheless, it was possible to show that EA3 induced cytotoxicity in some cancer cell lines, a response which began within 90 min of exposure. Given apoptosis is a relatively quick process, it was thought that this rapid response suggested cell death may have been via apoptosis. However, time constraints meant experiments had to be finalised before this impression could be confirmed.

The gold standard test of distinguishing apoptosis from necrosis is detecting specific morphological changes via electron microscopy (Huerta *et al.*, 2007). However, as already mentioned (Section 8.2.4.4), this is an expensive and time-consuming technique, but could be considered if other tests also suggested apoptosis taking place. Classical morphological features of apoptotic cells may be identified using simpler microscopic techniques, as outlined in Section 8.2.4.4, but this is subjective and tedious (Staunton & Gaffney, 1998). Furthermore, even if the characteristic morphological changes are evident, it is generally recommended that no single measure of apoptosis should be relied upon as definitive (Huerta *et al.*, 2007). Apoptosis can really only be inferred after it is verified by multiple, complementary techniques (Huerta *et al.*, 2007).

The definitive determinant of programmed cell death is the step-wise cleavage of nucleosomal DNA into 180-200bp fragments (Staunton & Gaffney, 1998; Saraste & Pulkki, 2000; Huerta *et al.*, 2007). Since the biochemical hallmark of apoptosis is initiation of a caspase cascade that ultimately leads to DNA fragmentation (see Appendix A), both caspase activation and DNA fragmentation must be demonstrated to determine that a cell has undergone apoptosis (Huerta *et al.*, 2007). Identification of DNA fragmentation is frequently by detection of “laddering” of the DNA fragments via DNA gel electrophoresis (Staunton & Gaffney, 1998; Huerta *et al.*, 2007). In this technique, DNA is first extracted from apoptotic cells and applied to a conventional agarose gel. Upon electrophoretic separation, DNA fragments are seen as a ladder of bands of the size equivalent to multimers of approximately 180-200bp (Zamai *et al.*, 1993). In contrast, the absence of a ladder pattern indicates necrosis has occurred as this process is associated with random (not ordered) DNA fragmentation due to the release of multiple endonucleases (Staunton & Gaffney, 1998). However, guidelines laid down by the Nomenclature Committee on Cell Death advise against using biochemical

analyses like DNA ladders to define apoptosis as the degree of DNA fragmentation is noticeably different depending on the cell type, which can result in false-negatives (Doonan & Cotter, 2008). Nevertheless, it remains a popular tool of detecting apoptosis when used in conjunction with other techniques (Ajiro *et al.*, 2010; Matsuda *et al.*, 2010). An alternative is to use flow cytometry to detect DNA fragmentation using the monoclonal antibody (mAb) B-F6, which detects internucleosomal breaks, or perhaps an enzyme-linked immunosorbent assay (ELISA) to detect histone associated DNA fragments (Huerta *et al.*, 2007). The terminal deoxynucleotidyl transferase-dUTP nick end labelling (TUNEL) assays can also be used to detect DNA fragmentation *in vitro* and are accepted as a reliable way to establish apoptosis if confirmed with other methods (Huerta *et al.*, 2007).

Another option is to ascertain activation of caspases. Given the essential role of caspases in apoptosis and the fact that they operate in a cascade system, cleaved caspases are markers for various stages in early apoptosis. For example, the initiator caspases, caspase-8 (extrinsic pathway) and -9 (intrinsic pathway), activate executioner caspases, like caspase-3 and -7, which would be detected later (Kim *et al.*, 2007; Huerta *et al.*, 2007). mAbs are available against all of the caspases for immunochemistry, immunohistochemistry, ELISA and even (in the case of caspase-3) for flow cytometry (Huerta *et al.*, 2007). The most informative assay for the activation of caspases involves detection of proteolytic cleavage of their target molecules (Huerta *et al.*, 2007).

Alternatively, cells could be cultured in the presence or absence of a caspase inhibitor, such as Z-DEVD-FMK to target caspases-3/7 or Z-IETD-FMK for caspase-8, to see if apoptosis still occurs when caspases are blocked, although these inhibitors can exhibit significant cross-reactivity towards non-target caspases (Berger *et al.*, 2006). The easiest way to infer apoptosis by assaying for caspase activation might be to employ one

of the various commercially available kits (e.g. Promega's Apo-ONE[®] Homogeneous Caspase-3/7).

There are many other techniques commonly employed to establish apoptosis (see Huerta *et al.*, 2007 for review). These include measuring changes in mitochondrial membrane potential with dyes like JC-1 and measurement of cytochrome C release from mitochondria via Western blotting (Mantena *et al.*, 2006; Sun *et al.*, 2007a). Regardless of which technique is settled on, confirming that EA3 is inducing cytotoxicity via apoptosis is a priority future direction of the current research.

8.2.6.4 Intracellular Ca^{2+} measurement

Other results that suggested (but did not prove) that EA3-induced cytotoxicity was mediated via an apoptotic pathway were obtained by measuring changes in intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$). Increased $[\text{Ca}^{2+}]_i$ is a universal second messenger system in cells which links receptor activation to signalling pathways downstream (Price *et al.*, 2003; Clapham, 2007). A transient increase in $[\text{Ca}^{2+}]_i$ has been implicated in apoptosis, while a sudden increase that remains elevated is indicative of a breach in cell membrane integrity and necrosis (Sylvie *et al.*, 2000; Baumgartner *et al.*, 2009). Therefore, in Chapter 6 the dynamics of changes to $[\text{Ca}^{2+}]_i$ in cancer cells exposed to EA3 were examined and compared to those of histamine, a known endogenous activator of Ca^{2+} signalling. It was shown that the Ca^{2+} response to EA3 was biphasic, consisting of a transient rise in $[\text{Ca}^{2+}]_i$, followed by a plateau phase. Histamine induced a transient Ca^{2+} response of similar speed and magnitude to EA3, although $[\text{Ca}^{2+}]_i$ gradually returned to baseline. Some of the transport mechanisms described in section 6.1.4 could account for the observed decline in $[\text{Ca}^{2+}]_i$ after the initial peaks in both the EA3- and histamine-induced Ca^{2+} response traces. For example, it is well known that an initial rise in $[\text{Ca}^{2+}]_i$ is mainly due to the mobilisation of Ca^{2+} from internal stores, while a

plateau is due to a sustained influx of Ca^{2+} from the extracellular fluid across the plasma membrane that is in equilibrium with processes that deplete intracellular Ca^{2+} levels, such as efflux or storage (Tilly *et al.*, 1990; Liou *et al.*, 2005; Chen *et al.*, 2010).

Therefore, a future direction of this study is to conduct further experiments with EA3 in which the cell bathing solution contains no added Ca^{2+} , as well as EGTA to chelate residual Ca^{2+} . If the plateau phase is still evident, then it is cannot be due to extracellular Ca^{2+} influx via capacitative Ca^{2+} entry (see Appendix J). Also, specific Ca^{2+} channel blockers (e.g. nifedipine) could be employed to attempt to prevent increases in $[\text{Ca}^{2+}]_i$, and ATPase inhibitors (e.g. bafilomycin A_1) could be used to block the plasma membrane Ca^{2+} -ATPase and impede Ca^{2+} efflux. This may help elucidate which channels and pumps are involved in the Ca^{2+} signalling processes at play. Moreover, in this vein, the Ca^{2+} response could be blocked and cell proliferation studies conducted to see if the anti-proliferative effect of EA3 is inhibited. If cells were able to proliferate normally in the absence of Ca^{2+} but presence of an appropriate concentration of EA3, this would provide further evidence for the involvement of Ca^{2+} in EA3-induced cytotoxicity. However, it must first be established that the cancer cells used in such studies can survive long periods without extracellular Ca^{2+} , although it has been shown that, in contrast to normal cells, the ability to proliferate at low calcium levels seems to be a general property of the neoplastic phenotype (Parsons *et al.*, 1983; Parsons *et al.*, 1985; Chan, 1989).

It has been shown that a key event in triggering some apoptotic signals is the release of Ca^{2+} from ER stores into the cytosol, followed by its entry into the mitochondria (Nutt *et al.*, 2002; Baumgartner *et al.*, 2009). Therefore, it would also be interesting to deplete ER Ca^{2+} stores with a sarco(endo)plasmic reticulum Ca^{2+} -ATPase inhibitor (e.g.

thapsigargin) and use a ratiometric mitochondria-targeted fluorescent Ca^{2+} -sensitive dye (eg. the pericams 2 mt8 or RPmt) to monitor subsequent Ca^{2+} entry into the mitochondria in a Ca^{2+} -free solution in the presence and absence of an inhibitor of the mitochondrial Ca^{2+} uniporter (e.g. RU-360) (Jackisch *et al.*, 2000; Nutt *et al.*, 2002; Baumgartner *et al.*, 2009). In this way, it should be possible to determine the relative roles of ER and mitochondria mobilisation of Ca^{2+} stores in producing the initial Ca^{2+} signal. Showing an increase in the amount of Ca^{2+} released by the ER and its subsequent uptake by the mitochondria would suggest that the observed Ca^{2+} transient is a death signal which triggers apoptosis (Rizzuto *et al.*, 2003).

Additionally, as cell apoptosis occurs over hours and not minutes, another worthwhile future endeavour would be to explore the temporal relationship between the initial EA3-induced elevation of $[\text{Ca}^{2+}]_i$ and activation of apoptosis by measuring changes in $[\text{Ca}^{2+}]_i$ over a longer term. However, the passive loading technique routinely used in this laboratory results in underestimations of $[\text{Ca}^{2+}]_i$ as, gradually over time, Fura-2 leaks out of cells (Putney, 2006). It is also compartmentalised inside the ER within hours (Jackisch *et al.*, 2000). Therefore, for longer time course experiments, it would be necessary to adopt a new loading technique, such as the microinjection of cells with Fura dye complexed to high molecular weight dextran that is resistant to leakage and compartmentalisation (Jackisch *et al.*, 2000). Of course, the adoption of any new method would have to be practicable within the limitations of the facilities and expertise available at this university.

Another worthy future experiment would be to track changes in $[\text{Ca}^{2+}]_i$ over time using a range of concentrations of EA3. Any dose-dependence could then be ascertained and compared to the results obtained using the MTT assay and CS system. Ideally, these experiments would be conducted over a longer period of time, preferably over at least

48 h. This would allow for the possibility of determining at what concentration EA3-induced cytotoxicity becomes due to necrosis rather than apoptosis (as inferred), since it is widely known that the mode of cell death is dependent upon the dose of a compound (Healy *et al.*, 1998; Mattson, 2008).

8.2.7 Chapter 7: Two Different Biodiscovery Strategies

8.2.7.1 *Random approach*

Two extracts of a marine sponge chosen randomly were shown to have strong, selective cytotoxicity against several cancer cell lines. If more material could be obtained, it would be worth investigating these particular extracts further in future experiments. Although not yet taxonomically identified, a voucher specimen was retained, and this could be used to correctly identify the species so that more can be collected for future extraction. Importantly, the geographical coordinates of the collection site were recorded to ensure consistency in sampling when different expeditions take place. These measures help to safeguard against possible pitfalls associated with the random collection method where subsequent expeditions can fail to relocate the same organism (Soejarto, 1996; Hildreth *et al.*, 2007).

8.2.7.2 *Guided approach*

Despite its use as a traditional medicine, extracts of *Haemodorum spicatum* displayed no bioactivity against a panel of several human cancer cell lines. This might have been because the levels of haemocorin in the extracts were too low, or because the compound was oxidised, destroyed in methanol, or in a biologically unavailable form. Alternatively, perhaps another compound in the crude extract masked, or even antagonised, the cytotoxic activity of the haemocorin. Then again, maybe haemocorin does not have any antitumour properties, regardless of unsubstantiated claims it does (Daw *et al.*, 1997; Harborne *et al.*, 1999).

It must be recognised that the traditional medical use of *H. spicatum* was not necessarily in the treatment of cancer. Despite haemocorin having purported antitumour properties (Daw *et al.*, 1997; Harborne *et al.*, 1999), no references could be found that state it was ever used by Aboriginal people to treat cancer. Of course, this was also the case for most of the plants initially screened for bioactivity in the current study as it is generally difficult to establish a direct link between traditional plant remedies and true cancer (Heinrich and Bremner, 2006). Cancer is not a well-defined disease state in Aboriginal traditional culture, although it is likely it was recognised as a wasting syndrome and may have had a specific name (Carrick *et al.*, 1996). However, McGrath *et al.* (2006) asserted there is not even an Aboriginal word for cancer. A problem is there is little or no historical data on cancer in traditional Aboriginal societies prior to white settlement to know for sure (Low, 1990; Cunningham *et al.*, 2008).

Most ethnomedicines have been developed to treat symptoms of infectious diseases like gastrointestinal problems, skin diseases and respiratory illnesses (Heinrich & Bremner, 2006) and this appears to be the case for Aboriginal traditional medicines on the whole, although certain plants were also commonly used as painkillers or to treat ailments like snakebites and wounds (ACNTA, 1988; Lassak & McCarthy, 2001). To complicate matters, the same plant was often used to treat many conditions, including symptoms that may be part of very different disease states. As an example, among its other medical uses, *Eremophila freelingii* (part of the current screen) was traditionally used to treat headache (Low, 1990; Latz, 1995; Lassak & McCarthy, 2001). However, headaches may be due to stress, tiredness, migraine attack or a brain tumour and so appropriate screening tests should cover all of the possible underlying biological causes. Conversely, a particular disease state might be characterised by an array of symptoms

which all must be addressed when searching for leads to treat that particular disease (Houghton, 2002).

In addition, many different plants could be used to treat the same symptoms. Using the same example, Latz (1999) reported seven different plants that were used to relieve headaches, just in Central Australia. Similarly, seven plants were traditionally used to treat wounds, burns and sores, while 58 were described as “general medicines”. Indeed, few plants were used for very specific purposes and this is thought to be related to the traditional nomadic lifestyle of Aboriginal people. That is to say, when on the move, it was better to concentrate on a range of plants with a broad spectrum of uses as the specific plant required for a particular ailment may be a long way away just when it was needed (Latz, 1995). Moreover, the season of the year could restrict the availability of useful plants as the effectiveness of traditional medicines was known to depend on using the appropriate plant parts or plants at a particular stage of maturation (Lassak & McCarthy, 2001; Latz, 1995).

This is why the plants in this study were selected based on their purported medicinal properties against a wide range of conditions, not any specific use against cancer. It is common practice to screen plants with any reputed ethnomedical use at all for bioactive compounds that may be useful in treating conditions that may or may not be related to their traditional use (Lewis *et al.*, 1999, Calderón *et al.*, 2006; Mazzio & Soliman, 2009; Rizwana *et al.*, 2010). For example, Pennachio *et al.* (1996, 2005) showed that methanolic extracts of the leaves of the Aboriginal “number one medicine”, *Eremophila alternifolia* (ACNTA, 1988; Low, 1990), significantly affected certain aspects of heart activity in hypertensive rats.

If screening is only performed against one disease target, compounds useful to treat other diseases may not otherwise be found. However, sometimes even if not specifically screened for, bioactivity against another disease state may be discovered during testing against a different target via serendipitous means. A famous example is the discovery of the powerful antileukemia drugs, vincristine and vinblastine, after researchers investigating the reputed antidiabetic activity of *Catharantus roseus* observed that treated test animals had reduced leukocyte counts (see Section 1.4.4.1.2). For this reason, the more generalised screening approach to ethnopharmacology is advocated by many prominent scientists in the field, including Lars Bohlin who advised researchers at a conference not to discard samples if they show inactivity against one disease target as they may prove to be useful against another target (Bohlin, 2008).

While this study did not uncover a NCE, the bioactivity of many plants traditionally used as medicines was supported. Thus, it was concluded that the ethnopharmacological approach to drug discovery has some merit because the potential for discovering new compounds exists. However, it may not necessarily be a superior strategy to random selection when plants are screened against disease targets that are unrelated to their traditional medical use. This conclusion is endorsed by many, including the editor of *The Practice of Medicinal Chemistry*, who concluded that “all strategies resulting in identification of lead compounds are *a priori* equally good and advisable, provided that the research they subsequently induce is done in a rational manner” (Wermuth, 2008). And in the words of Bohlin’s colleague, Tulp (1999), “ethnopharmacology is one road, but certainly not the only one, and most likely not the best one”. At least anymore. As Gertsch (2009) pointed out, the ethnopharmacological approach has historically been very successful, but few significant discoveries have been made in recent years. He wonders if the golden years of ethnopharmacology are in the past because the most

relevant plant constituents have already been found, “the low-hanging fruits have already been picked”, or if ethnopharmacologists just have to work harder to find a new therapeutically useful molecule (Gertsch, 2009, p178).

8.3 OTHER CONSIDERATIONS

It was beyond the scope of this thesis to discuss many other important aspects of ethnopharmacological research. However, it is important to at least acknowledge the existence of a few.

8.3.1 Traditional usage

The first point is that it must be recognised that traditional methods of making herbal medicines, treatment of the extract prior to administration, the effects of other substances used in herbal mixtures and the dose used should all be taken into account when scientifically evaluating the efficacy or mechanism of action of traditional medicines (Houghton *et al.*, 2007). For example, sometimes, a traditional medicine is prescribed for a substantial length of time, often with no significant improvement expected for weeks or even months. This type of prolonged dosage is usually not considered in new drug screening programmes, largely due to a lack of awareness on the part of the researchers but also because of practical reasons. *In vitro* technologies are not suitable for assessing the effects of repeated interactions with tissues or molecular targets, which also requires large quantities of test materials. Thus, traditional claims of efficacy may be falsely rejected (Etkin & Elisabetsky, 2005).

8.3.2 Holistic medicine

Secondly, and related to this, is that, traditional medical practices are generally holistic in nature. For example, in traditional Chinese medicine, the essence of illness is viewed as symptom-complex (*zheng*) of the whole body, in contrast to modern scientific medicine which views it as anatomicopathological (Fan, 2003).

Additionally, many traditional remedies have been shrouded in religion and even magic over the centuries (Dubick, 1986). Religion and mysticism can often play a big part in the lives of indigenous cultures and their beliefs, influencing their medical treatments much more commonly than in conventional, western therapies. While western medicine often seems to underestimate or ignore the power of the human spirit (Cassell, 1998), in a 1996 survey of 296 practicing US physicians, 99% were convinced that religious beliefs can heal, and 75% believed that prayers of others could promote a patient's recovery (Sloan *et al.*, 1999).

However, who are we to judge which philosophy is correct? Surely, if a treatment works, it works; if it doesn't, it doesn't. So what if it is due to suggestibility or the placebo effect if a patient's condition is improved? Both systems of healthcare have their place and should be free to operate according to their own medical standards (Fan & Holliday, 2007). However, if the goal of ethnopharmacological research is to discover a new drug, then evidence-based, placebo-controlled experiments are an absolute requirement.

It has been suggested that because of the holistic approach of traditional medicine, a holistic approach to study drug activity of these medicines seems more appropriate than a reductionist approach (Verpoorte *et al.*, 2006). Etkin & Elisabetsky (2005) argue for a *transdisciplinary* approach to ethnopharmacology, spanning both the biological and social sciences, creating a dynamic tension that encourages dialogue and collaboration. At the moment, ethnopharmacology is heavily focussed on the biology and pharmacology of the (mostly) botanical sources of potential drugs, only sometimes touching on areas like cultural anthropology (Gertsch, 2009). A truly integrated

approach would aid in facing the particular challenges of ethnopharmacology that were described in Section 1.4.4.2 of the General Introduction.

8.3.3 Endophytes

Thirdly, it has been shown for many bioactive plant extracts that the active constituents are also produced by bacteria or fungi living inside the plant (Tan & Zou, 2001; Strobel & Daisy, 2003). These microorganisms, which do not overtly harm the plant, are known as endophytes and are ubiquitous in nature (Tan & Zou, 2001; Guo *et al.*, 2008). In fact, it is believed that of the nearly 300,000 known plant species, each individual plant is host to one or more endophytes (Strobel & Daisy, 2003). It is thought that the reason why some endophytes produce phytochemicals originally characteristic of the host might be due to a recombination of the endophyte and host genes during evolution of the symbiotic or commensal relationship (Stierle *et al.*, 1993; Tan & Zou, 2001). As very few plants have been studied in terms of their endophyte biology, great opportunities exist to find new and interesting endophytic microorganisms that may prove useful in drug discovery and development (Strobel & Daisy, 2003). Indeed, if endophytes can produce the same rare and important bioactive compounds as their host plants, this would reduce the need to harvest slow growing and/or rare plants, in the process helping to preserve the world's ever-diminishing biodiversity. Moreover, as a microbial source of a valued product may be easier and more economical to produce, its market price would be effectively reduced (Strobel & Daisy, 2003). Already, isolates of different fungal endophytes, obtained from various *Taxus* species have been shown to produce the potent and expensive anticancer drug paclitaxel (see Appendix M) in culture (Stierle *et al.*, 1993; Strobel *et al.*, 1996; Li *et al.*, 1996). Paclitaxel isolated from these sources is biologically active against certain cancer cell lines, is spectroscopically identical to authentic paclitaxel, and accumulates in cultures at the level of micrograms per litre. Hence, via fermentation technology, endophytes may present a dependable and

cheaper source of NPs than their corresponding host plants and this potential should be further investigated.

8.3.4 Drug development

Finally, there remains a gulf between converting potential drug leads into clinically effective drugs. The high failure rate of drug candidates during drug discovery and development is often due to their lack of “drug-like properties”, including physicochemical, (e.g. solubility, stability) and biological (e.g. absorption, metabolism, toxicity) characteristics that are consistent with good clinical performance (Borchardt *et al.*, 2004; see Section 1.3.5). Inadequate drug-like properties can mean increased development time and costs and even project cancellation. For example, an elaborate formulation may be required for poorly soluble compounds, an expensive delivery vehicle may be needed for poorly permeable compounds, low bioavailability may create concerns about patient variability, rapid clearance may mean many doses per day are required, and clinical drug-drug interactions and toxicity could all result in an abrupt end to further clinical development (Borchardt *et al.*, 2004). Thus, there are arguments that it is better for a drug candidate to “fail early and cheaply”, but the reality is that active pharmacophores are rare and precious and cannot be discarded lightly. Therefore, the newer strategy is to try to fix pharmaceutical properties *during* the discovery process via structural modifications (Borchardt *et al.*, 2004).

Orally administered drugs account for more than 75% of the drug market. However, the inability to predict absorption, distribution, metabolism, excretion and toxicity (ADMET) properties has been the main reason why clinical testing of drug candidates has been terminated (Hodgson, 2001; Artursson & Mattsson, 2004; Lee & Dordick, 2006). If a drug cannot be administered orally due to low absorption, it will not be considered for further development simply because alternative administration routes are

too complex (Artursson & Mattsson, 2004). The rate limiting steps of oral drug absorption are drug solubility in the intestinal lumen and passive drug permeability across the intestinal wall (Artursson & Mattsson, 2004; Bergström, 2005). Therefore, drug delivery strategies are an increasingly important part of current development tactics. For example, within the same School as the current studies were undertaken, there is ongoing research on the prospect of using nanoparticles to deliver drugs into cells (Ren *et al.*, 2007; Cheng & Lim, 2009). Potentially, future collaborations between these two laboratories could result in the optimisation of any potent anticancer drug candidate that is identified via ethnopharmacological knowledge, regardless of poor ADMET properties.

This is a similar situation to what is occurring at the University of Wollongong, where cancer researchers based in the School of Biological Sciences are collaborating with chemists in the same Faculty (Vine *et al.*, 2007; Matesic *et al.*, 2008; Locke *et al.*, 2009). These researchers, who have patented their ideas (Australian Patent No. AU2007/001966, Perrow *et al.*, 2008), hope to develop a new generation of targeted cancer therapeutics based on potent toxins coupled to an improved delivery agent that selectively identifies and targets a critical biomarker of cancer malignancy in order to deliver novel toxins directly to tumours, thus minimising toxicity and adverse side effects ("Identifying malignancy markers," 2009). Meanwhile, related theoretical research is being carried out by members of the Nanomechanics Group in the School of Mathematics and Applied Statistics within the same university, providing further opportunity for future collaborations. These researchers are investigating the feasibility of using carbon nanotubes as drug carriers (Hilder & Hill, 2008). Nanotubes are an improvement on nanoparticle delivery methods, offering the perfect isolated environment for a drug, until it reaches its target site, protecting it from degradation as

well as from reactions with healthy cells. Efficient intracellular delivery of a drug can allow a stronger drug to be used at a smaller dosage and can reduce nonspecific effects and toxicity, as well as enhance the effectiveness of drugs incapable of reaching their *in vivo* therapeutic targets (Hilder & Hill, 2009). The use of such targeted drug delivery techniques means that the “magic bullet” concept proposed by Paul Ehrlich over a century ago is fast becoming reality for cancer therapy (Strebhardt & Ullrich, 2008).

8.4 FINAL WORD

Even though this project did not result in the discovery of a NCE to treat cancer, it is argued that the exercise was worthwhile. Several of the main constituents of EA3 have known antitumour properties, although these were likely not responsible for all of its observed cytotoxicity. The active principle/s of this and other promising extracts were not identified as part of the present study, but it was only possible to evaluate three constituents. Any of the compounds not tested may yet be shown to be therapeutically useful, or even be a NCE. Additionally, all the extracts could be tested for bioactivity

against a plethora of other targets, including diseases that the plants are used to treat in traditional Aboriginal societies. Moreover, simply scientifically verifying the medicinal value of the extracts of plants traditionally used as medicines by Aboriginal people has its own merit. The validation of the bioactivity of their medicines will hopefully give the Indigenous communities who provided the TK a sense of empowerment and can only add to their rich heritage. It might also inspire other Aboriginal communities to share their TK of plant medicines to aid in the never-ending search for new compounds to combat diseases.

Bibliography

ACRONYMS

ABS	Australian Bureau of Statistics
ACNTA	Aboriginal Communities of the Northern Territory of Australia
AIHW and AACR	Australian Institute of Health and Welfare and Australasian Association of Cancer Registries
ANSPA	Australian Native Plants Society Australia
CBD	Convention on Biological Diversity
CCWA	Chemistry Centre of Western Australia
DKCRC	Desert Knowledge Cooperative Research Centre
NEPAD	New Partnership for Africa's Development

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List of Abbreviations (Appendices)

5FU	5-Fluorouracil
Apaf-1	Apoptosis protease activating factor-1
APC	Anaphase-promoting complex
ATP	Adenosine tri-phosphate
bFGF	Basic fibroblast growth factor
CAK	Cdk activating kinase
CCE	Capacitative Ca ²⁺ entry
Cdk	Cyclin-dependent kinases
CI	Confidence interval
CICR	Ca ²⁺ -induced Ca ²⁺ release
CKI	Cdk inhibitor
CML	Chronic myelogenous leukemia
COX-2	Cyclooxygenase-2
CRAC	Calcium release activated channel
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide

Aiv	
DNA	Deoxyribose nucleic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HPMA	N-(2-Hydroxypropyl) methacrylamide
hTR	Human telomerase RNA
IAP	Inhibitor of apoptosis
IGF	Insulin-like growth factor
InsP ₃	Inositol 1,4,5-tri-phosphate
mAb	Monoclonal antibody
MCU	Mitochondrial calcium uniporter
MDR	Multidrug resistance
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NCI	National Cancer Institute
NCX	Sodium calcium exchanger
NF- κ B	Nuclear factor kappa light-chain-enhancer of activated B cells
PAF	Platelet-activating factor
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PFT α	Pifithrin- α
PMCA	Plasma membrane Ca ²⁺ -ATPase
PNA	Purine nucleoside analogue

Pot1	Protection of telomeres 1
Pot-1	Protection of telomeres 1
Rb	Retinoblastoma
REDOX	Reduction-oxidation
RNA	Ribose nucleic acid
ROC	Receptor-operated Ca^{2+} channel
RTK	Receptor tyrosine kinase
RyR	Ryanodine receptor
SDS	Sodium dodecyl sulfate
SERCA	Sarco(endo)plasmic reticulum Ca^{2+} -ATPase
SOC	Store-operated Ca^{2+} channel
TEP1	Telomerase-associated protein 1
TERT	Telomerase reverse transcriptase
TGF- β	Transforming growth factor β
TK	Tyrosine kinase
TNF	Tumour necrosis factor
TNQX	2,3,7-Trichloro-5-nitroquinoxaline
Topo	Topoisomerase
TRP	Transient receptor potential
TTA	Telomere targeting agents
UP-S	Ubiquitin-proteasome system
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VOC	Voltage-operated Ca^{2+} channel

Appendix A. Basic Cell Biology

A1 The cell cycle

Under normal circumstances, a cell reproduces by duplicating its genome and cytoplasm and dividing into two daughter cells in a tightly regulated and orderly sequence of events known as the cell cycle (Alberts *et al.*, 2004). While the details of the cell cycle vary for different organisms, and even for different cells and at different times in an individual organism's life, certain characteristics are universal. The fundamental task of a cell is to pass on its genetic information to the next generation. This means the DNA in each chromosome must be faithfully replicated to produce two complete copies and the duplicated chromosomes must then be segregated to the two daughter cells so that they both receive an identical copy of the entire genome (Fig. A1.1).

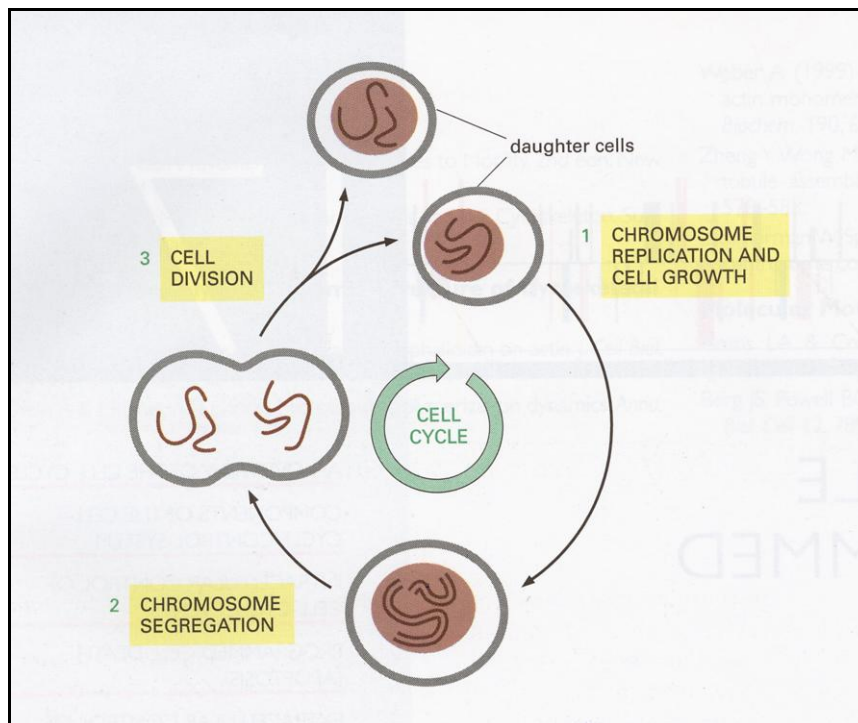


Figure A1.1 The cell cycle.

Note. From *Molecular biology of the cell* p.984, by B. Alberts, *et al.*, 2002, New York: Garland Science.

The cell cycle of a eukaryotic cell is traditionally divided into four phases: G_1 , S, G_2 and M (Fig A1.2). During G_1 (Gap 1), the cell prepares for DNA replication, which occurs during S (synthesis) phase. This is followed by another gap phase (G_2), in which the cell checks the accuracy of DNA replication and prepares for mitosis. After G_2 , chromosome segregation and cytokinesis (cytoplasmic division) occur in M (mitosis) phase (Johnson and Walker, 1999; Park and Lee, 2003; Maddika *et al.*, 2007).

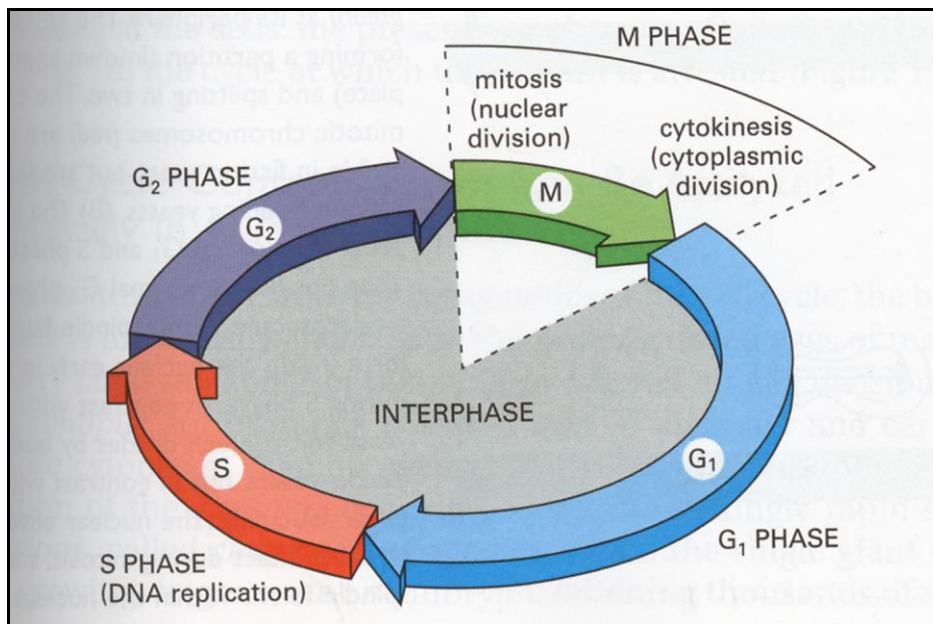


Figure A1.2 The phases of the cell cycle.

Note. From *Molecular biology of the cell* p.985, by B. Alberts, *et al.*, 2002, New York: Garland Science.

A1.1 G_1 phase

In G_1 (Gap 1) phase, the entire mass of proteins and organelles of the cell doubles. This is important so that each generation of cells does not become progressively smaller with each division. However, besides serving as a simple time delay to allow cell growth, G_1 phase also provides time for the cell to monitor both the intracellular and extracellular environments to ensure conditions are suitable and preparations complete before the cell commits to the risks associated with S phase. The length of G_1 can vary greatly depending on extracellular

conditions. Unfavourable conditions result in extracellular signals being released from other cells that delay the cell's progress through G_1 . It may enter a specialised resting state, known as G_0 (G zero), and remain there for days or weeks before resuming proliferation. Indeed, *in vivo*, most non-growing, non-proliferating mammalian cells are quiescent (in G_0) at any one time (Ford & Pardee, 1999; Vermeulen *et al.*, 2003), and some cells, such as nerve and muscle cells, even remain arrested in G_0 for their entire lives. On the other hand, if extracellular conditions are favourable, signals are released which tell the cell in early G_1 or G_0 to progress through a commitment point known as the restriction (R) point in mammalian cells. If growth factors or nutrients are insufficient, a cell cannot pass beyond this R point. However, after the R point, the cell is committed to DNA replication, even if the extracellular signals stimulating growth and division are no longer present (Alberts *et al.*, 2002; Ford & Pardee, 1999; Vermeulen *et al.*, 2003; Park & Lee, 2003).

A1.2 S phase

DNA is synthesised during S phase, which accounts for approximately half of the cell cycle time in a typical mammalian cell. At the beginning of this phase, each chromosome is composed of one coiled double helix molecule, known as a chromatid. By the end of S phase, there are two identical “sister” chromatids. The centrosome is also duplicated during S phase. Centrosomes are large, complex organelles containing two cylindrical centrioles, each composed of microtubules. Centrosomes are responsible for nucleating microtubules that form the mitotic spindle and thus control the equal division of chromosomes. Unlike DNA synthesis, no cell cycle checkpoints exist for centrosome duplication (Sankaran & Parvin, 2006).

A1.3 G_2 phase

The second gap phase, G_2 , provides an opportunity for the cell to monitor extracellular conditions before committing to the radical changes of M phase. During G_2 , the centrosomes also elongate and mature, dramatically increasing in size (Sankaran & Parvin, 2006). Together, G_1 , S and G_2 phase are called interphase and collectively account for approximately 23 hours of a 24 hour cycle in a typical human cell proliferating in culture (Alberts *et al.*, 2002; Collins *et al.*, 1997; Sandal, 2002).

A1.4 M phase

M phase involves a series of prominent events, beginning with mitosis, which is simply nuclear division, and ending in cytokinesis, or cell division. M phase is the shortest phase of the cell cycle, typically lasting less than an hour in a mammalian cell. During mitosis, duplicated DNA strands are packaged into elongated chromosomes and condensed into much more compact chromosomes necessary for segregation. Next, the microtubule network is disassembled and rearranged into mitotic spindles and the cytoskeleton is reorganised (Ford & Pardee, 1999). This is followed by the nuclear envelope breaking down and the replicated chromosomes, each consisting of a pair of sister chromatids, becoming attached to the microtubules of the mitotic spindle. The cell then pauses briefly in a state known as metaphase, in which the chromosomes align at the equator of the mitotic spindle, ready for segregation. In anaphase, the sister chromatids suddenly separate and move to opposite poles of the mitotic spindle, where they decondense to reform intact nuclei. The cell is then pinched in two by cytokinesis and cell division is complete. These processes are summarized in Figure A1.3.

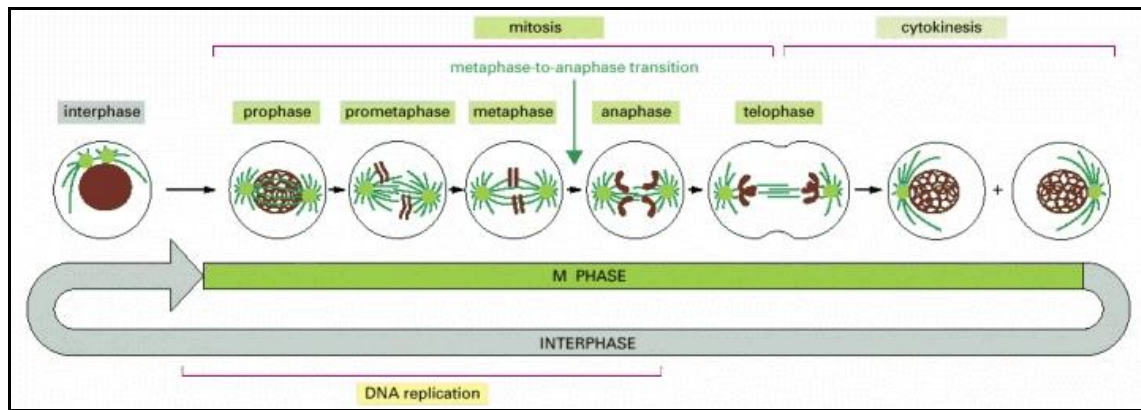


Figure A1.3 The events of eukaryotic cell division as seen under a microscope.

Note. From *Molecular biology of the cell* p.985, by B. Alberts, *et al.*, 2002, New York: Garland Science.

A2 Cell cycle control

While some features of the cell cycle vary from organism to organism and even cell type to cell type, the basic organization of the cell cycle and its control system are consistent for all eukaryotic cells. In most cells there are several points in the cell cycle, known as checkpoints, at which the cycle can be halted if previous events are not completed. For example, entry into mitosis is prevented if DNA replication is not complete and chromosome segregation is delayed if some chromosomes are not properly attached to the mitotic spindle (Alberts *et al.*, 2002).

According to Sandal (2002), there are two main classes of regulatory mechanisms driving the cell cycle: a) the intrinsic mechanisms occurring every cycle, and b) the extrinsic mechanism, acting only when defects are detected.

A2.1 Cyclin dependent kinases (Cdks)

The basis of the cell cycle control system is a family of protein serine/threonine protein kinases known as cyclin-dependent kinases (Cdks) (Vermeulen *et al.*, 2003; Maddika *et al.*, 2007). The

activity of these kinases oscillates as the cell progresses through the cycle, directly leading to cyclical changes in the phosphorylation of intracellular proteins that trigger or regulate DNA replication, mitosis and cytokinesis. These cyclical changes in Cdk activity are controlled by an intricate set of enzymes and other proteins, the most important of which are cyclins. Unless Cdks are tightly bound to a cyclin, they have no protein kinase activity. While cyclins undergo a cycle of synthesis and degradation in each cell cycle, Cdk levels are constant. The cyclical changes in cyclin levels result in the cyclic assembly and activation of cyclin-Cdk complexes, triggering cell cycle events (Fig. A2.1) (Alberts *et al.*, 2002; Sandal, 2002; Vermeulen *et al.*, 2003; Park & Lee, 2003).

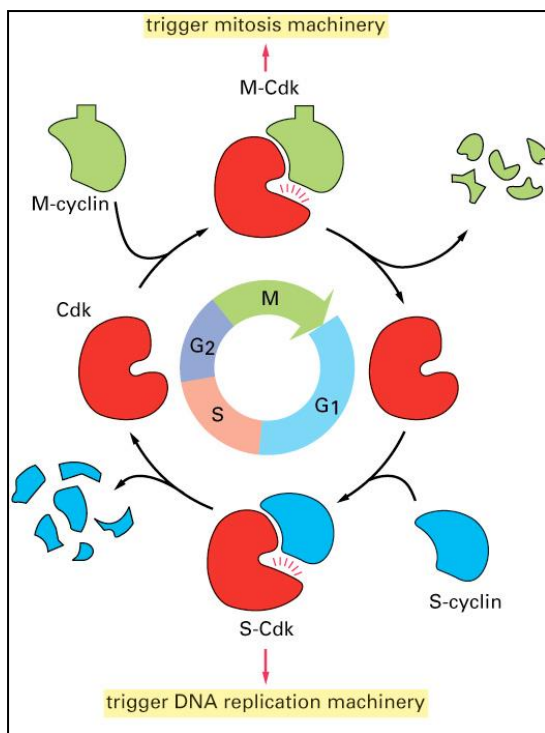


Figure A2.1 The core of the cell-cycle control system.

Note. From *Molecular biology of the cell* p.993, by B. Alberts, *et al.*, 2002, New York: Garland Science.

Although sixteen cyclins have been identified, not all of them are implicated in the cell cycle (Vermeulen *et al.*, 2003; Johnson & Walker, 1999). There are four classes of cyclins, named according to the stage of the cell cycle at which they bind Cdks and function:

1. G_1 /S-cyclins, which bind Cdks at the end of G_1 and commit the cell to replication;
2. S-cyclins, which bind Cdks during S phase and initiate DNA replication;
3. M-cyclins, which promote mitosis; and
4. G_1 -cyclins, which assist the cell to pass through the R point.

At least nine different Cdks are known, but only five are known to be involved in cell cycle progression (Johnson and Walker, 1999; Sandal, 2002; Vermeulen *et al.*, 2003). Two Cdks interact with G_1 -cyclins, one with G_1 /S- and S-cyclins, and one with M-cyclins. Cdk7 combines with cyclin H to form Cdk activating kinase (CAK) (Vermeulen *et al.*, 2003). The names of the individual cyclins and their Cdk partners involved in cell cycle regulation are given in Table A1.1. Other Cdk/cyclin complexes are involved in regulation of transcription, DNA repair, differentiation and apoptosis (Johnson & Walker, 1999).

Table A1.1 Major Cyclins and Cdks of Humans Used in the Cell Cycle
(adapted from Alberts *et al.*, 2002, p994; Sandal, 2002; Vermeulen *et al.*, 2003).

Cell cycle phase activity	Cyclin-Cdk Complex	Cyclin	Cdk Partner
G_1	G_1 -Cdk	cyclin D1, D2, D3	Cdk4 and Cdk6
G_1 /S	G_1 /S-Cdk	cyclin E	Cdk2
S	S-Cdk	cyclin A1, A2	Cdk2
M	M-Cdk	cyclin B1, B2	Cdk1
All	All-Cdk	cyclin H	Cdk7

The activities of cyclin-Cdk complexes are tightly regulated by several mechanisms.

Phosphorylation of the Cdk subunit by a protein kinase (e.g. Wee1 or Myt1) inhibits the activity of a cyclin-Cdk complex, while dephosphorylation by a phosphatase (e.g. Cdc25) increases activity. The binding of a Cdk inhibitor (CKI) protein to a Cdk or Cdk-cyclin complex also inactivates a Cdk, usually in G₁ or S phase (Vermeulen *et al.*, 2003). CKIs are divided into two distinct families according to their substrate specificity, the Cip/Kip family and the INK4 family (Park & Lee, 2003). The Cip/Kip family includes p21 (Waf1, Cip1), p27 (Cip2) and p57 (Kip2) which inhibit G₁-Cdk-cyclin complexes and, to a lesser extent, Cdk1-cyclin B complexes. The INK4 family of CKIs include p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d), which are potent inhibitors of the Cdk4/Cdk6/cyclin D complex (Sandal, 2002; Vermeulen *et al.*, 2003; Ford & Pardee, 1999; Park & Lee, 2003). p21 also inhibits DNA replication by binding to and thereby inhibiting proliferating cell nuclear antigen (PCNA) (Ford & Pardee, 1999). CKI activities are controlled by internal and external signals. For example, p21 expression is under transcriptional control of the *p53* tumour repressor gene (see section A6.2.1), while p15 expression increases in response to transforming growth factor β (TGF- β) (Vermeulen *et al.*, 2003).

In addition, two ubiquitin ligase complexes, SCF and anaphase-promoting complex (APC), are also critical to cell cycle control. These enzyme complexes add ubiquitin to specific regulators of the cell cycle, inducing their proteolysis and thereby triggering cell cycle events. In the case of SCF, G₁/S-cyclins and certain CKI proteins controlling the initiation of S-phase are destroyed, while APC is responsible for the destruction of M-cyclins and other regulators of mitosis (Alberts *et al.*, 2002).

The different cyclin-Cdk complexes act as molecular switches for specific cell cycle events (Fig. A1.4). In early G_1 phase most Cdk activity is suppressed to allow the cell time for growth and regulation by extracellular signals before S phase. Cdk suppression is due to a combination of APC activation, CKI accumulation and a decrease in production of cyclins (other than D). Entry into S phase usually occurs through the accumulation of G_1 -cyclins, leading to an unopposed increase in G_1 -Cdk activity. This leads to increased synthesis of G_1 /S-cyclins, which in turn leads to increased G_1 /S-Cdk activity resulting in initiation of the events that commit the cell to enter S phase. S-Cdks initiate DNA replication, ensuring genes are copied only once per cell cycle. (Alberts, 2002; Johnson & Walker, 1999).

The activation of M-Cdks, after S phase is complete, triggers a cell's preparation for and entry into mitosis. However, if DNA replication is not complete at the end of S phase, a negative feedback signal at the DNA replication checkpoint (see section A2.3) results in M-Cdk activation being blocked, saving the cell from entering into a suicidal mitosis. The ubiquitin ligase APC launches the separation of sister chromatids by destroying several mitotic regulatory proteins, provided chromosomes are properly attached to the mitotic spindle at the spindle-attachment checkpoint (section A2.3). Exit from mitosis occurs by a reversal of the above processes through inactivation of M-Cdk via ubiquitin-dependent proteolysis of cyclin B (Ford & Pardee, 1999).

A2.2 Other signal transduction molecules

The signals for a cell to proliferate are generally growth factors, such as epidermal growth factor (EGF), transforming growth factor α (TGF- α) and insulin-like growth factor (IGF), which act by binding extracellularly to their specific transmembrane receptor proteins thereby activating the receptor's intracellular kinase and initiating a multistep signal transduction

kinase cascade involving the products of many genes (e.g. ras, fos, myc, and the MA and PI-3 kinases) (Ford & Pardee, 1999; Hellawell & Brewster, 2002). The fibroblast growth factor (FGF) family and the endothelial growth factors, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), are other key stimulatory regulators of proliferation. However, other growth factors, such as transforming growth factor β (TGF- β) family have an inhibitory effect on cell growth and proliferation (Hellawell & Brewster, 2002). Still others, such as tumour necrosis factor (TNF) and the Fas ligand, are used as “death signals”, triggering a cell to undergo apoptosis (Nagata & Golstein, 1995). There are at least 17 known signal transduction pathways, each including some form of receptor, proteins (e.g. kinases and phosphatases) to convey the signal and downstream transcription factors which up- or down-regulate expression of specific genes (Nebert, 2002). It is not possible to cover all of these here, so the reader is directed to an excellent review by Maddika *et al.*, (2007) who describe the interconnections between these signalling pathways that contribute to regulating homeostatic circuits. Figure A2.2 summarises the major molecules involved in cell cycle regulation.

A2.3 Checkpoints

Chromosomes can sustain damage from chemicals or radiation or through spontaneous mutation. It is essential that any such damage to chromosomes is repaired before the cell attempts to duplicate or segregate them. Therefore, most cells have at least two DNA damage checkpoints where DNA damage can be detected and the cell cycle arrested if necessary, generally via negative intracellular signals. These DNA damage checkpoints exist in late G₁ and late G₂, controlling entry into S phase and M phase, respectively. However, there also appear to be DNA damage checkpoints during S and M phases too (Vermeulen *et al.*, 2003).

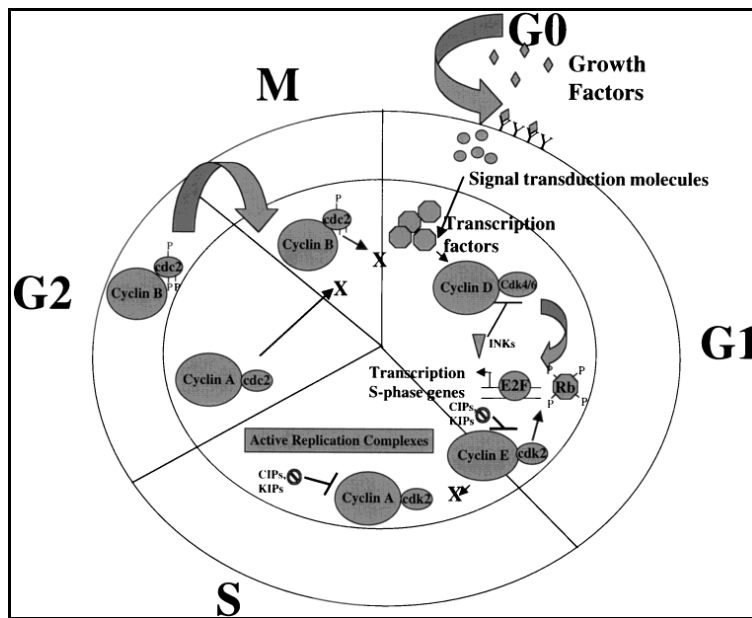


Figure A2.2 Some of the many molecules involved in cell cycle control.

Note. From “The apoptosome: signalling platform of cell death.” by H.L. Ford and A.B. Pardee, 1999, *Journal of Cellular Biochemistry, Supplements* 32/33, p. 167.

Progression into S phase is blocked at the G₁/S checkpoint by inhibiting the activation of G₁/S-Cdk and S-Cdk complexes. One way this can occur is via p53, which stimulates the transcription of a gene encoding the CKI p21, which binds to G₁/S-Cdk and S-Cdk to inhibit their activities. The mechanisms of the DNA damage checkpoint in S phase are not well understood, but may involve suppression of both the initiation and elongation phases of DNA replication.

The G₂/M checkpoint operates in a similar manner to the DNA replication checkpoint by maintaining Cdk1 in its inhibited form via phosphorylation or by sequestering components of the Cdk1-cyclinB complex outside the nucleus. p53 may also play a role in the regulation of the G₂/M checkpoint through p21, 14-3-3σ and Gadd45, although cells without p53 are also able to arrest in G₂ in response to DNA damage (see Vermeulen *et al.*, 2003; Ford & Pardee, 1999).

There is also a “spindle checkpoint” in M phase that detects any improper chromosome alignments on the mitotic spindle and arrests the cell cycle in metaphase. Examples of spindle checkpoint-associated proteins include Mad and Bub, which are activated when defects in microtubule attachment are detected. These proteins inhibit the Cdc20 subunit of APC, thereby preventing the transition between metaphase and anaphase (Vermeulen *et al.*, 2003; Ford & Pardee, 1999).

The R point in G₁ is regulated by the INK4 proteins, which block cyclin D-dependent kinase activity, rendering the unphosphorylated Rb protein inactivated (see section A6.2.2). This prevents the synthesis and activity of cyclin E, meaning the cell cycle cannot progress past the R point (Ford & Pardee, 1999). An overview of the cell cycle control system is presented in Figure A.2.3.

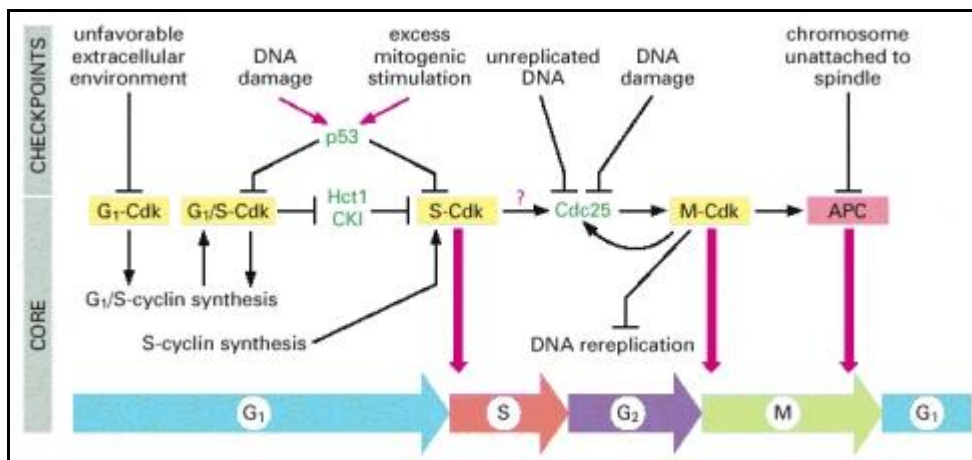


Figure A2.3 An overview of the cell cycle control system.

Note. From *Molecular biology of the cell* p.1009, by B. Alberts, *et al.*, 2002, New York: Garland Science.

A3 Apoptosis

Sometimes, however, DNA damage is so severe that it simply cannot be repaired. In this case, permanent cell cycle arrest (senescence) may be triggered by p53 (Levesque & Eastman, 2007). Alternatively, the cell may commit suicide by undergoing apoptosis rather than pass on

the damaged DNA to its progeny. This is because genetic damage to key cell regulatory proteins (protooncogenes or tumour repressor genes) can often lead to cancer, and in a multicellular organism, the life of the whole organism must have priority over an individual cell.

In contrast to cells that die as a result of acute injury via necrosis, cells that undergo apoptosis die neatly, without bursting and leaking their potentially inflammatory-inducing cell contents over neighbouring cells (Kerr *et al.*, 1972). Firstly, the cell shrinks and condenses, then the cytoskeleton collapses, the nuclear envelope disassembles, and the DNA inside the nucleus fragments (into 180-200 bp pieces) (Doonan & Cotter, 2008). Importantly, the cell surface changes so that it can be recognised as dying and quickly engulfed by a phagocytotic cell before any damaging leakage occurs (Li *et al.*, 2003a; Wang *et al.*, 2003).

Apoptosis is primarily mediated by an intracellular proteolytic cascade involving a family of proteases known as caspases. Caspases are signalling proteases intended for specific protein cleavage, not protein degradation (Riedl & Salvesen, 2007). Caspases are synthesised in the cell as inactive procaspases, which are cleaved and thereby activated by other caspases, which in turn activate other procaspases in an amplifying proteolytic cascade. Some of the activated caspases then cleave and activate other intracellular proteins such as nuclear lamins or DNase, resulting in the rapid and neat dismantling of the cell. In mammals, the *extrinsic* death receptor pathway and the *intrinsic* mitochondrial pathway are the two main signalling systems that trigger the activation of caspases and the consequent induction of cell suicide (Fig. A3.1). However, mounting evidence indicates that these two pathways involve significant cross-talk (Khosravi-Far & Esposti, 2004). The (extrinsic) death receptor-dependent pathway is mediated via the TNF-family ligands, while the (intrinsic) mitochondrial pathway is induced by different

factors, including UV radiation, free radicals, chemotherapeutics or DNA damage (Maddika *et al.*, 2007).

It is important to understand that activation of the protease chain reaction is usually irreversible once the cell reaches a point of no return along the pathway to suicide (Riedl & Salvesen, 2007). Therefore, initiation of the cascade is tightly controlled by intracellular adaptor proteins (e.g. Apoptosis protease activating factor 1 = Apaf-1), which aggregate and activate procaspases (intrinsic pathway), or by activation of death receptor proteins (e.g. Fas, TNF α), on the cell's surface (extrinsic pathway). The main regulators of the cell death programme are the Bcl-2 family of intracellular proteins, members of which either inhibit apoptosis by blocking (e.g. *Bcl-2* and *Bcl-X_L*) or stimulating (e.g. *Bax* and *Bak*) the release of the electron carrier protein cytochrome C from mitochondria into the cytosol where it binds to Apaf-1 to accelerate and amplify the caspase cascade. Activation of transcription genes that encode proteins promoting the release of cytochrome C from mitochondria usually requires the gene regulatory protein p53 (see section A6.2.1). Other members of the Bcl-2 family (e.g. *Bad*) promote procaspase activation by binding and inactivating the death-inhibiting members. The inhibitor of apoptosis (IAP) family of intracellular apoptosis regulators also, as their name suggests, inhibit apoptosis. They do so either by binding to procaspases to prevent their activation, or binding to caspases to inhibit their activity (Khosravi-Far & Esposito, 2004; Alberts, 2002).

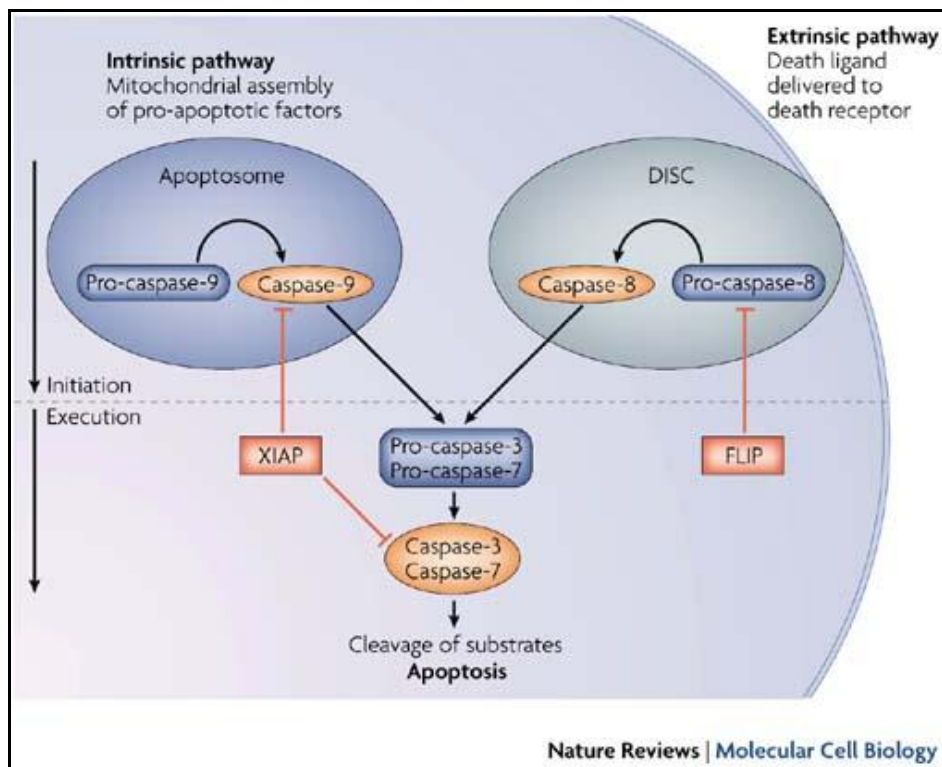


Figure A3.1 The proteolytic caspase cascade in the initiation and execution of apoptosis.

In the intrinsic pathway, an apoptotic stimulus in the cell leads to the assembly of a wheel-shaped signalling platform, the *apoptosome*, which activates the initiator caspase-9. In contrast, an extrinsic apoptotic signal is mediated by binding of an extracellular ligand to a transmembrane receptor, leading to the formation of the death-inducing signalling complex (DISC), which is capable of activating the initiator caspase-8. Once activated, either caspase-8 or caspase-9 cleaves executioner caspase-3 and caspase-7, which represents the execution level of the caspase cascade and leads to apoptosis of the doomed cell. Negative regulators of the caspase cascade can be found at both levels. Whereas FLIP blocks the activation of the initiator caspase-8 in the DISC, XIAP blocks both the initiation phase, by inhibiting caspase-9, and the execution phase, by blocking caspase-3 and caspase-7, of the cascade.

Note. From “The apoptosome: signalling platform of cell death.” by S.J. Riedl and G.S. Salvesen, 2007, *Nature Reviews Molecular Cell Biology*, 8(5), p. 406.

A4 Ubiquitin-mediated protein

In mammalian cells, the steady state levels of almost all cellular proteins is the balance between degradation and *de novo* synthesis. Degradation is also responsible for eliminating damaged, mis-folded or mutant proteins, the accumulation of which may harm the cell. In contrast to cell-surface proteins that are endocytosed and degraded in lysosomes, most cellular

proteins (80%) are degraded by the proteasome in the cytoplasm and nucleus after being tagged with ubiquitin. The destruction of regulatory proteins is irreversible and has an essential role in the regulation of signal transduction, transcription, cell cycle and antigen processing (Burger & Seth, 2004). For example, cyclins, Cdks and CKIs, including p21, are degraded during the cell cycle by the ubiquitin-proteasome pathway. Other important targets of the ubiquitin-proteasome system (UP-S) are p53 (see section A6.2.1), p27, nuclear factor kappa B (NF- κ B, see Appendix B2.4.2) and Bcl2 (section A3) (O'Connor *et al.*, 2004). Hence, intracellular proteolysis is a critical regulator of protein function and aberrations in this pathway cause a range of pathological phenotypes, including cancer (Burger and Seth, 2004; Chin *et al.*, 2005).

A5 Replicative senescence

Normal human cells in culture have a limited replicative potential, ceasing to grow after about 50 cycles (Weedon & Chick, 1976; Ford & Pardee, 1999). This “counting” mechanism for cell divisions is known as the *Hayflick limit* and is generally believed to happen via telomere attrition (Sitte *et al.*, 1998). Telomeres are specialised, complex structures that cap the ends of eukaryotic DNA strands, acting as a disposable buffer to delay what is known as the *end-replication problem* of linear genes (Levy *et al.*, 1992; Olovnikov, 1996). They are comprised of a repetitive sequence of DNA, (TTAGGG) n in vertebrates, and various associated proteins, some of which may be involved in controlling telomere length. Telomere repeats are synthesised by the enzyme telomerase, a RNA-protein complex. The activity of this enzyme is tightly controlled by limiting the expression of the catalytic subunit, telomerase reverse transcriptase (TERT) (Possemato & Hahn, 2007). Besides TERT, five other subunits composing the telomerase complex have been cloned: hTR (human telomerase RNA), TEP1 (telomerase-associated protein 1), hsp90

(heat shock protein 90), p23, and dyskerin. However, whereas TERT is regulatable, the other components are expressed more constantly in cells (Chang *et al.*, 2002).

A guanine-rich telomere extension of a few hundred bases, when exposed, induces cellular senescence, but usually this region is protected by proteins (e.g. protection of telomeres 1 = Pot1) and by telomerase and is folded back into the T-loop. Because DNA polymerases cannot replicate the ends of linear strands, 50-200 bases of telomeric DNA are lost every cell division, eventually resulting in the loss of vital genetic information required for cell survival (Feng *et al.*, 1995; Hellawell & Brewster, 2002; Kelland, 2005).

A6 What goes wrong in cancer?

Most cancers arise from a single abnormal somatic cell that has had multiple “hits” to its genome. There is much evidence to indicate that multiple, independent, random mutations in many (maybe thousands of) genes must have occurred in the lineage of the cell to cause it to become cancerous (Sandal, 2002; Nebert, 2002). One telling piece of evidence is that the incidence of cancer increases sharply as a function of age, suggesting the accumulation of mutations over time. Moreover, in a human’s lifetime, every single gene is likely to have undergone spontaneous mutation over 10^{10} separate times (due to the inherent limitations on the accuracy of DNA replication and repair) and many of these mutations are likely to disturb genes that regulate cell cycling, leading to unrestricted cell proliferation, the major trait of cancer. Clearly then, as the Knudson hypothesis (Knudson, 1971), first postulated by Nordling (1953), states, more than a single mutation in a single gene is required for carcinogenesis. The initial population of slightly abnormal cells, the descendants of a single mutant ancestor, is conferred a growth advantage but must undergo successive cycles of additional mutation and natural selection to form a tumour (Alberts *et al.*, 2002). It is important to realise that damage is caused by mutations to normal protooncogenes (dominant, gain of function) which act as

accelerators to activate the cell cycle and/or mutations to tumour suppressor genes (recessive, loss of function) serving to slow cell proliferation (Nebert, 2002). Generally, mutations in both types of genes are necessary for cancer to occur as a mutation to a single oncogene would simply be suppressed by normal cell cycle control and tumour repressor genes. Similarly, a damaged tumour repressor gene does not lead to cancer unless growth is stimulated by an activated oncogene. However, regardless of the genetic damage or the type of cancer, the common feature is frequently the deregulation of the cell cycle (Sandal, 2002; Park & Lee, 2003).

A6.1 Protooncogenes

Protooncogenes are normal cellular genes which promote cell growth, such as those that produce hormones. Mutations in protooncogenes can modify or amplify their expression, converting them to oncogenes. Dysfunction of the protein products of protooncogenes causes abnormal regulation of signalling pathways controlling the cell cycle, apoptosis, genetic stability, cell differentiation and morphogenetic reactions (e.g. contact inhibition of proliferation). In addition, expression of oncogenes can result in increased stimulation of angiogenesis, acquisition of metastasising ability and immortalisation, key features of neoplasms (see section 1.2.2). Examples of changed protooncogenes found in human tumours include EGFR and HER2 (see Appendix B2.1.1), Bcl2 (see Appendix B2.4.3), Ras (see Appendix B2.6.2) and ABL (see Appendix B2.1.2) (Kopnin, 2000).

A6.2 Tumour suppressor genes

Tumour suppressors are also major players in regulation of the cell cycle and other processes associated with cell growth. Some only act if the cell is damaged. In relation to cancer, the two most important are the Retinoblastoma (Rb) protein and the transcription factor, p53 (Sandal, 2002; Gali-Muhtasib & Bakkar, 2002). It is thought that tumour suppressor genes have evolved

to protect multicellular organisms from uncontrolled cell division caused by mutations, a concept supported by the fact that the corresponding pathways or proteins are mutated or inactivated in most human cancers (Maddika *et al.*, 2007).

A6.2.1 p53

The gene regulatory protein p53 acts as a negative regulator of cell growth in response to various stresses, which can be genotoxic (DNA changes induced by irradiation, UV light, carcinogens or cytotoxic drugs) or not (hypoxia, oncogene activation, microtubule disruption) (Lacroix *et al.*, 2006). p53 inhibits cell proliferation by inducing reversible or irreversible (senescence) cell cycle arrest, or apoptosis, and may also enhance DNA repair and inhibit angiogenesis (section A6.2.2) (Maddika *et al.*, 2007). It does so mainly through its ability to act as a transcription factor and is required for the transcription of several genes involved in cell cycle control and DNA synthesis (Lacroix *et al.*, 2006). For example p53 stimulates the transcription of a gene encoding a CKI protein (p21) which inhibits the activities of G₁/S-Cdk and S-Cdk, blocking a cell's entry into S phase (section 1.2.2.2.4). In the case of severely damaged cells, p53 activates the transcription of genes encoding proteins that promote the release of cytochrome C from mitochondria into the cytosol, thereby triggering the caspase cascade involved in apoptotic cell death (section A3) (Vermeulen *et al.*, 2005; Sullivan *et al.*, 2004). Other genes regulated by p53 include those encoding the cell cycle regulatory proteins 14-3-3 σ , TERT, cyclin A2 and cyclin B1 (see section A2.1), proteins involved in apoptosis and survival, including Apaf-1, Bax, Bac1, Bcl2 and Fas (see section A3) and angiogenesis, such as endostatin and VEGF (see Appendix B2.6.1 and B2.6.2) (Sullivan *et al.*, 2004; Lacroix *et al.*, 2006). For a more comprehensive list, see Lacroix *et al.*, 2006.

A6.2.2 Rb

The retinoblastoma protein family (pRb, p107 and p130) are the primary substrates of Cdk2, Cdk4 and Cdk6 in G₁ progression, functioning as negative regulators at the R point. The E2F

family of transcription factors are important targets of pRb for regulating early G₁ progression. E2Fs control the expression of many genes that mediate progression through the R point (e.g. cyclin E) and S phase (e.g. dihydrofolate reductase) (Park & Lee, 2003). E2F functioning as a transcription factor is prevented by its interaction with Rb, but Rb is only able to bind E2F when it is unphosphorylated. Some Rb mutants are constitutively phosphorylated so cannot bind E2F, meaning cell division is uncontrolled at the R point and cells may become cancerous. A growth advantage is also conferred by direct mutation and/loss of function of Rb in a subset of human cancers (Sandal, 2002). Phosphorylation of Rb is catalysed by the activity of Cdk/cyclin complexes. Mitogenic signals generally lead to activation of Cdk/cyclin complexes, whereas antimitogenic signals inhibit the activation of G₁ Cdk/cyclin complexes (Maddika *et al.*, 2007; Sandal, 2002). Rb is also crucial for stem cell maintenance, tissue regeneration, differentiation and developmental programmes and it has been shown that the Rb pathway can affect the activity of the p53 pathway and vice versa (Maddika *et al.*, 2007).

Appendix B. Chemotherapeutic Strategies to Combat Cancers

B1 Cytotoxic drugs

B1.1 Drugs that impair DNA synthesis

In cancer cells, the control of G_1 progression and the initiation of S phase is often disrupted, resulting in the premature transition through the R-point, leading to unrestrained cell proliferation. To this end, drugs that target the synthesis of DNA have been developed.

B1.1.1 Antimetabolites

Antimetabolites are analogues of purines, pyrimidines and folates that prevent cells from making nucleosides, the building blocks of DNA (Galmarini *et al.*, 2002). Purine nucleoside analogues (PNAs) are incorporated into DNA during replication instead of adenine and guanine, preventing extension by DNA polymerase and leading to chain termination. PNAs have particularly high activity against lymphoid and myeloid cancers and all have similar chemical structures and mechanisms of action. Several mechanisms could be responsible for their cytotoxicity, including inhibition of DNA synthesis and repair, and accumulation of DNA strand breaks (Robak *et al.*, 2005). Antimetabolites are cell cycle specific, primarily acting on cells undergoing DNA synthesis and so are most useful during S-phase. Purine analogues that have been Food and Drug Administration (FDA)-approved for the treatment of cancer include mercaptopurine, thioguanine, fludarabine monophosphate, deoxycoformycin and cladibrine (Parker *et al.*, 2004), while three other compounds, clofarabine, nelarabine and immucillin-H, are under clinical evaluation (Robak *et al.*, 2005). Synergistic interactions between PNAs and other cytotoxic agents have been demonstrated clinically (Robak *et al.*, 2005).

Pyrimidine antagonists include 5-fluorouracil (5FU), gemcitabine (Gemzar[®]) and arabinosylcytosine which either block pyrimidine nucleotide formation or cause premature termination by themselves being incorporated into DNA. 5FU also inhibits thymidylate synthase, the enzyme that catalyses the synthesis of thymine nucleotides from uracil nucleotides (Longley *et al.*, 2003; Luqmani, 2005).

Another anticancer chemotherapeutic that inhibits the folate pathway is the folate analogue methotrexate, which inhibits dihydrofolate reductase (Schweitzer *et al.*, 1990).

Methotrexate has been widely used to treat a variety of malignancies including acute lymphocytic leukaemia, non-Hodgkin's lymphomas, squamous cell carcinoma choriocarcinoma and cancers of the breast, lung, bladder, head and neck and bone (Bleyer, 1978; Luqmani, 2005).

B1.1.2 Camptothecins

Another class of anticancer drugs, the camptothecins (e.g. topotecan), selectively target topoisomerase (topo) I by reversibly stabilising a covalent DNA-topo I intermediate (van Waardenburg *et al.*, 2004). Topo I is the enzyme responsible for producing a transient single-strand break (nick) in DNA, allowing the two sections of the DNA helix on either side of the nick to rotate freely relative to each other, using the phosphodiester bond in the strand opposite as a swivel point, until the enzyme leaves and the phosphodiester bond reforms (Alberts, 2002). Camptothecins prevent DNA strand breaks resealing, resulting in DNA damage and arrest of the cell cycle between S phase and G₂. They do this by forming a reversible complex with DNA and topo I, which prevents the re-ligation of the topo I-induced transient nick in the DNA strand, allowing strand passage and thus reducing twists and consequent strain in DNA during replication and transcription. The persistence of this so-called cleavage complex on DNA increases the chance that the complex will collide with the DNA fork machinery, leading to a

lethal double strand break (Adams, 2005). Interestingly, all of the topo I inhibitors clinically evaluated to date are analogues of camptothecin, an extract of the Chinese tree *Camptotheca acuminata* (Sriram *et al.*, 2005).

B1.1.3 Anthracyclines

Anthracycline antibiotics (e.g. doxorubicin and daunorubicin) impair the function of topoisomerase II, the enzyme responsible for transiently nicking double-strands of DNA. By reversibly breaking one double helix to create a DNA gate through which the second double helix can pass, and resealing the break before dissociating from the DNA, topo II can efficiently separate two interlocked circles of DNA (Alberts, 2002).

Anthracyclines are one of the most utilised anticancer drugs ever developed and are active against a variety of tumours. However, due to their mechanism of action, they are also toxic towards normal proliferating cells. Their main serious side effect is cardiotoxicity, which can lead to arrhythmia and heart failure, so this limits the dosage that can be used (Kwok & Richardson, 2002; Xu *et al.*, 2005; Barry *et al.*, 2007). Anthracyclines transform topo II into potent nuclear toxins that lethally damage the DNA double helix. They do so by stabilising the topo II-DNA covalent complex and preventing the resealing of nicks. It is known that drug intercalation is necessary, but not sufficient, for topo II inhibition and the removal of certain substituents greatly increases activity. The action of topo II poisons is generally DNA sequence specific in that each compound can cleave DNA at only certain sites recognised by the enzyme and this specificity is dependent upon the 3' substituent of the sugar moiety. In addition, some anthracyclines can also inhibit topo I. This information has been used to help find new anthracycline drugs with lower side effects and higher activity against resistant cancer cells. In the search for “a better doxorubicin”, more than 2000 analogues have been reported, the most promising being MEN10755, which is itself the progenitor of a third generation of synthetic

anthracycline oligosaccharides with different chemical and biological characteristics (Binaschi *et al.*, 2001).

B1.2 Drugs that impair DNA integrity

B1.2.1 Platinum compounds

The aforementioned drug classes are all cell cycle specific, i.e. they have activity only in specific phases of the cell cycle (Fig. B1). Another class of anticancer drugs, the platinum compounds (e.g. cisplatin), have cytotoxic activity throughout the entire cell cycle. This is because these compounds cross-link with DNA to form adducts, causing strand breaks which damage the integrity of the DNA and result in cell cycle arrest at G₂ and apoptosis. Toxicity seems to be more dependent on the inhibition of transcription rather than the inhibition of DNA synthesis as cells proficient in DNA repair are able to circumvent G₂ arrest and transcribe damaged genes to make mRNA required for passage into mitosis (Sorenson & Eastman, 1988a; Sorenson & Eastman, 1988b). However, the DNA binding model does not explain all cellular and molecular events observed in experiments, so a complete understanding of the mechanisms of action is still to be determined (Bose, 2002). It has been reported that platinum-DNA adducts also act as topo I poisons to enhance the stability of covalent topo I-DNA complexes, accounting for an observed synergistic effect of platinum drugs in combination with a camptothecin (van Waardenburg *et al.*, 2004).

Cisplatin is the treatment of choice for many cancers, including testicular and ovarian cancer, but is ineffective against some of the most common tumours, including breast and colon cancer. Other limitations include toxic side effects, such as nausea, hearing loss, kidney toxicity and loss of sensation in hands, and acquired drug resistance in certain tumours. Efforts to overcome the shortcomings of cisplatin have led to the development of thousands of platinum

compounds, only four of which are currently approved in at least one developed nation (carboplatin, oxaliplatin, nedaplatin and Lobaplatin). Other metal ions such as nickel and palladium are also being studied for antitumour effects, but they may have different target sites than DNA (Abu-Surrah & Kettunen, 2006; Pasetto *et al.*, 2006).

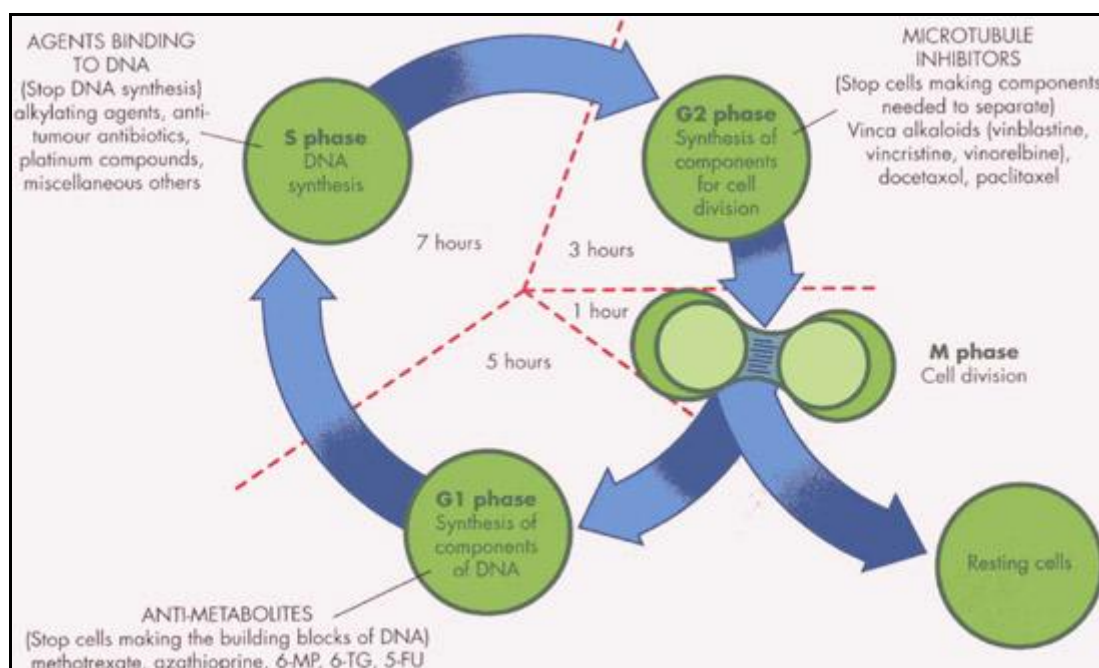


Figure B1 Cell cycle specificity of cytotoxic anticancer drugs.

Note. From "Zactima," 2010 Retrieved 26th May, 2010, from <http://www.drugdevelopment-technology.com/projects/zactima/>.

B1.2.2 Alkylating agents

In contrast to platinum compounds which intercalate with DNA and induce strand breaks, alkylating agents (e.g. cyclophosphamide) directly damage the 3-dimensional structure of DNA by covalently binding alkyl groups to DNA, introducing cross-links that inhibit replication. They can also cause abnormal base pairing of nucleotides or DNA strand breaks, thus preventing cell division (Ralhan & Kaur, 2007). Although alkylating agents are active in all stages of the cell cycle, as with most cytotoxic drugs, proliferating cells are

more sensitive, especially those in G₁ and S phase. However, they are commonly more effective against slow-growing cancers (Ralhan & Kaur, 2007).

There are six classes of alkylating agents: (i) nitrogen mustards (e.g. mechlorethamine and melphalan), (ii) nitrosureas (e.g. carmustine, streptozocin), ethylenimes and methylmelamines (e.g. altretamine), (iii) alkylsulfonates (e.g. busulfan), (v) triazenes (e.g. dacarbazine and temozolomide) and (vi) piperazines (Pauwels *et al.*, 1995; Ralhan & Kaur, 2007).

B1.3 Drugs that impair mitosis

Some of the most powerful anticancer drugs stop cells from making the components needed for cell division. Microtubules, long cylindrical polymers consisting of tubulin monomers (α and β), represent the principal target as cell cycle progression is dependent on microtubule dynamics. There are two classes of tubulin-interacting antimitotic agents- one that promotes microtubule polymerisation, thereby stabilising them and another class that inhibits microtubule polymerisation (Nagle *et al.*, 2006).

B1.3.1 Tubulin destabilising agents

The vinca alkaloids (e.g. vinblastine and vincristine) cause cells to arrest at metaphase by inhibiting microtubule assembly and inducing the self-association of tubulin into coiled spiral aggregates (Lobert *et al.*, 1996). Compounds that bind to the “vinca domain” of tubulin generally function as quick, reversible and temperature-independent inhibitors of tubulin assembly. Vinblastine and vincristine were the first drugs of this group to be developed, after being isolated from the periwinkle plant, *Catharanthus roseus* G. Don (or *Vinca rosa* Linn) and immediately had an impact in oncology, vincristine, for example, increasing the survival rate of childhood leukemia by 80% (Mukherjee *et al.*, 2001; Kong *et al.*, 2003). Derivatives and relatives include vinflunine, dolastatin-10, rhizoxin and spongiostatins, some of which show

promising anticancer activity *in vivo*, but have not yet been approved as drugs (Nagle *et al.*, 2006).

Other microtubule inhibitors that inhibit tubulin polymerisation bind to a different site on tubulin, retarding the addition of new tubulin and causing the mitotic spindle to disassemble during metaphase. This subclass has a colchicine binding site and includes the compounds colchicine, combrestatin A4, podophyllotoxin, halichondrin B and curacin A and their respective analogues. While colchicine itself lacks cancer efficacy *in vivo*, analogues of these lead compounds have shown clinical promise (Nagle *et al.*, 2006).

B1.3.2 Microtubule stabilising agents

Taxanes (e.g. paclitaxel = Taxol[®] and docetaxol) inhibit microtubule function by binding to β tubulin, thereby stabilising microtubules and altering the formation of the mitotic spindle. This induces cell cycle arrest at the metaphase/anaphase transition and subsequent apoptosis. Today, taxanes are one of the most commonly prescribed classes of cytotoxic agents in the treatment of a wide variety of cancers. Furthermore, while their mechanism of action and anticancer activities are very similar, key differences exist which means there is little cross-resistance between taxanes clinically (Goodin *et al.*, 2004; Montero *et al.*, 2005).

However, they do have their shortcomings, such as susceptibility to resistance via drug efflux conferred by P-glycoprotein and difficulties with formulation and administration. Furthermore, the mechanisms underlying cancer cell selectivity are not well understood and not all molecules that affect microtubule function are useful as anticancer drugs (e.g. colchicine). For this reason, there has been considerable interest in identifying new, structurally distinct, molecules that interfere with microtubule function. This has led to the discovery of the epothilones, originally discovered as cytotoxic metabolites from the myxobacterium *Sorangium cellulosum*. This class

of microtubule-stabilising drugs competitively inhibit binding of paclitaxel to microtubules *in vitro*, and preclinical studies indicate a broad-spectrum of action, even in paclitaxel-resistant models, with tolerable side effects (Goodin *et al.*, 2004). Other drugs, including eleutherobins, laulimalide, sarcodictyins and discodermolide, bind to different sites on tubulin and at different positions within the microtubule, meaning they have differing suppressive effects on microtubule dynamics but still block mitosis and induce cell death (Jordan, 2002).

B2 Targeted therapies

B2.1 Drugs that target molecular abnormalities (oncogenes and tumour suppressors)

B2.1.1 Molecular antibodies

Because cancer arises as a result of a series of genetic changes in a cell, drugs targeting these molecular abnormalities (oncogenes and tumour repressors) have recently been developed. For example, trastuzumab, more commonly known as Herceptin[®], is a humanised monoclonal antibody (mAb) that has antitumour activity against HER2-overexpressing human breast tumour cells. Overexpression of this oncogene occurs in 20-30% of breast cancer cases (Albanell *et al.*, 2003) and is associated with a more aggressive course of the disease (Menard *et al.*, 2003; Nahta & Esteva, 2006). HER2, also known as erbB2, is a member of the epidermal growth factor receptor (EGFR), erbB, or HER, subfamily of receptor tyrosine kinases (RTKs) (Menard *et al.*, 2003; Zwick *et al.*, 2000). All these receptors are comprised of an extracellular ligand-binding domain, a transmembrane lipophilic domain, and an intracellular tyrosine kinase (TK) domain, but unlike other RTKs in the EGFR family, HER2 does not bind to receptor-specific ligand (Ono & Kuwano, 2006; Albanell *et al.*, 2003; Zwick *et al.*, 2000; Menard *et al.*, 2003). Activation of TK enzymes and receptors triggers intracellular signal transduction pathways that control normal cell proliferation, differentiation, motility and adhesion of epithelial

cells (Menard *et al.*, 2003). Aberrant expression of RTKs undermines the normal controls of these signalling pathways, leading to increased cell proliferation, differentiation and survival, as well as the promotion of cell cycle progression and angiogenesis (Ono & Kuwano, 2006; Herbst, 2004; Menard *et al.*, 2003). By forming heterodimers, HER2 has a central, amplifying role in this signalling network and was therefore identified as a potential target for cancer therapy (Albanell *et al.*, 2003; Zwick *et al.*, 2000). AntiHER2 mAbs were actively sought (Raymond *et al.*, 2000) and in 1998 FDA approval of trastuzumab, which induces HER2 receptor downmodulation, was gained (Adams & Weiner, 2005; Albanell *et al.*, 2003).

Another mAb specifically targeting a different RTK, EGFR (also known as HER1 or erbB1), is the human-murine chimeric antiEGFR drug, cetuximab (Erbix[®]), which is used clinically for colorectal cancer where EGFR is overexpressed in 70-80% of cases (Herbst, 2004; Adams & Weiner, 2005; Ciardiello *et al.*, 2005; Zhang *et al.*, 2006), non-small cell lung cancer and head and neck cancer (Mendelsohn & Baselga, 2006; Astsaturov *et al.*, 2006). Examples of other FDA approved mAbs in cancer therapy targeting different oncogenes include bevacizumab (Avastin[®]) used in colorectal and lung cancers, rituximab (Rituxan[®]) against lymphoma and alemtuzumab (Campath-1H[®]), to combat chronic lymphocytic leukemia (Adams & Weiner, 2005; Rajpal & Venook, 2006).

B2.1.2 Small molecule inhibitors

At present, the two ways of clinically targeting the EGFR pathway are mAbs against EGFR and inhibitors of the EGFR TK (Ganti & Potti, 2005; Lenz, 2006; Vokes & Chu, 2006). MABs, such as cetuximab, work by blocking ligand binding to the extracellular domains of RTKs. Small molecule inhibitors of TKs, on the other hand, prevent TK phosphorylation and subsequent activation of signal transduction pathways by acting on the intracellular portions of

RTKs. An example of a small molecule inhibitor of EGFR is imatinib mesylate (Gleevec[®] or Glivec[®]), which received FDA approval in 2001. Imatinib has unprecedented efficacy for the treatment of chronic myelogenous leukemia (CML), the hallmark of which is the Philadelphia chromosome, or translocation, present in over 90% of cases (Druker & Lydon, 2000). In the Philadelphia translocation, genes of two chromosomes (BCR on chromosome 22 and ABL on chromosome 9) swap places. The fused gene (BCR-ABL) results in a chimerical protein (*bcr-abl*) expressed by CML cells which has constitutive TK activity. This means several signal transduction pathways are perpetually activated, leading to increased cell proliferation, reduced apoptosis and increased tumour migration. As imatinib is an ATP-competitive selective inhibitor of *bcr-abl*, TK activity is reduced and hence the pathogenesis of CML is decreased by treatment with this drug (Deininger & Druker, 2003).

Other small molecule EGFR-specific TK inhibitors include gefitinib (Iressa[®]) and erlotinib (Tarceva[®]) for combination therapies against pancreatic and non-small cell lung cancer (Herbst, 2004; Paez *et al.*, 2004; Ono & Kuwano, 2006; Mendelsohn & Baselga, 2006; Ducreux *et al.*, 2007)

B2.3 Drugs that target cell cycle control

B2.3.1 Inhibitors of Cdks

The frequent disruption of cell cycle regulation in cancer has flagged many molecules as potential therapeutic targets. Indeed, tumourigenesis is often associated with aberrant expression of various cyclins, Cdks and CKIs. Designing inhibitors of Cdks represents the most direct and promising strategy and several potent Cdk inhibitors have entered clinical trials, including flavopiridol and UCN01 (7-hydroxystaurosporine). Cdk inhibitors possess strong

antiproliferative activity, arresting cells in G₁ or G₂/M and sometimes promoting cell differentiation and triggering apoptosis (Park & Lee, 2003; Sandal, 2002).

B2.3.2 *Activators of the Rb pathway*

The most frequently altered cell cycle regulatory genes occurring in tumours are those involved in controlling the G₁-S transition regulated via the Rb pathway (see Appendix A6.2.2). Interestingly, other G₁-S regulators, such as E2F, cyclin E and the Cip/Kip family members of CKIs are rarely lost or mutated in human cancers (Sandal, 2002). pRb also has Cdk-independent functions, suggesting the re-introduction and expression of pRb into cancer cells may arrest growth by mechanisms that do not depend on the formation of Cdk complexes (Park & Lee, 2003). Restoring the Rb pathway has potential for efficiently treating cancer, but due to tumourigenesis being a multistep process and crosstalking between the components of the different cell cycle phases means that pathways can sometimes be bypassed. While many agents have shown promise in preclinical studies, no drugs have yet been developed to target this pathway (Knudsen & Wang, 2010).

B2.4 *Drugs that target resistance to apoptosis*

Defective apoptosis is one of the characteristics of cancer cells and has been implicated in various stages of cancer development and progression. Furthermore, it is the ability to evade apoptosis that appears to provide tumour cells with their capacity to resist conventional chemotherapy and radiotherapy (Khosravi-Far & Esposti, 2004). However, while cancer cells inactivate elements of the apoptotic pathway, they never disable the complete signalling cascade. This implies that at least some molecules share function between cell proliferation and cell death (Maddika *et al.*, 2007). These authors go on to suggest that since cell survival, cell death and cell cycle progression pathways are interconnected, it should be possible to develop pharmacological agents that can selectively harness cell proliferation pathways and redirect

them into the apoptotic process. They mention several viral molecules that can selectively kill cancer cells, but as yet, no drugs have been developed based on this theory. Other strategies exploiting cancer cells' resistance to apoptosis, however, have yielded promising new chemotherapeutics.

B2.4.1 *Activators of the p53 pathway*

Normally, stressful stimuli such as DNA damage or hypoxia leads to the activation of the transcription factor p53. As outlined in Appendix A6.2.1, p53 activates the transcription of genes encoding proteins involved in the control of cell cycle progression and is important in DNA damage-induced arrests at both the G₁/S and G₂/M checkpoints, suggesting cells lacking p53 may be more susceptible to genetic instability (Ford & Pardee, 1999). In addition, p53 is involved in the induction of apoptosis (see Appendix A3) (Sullivan *et al.*, 2004). As cancer is associated with decreased apoptosis, p53 is a noted tumour suppressor (Sun, 2006) and cells that lack p53 might be expected to be more vulnerable to cancer. Indeed, mutations of the *p53* gene are the most common genetic abnormality found in human cancers so far (Greenblatt *et al.*, 1994; Vermeulen *et al.*, 2005), with mutational inactivation of *p53* detected in over 50% of cases (Sun, 2006; Levesque & Eastman, 2007). Furthermore, almost all *p53* mutants associated with human tumours are defective for apoptosis (Sullivan *et al.*, 2004). Tellingly, the p53 protein is absent in 30-70% of clinical tumour samples and the absence of the wild-type *p53* gene in a tumour correlates with increased resistance to chemotherapy because of the development of apoptosis resistance (Huang & Oliff, 2001a; Koike *et al.*, 2004; Sullivan *et al.*, 2004; Sun, 2006). Thus, strategies aimed at pharmacologically reactivating p53, preventing p53 degradation or modulating other components in p53 signalling pathways are promising areas of anticancer research (Sun, 2006). While no clinical drugs have so far been developed, research so far is encouraging. For example, Ventura *et al.* (2007) have shown that the restoration of endogenous p53 function leads to tumour regression in mice *in vivo*, without affecting normal

tissues. Interestingly, the mechanism of tumour regression differed between tumour types. Reactivation of p53 function caused widespread apoptosis in lymphomas, but cell cycle arrest and senescence was induced in sarcomas. Nevertheless, provided human cancers are also dependent on sustained p53 inactivation for tumour maintenance, these results add support for endeavours towards treating cancers by exploiting the prevalence of p53 pathway inactivation in human cancer cells (Ventura *et al.*, 2007).

Notwithstanding these observations, Levesque and Eastman (2007) point out that p53-defective cells can undergo apoptosis and the loss of p53 can even sensitise cells to DNA damage. Furthermore, anticancer drugs and radiotherapy have been used to successfully treat many patients with p53-defective tumours. For these reasons, an alternative approach to cancer therapy in which p53 is inhibited has been proposed. Under this strategy, a p53 inhibitor (e.g. pifithrin- α = PFT α) is used in combination with a cytotoxic drug or radiotherapy against p53-defective tumours. This tactic could also be used to protect normal cells from p53-induced apoptosis and hence, healthy tissues from the adverse effects of the anticancer therapy. However, if used in combination with DNA damaging agents to treat p53-defective tumours, PFT α might protect some normal tissues from apoptosis, but it would also prevent p53-dependent cell cycle arrest so that normal cells with damaged DNA might proliferate, potentially causing more cancer. Also, while PFT α suppresses caspase activation and decreases levels of nuclear p53, it also suppresses apoptosis in p53-defective cell lines and can even induce apoptosis. A more effective drug strategy may be to develop p53 inhibitors that target either the p53-dependent apoptotic pathway or the p53-dependent cell cycle arrest separately. A new small molecule, PFT μ , fitting these criteria has recently been identified (Levesque and Eastman, 2007). Actinomycin D, already used as an antibiotic, has been approved for use in

activating wild-type p53, while various other small molecules targeting the p53 pathway (e.g. PRIMA-1, Nutlin, RITA) are still in Phase 1 or pre-clinical trials (Brown *et al.*, 2009).

B2.4.2 *Inhibitors of the ubiquitin-proteasome pathway*

As outlined in Appendix A4, the proteasome is a ubiquitous enzyme complex that plays a crucial role in the controlled degradation of many proteins involved in cell cycle regulation and apoptosis, including p53. Since these pathways are fundamental for cell survival and proliferation, especially in cancer cells, and because proteasome inhibition has been shown to sensitise cancer cells to traditional anticancer agents, the ubiquitin-proteasome system (UP-S) provides a rich source of molecular targets for specific intervention. Therefore, targeting the UP-S has emerged as a promising approach to developing novel anticancer chemotherapeutics (Mitsiades *et al.*, 2005; Montagut *et al.*, 2006; Giuliano *et al.*, 2003; Burger and Seth, 2004).

Proteasomes, which account for approximately 1% of the cellular protein content of eukaryotes, are localised in the cytosol and nucleus. The functional large 26S proteasome is comprised of ring-shaped 19S and 20S particles, which are composed of numerous polypeptide subunits, each having distinct enzymatic activities. The level of 20S proteasome core in the plasma of patients with solid tumours has been shown to be up to 1,000 times higher than in normal subjects, highlighting the significance of the proteasome to cancer. However, little is currently understood about the role of the individual components of the UP-S in tumourigenesis and few ubiquitin-conjugating enzymes and ligases have been evaluated for their expression patterns in many human tumours (Burger and Seth, 2004).

The UP-S also plays a critical role in regulating the important transcription factor nuclear factor kappa B (NF- κ B), which is responsible for activating several genes that contribute to the malignant phenotype, including genes that promote cell proliferation, cytokine release,

antiapoptosis, and changes in cell surface adhesion molecules. The activity of NF- κ B is tightly controlled by the UP-S via the accumulation or degradation of I κ B, which binds to and inactivates NF- κ B. Cell adhesion molecules are proteins regulated by NF- κ B and are involved in tumour metastasis and angiogenesis *in vivo* (O'Connor *et al.*, 2005).

The dipeptide boronic acid analogue, bortezomib (Velcade®), an extremely potent, highly selective and reversible proteasome inhibitor, is the FDA-approved prototype drug of this class of anticancer agents, showing strong activity *in vitro* and *in vivo* against a broad range of cancer cell types (Montagut *et al.*, 2006; Cardoso *et al.*, 2004). Better still, it has only a few toxic effects on normal cells, although the few side effects are grade 3/4 (Burger and Seth, 2004). It seems to cause apoptosis either by blocking tumour necrosis factor (TNF) α -induced activation of NF- κ B (observed in multiple myeloma cells), or G₂-M-phase arrest and induction of p21 (prostate cancer cells), or by stabilisation of p53 (lung cancer cells) (Burger and Seth, 2004).

B2.4.3 Inhibitors of other signalling pathways

Other signalling pathways in apoptosis have also been identified as potential targets for cancer therapy. These include the antiapoptotic Bcl2 family of proteins (e.g. Bcl-2 and Bcl-xL) and their upstream regulators or downstream effectors and antagonists of IAPs (e.g. Smac). Agents that have shown preclinical promise in targeting Bcl-2 include suprafenacine, miR-181b (Choi *et al.*, 2010; Zhu *et al.*, 2010), while clinical trials are ongoing on several investigational drugs including AT-101, ABT-263, GX15-070 and oblimersen sodium (Kang & Reynolds, 2009). However, as yet no anticancer therapies have been developed based on this knowledge (Huang & Oliff, 2001a).

B2.5 Drugs that target tumour cell immortality

A key property of cancer cells is their immortality. In tumours, cell replication is associated with the maintenance of telomere length and integrity, usually through the reactivation of a reverse transcriptase mechanism, whereby telomerase adds TTAGGG units to telomeres (see Appendix A5). Telomerase is constitutively overexpressed in the vast majority of human cancers and telomeres are critically shorter in most tumours compared to normal tissues. This makes targeting telomeres, or the telomerase machinery, an attractive, potentially broad-spectrum, approach to cancer therapy (Kelland, 2005).

The genes encoding six of the subunits comprising human telomerase have been cloned. These include hTERT (human telomerase reverse transcriptase), hTR (human telomerase RNA), TEP1 (telomerase-associated protein 1), hsp90 (heat shock protein 90), p23, and dyskerin. (Feng *et al.*, 1995; Nakamura *et al.*, 1997; Chang *et al.*, 2002). Targeting the active site of hTERT has been reported using small molecules including AZT (3'-azido-2',3'-dideoxythymidine), but this approach has had limited success due to a lack of sensitivity for telomerase compared to other polymerases (Ji *et al.*, 2005). BIBR1532, a non-competitive inhibitor of telomerase, is the most extensively studied non-nucleoside molecule (El-Daly *et al.*, 2005). It has shown potent inhibition of telomerase *in vitro*, but *in vivo* studies have revealed a lag between enzyme inhibition and cellular growth arrest, apoptosis and tumour growth delay. A second molecule, 2,3,7-trichloro-5-nitroquinoxaline (TNQX), with similar properties to BIBR1532 also showed growth inhibition and induction of senescence, but only after extensive periods of exposure (Kelland, 2005).

Telomerase can also be directly targeted by various antisense molecules to hTERT or hTR. The most advanced is GRN163L, an oligonucleotide targeted to the template region of hTR. This molecule has shown promising effects *in vitro* and *in vivo* and is currently undergoing late-stage preclinical development. In addition, it may be possible to induce antitumour effects via indirect mechanisms that regulate telomerase expression, such as inhibitors of chaperones, hypoxia-inducible factors 1 or the nuclear factor κ B pathway (Kelland, 2005).

Targeting the telomeres themselves is another avenue being explored and various telomere targeting agents (TTAs) have been described. Guanine-rich sequences of DNA, such as telomeres, tend to fold into quadruplex intramolecular structures. Hence, compounds that interact with the G-quadruplex, such as trisubstituted acridines (e.g. BRACO19), ethidium derivatives and the natural product telomestatin (aka SOT-095) can inhibit telomerase activity. While some first generation G-quadruplex ligands exhibited a lack of selectivity for 4- versus 2-stranded DNA, resulting in non-specific acute cytotoxicity and similar inhibition of telomerase activity *in vitro*, improved molecules now exist (e.g. BRACO19) that have shown encouraging *in vivo* data. The antitumour effects from G-quadruplex ligands are generally much more rapid than those observed using direct hTERT inhibitors and there is considerable potential for broad-spectrum antitumour activity, especially in tumours with short telomeres. There is also the evidence for synergistic effects when used in combination with radiotherapy and cytotoxic drugs, particularly those that induce DNA damage (Kelland, 2005). However, despite the great potential, most molecules that have shown specific telomerase activity have been too toxic at the doses required. Two telomerase-targeting drugs in clinical development are telomelysin and imetelstat, which will both begin Phase II clinical trials in 2010 (Brower, 2010).

B2.6 Drugs that target angiogenesis

The generation of a lethal tumour requires more than excessive tumour cell proliferation. A solid tumour must also have an adequate network of blood vessels (vasculature) from normal tissue to supply nutrients and oxygen and to remove waste products (Nishida *et al.*, 2006; Yano *et al.*, 2006). However, when a tumour becomes too large ($\sim 1\text{-}2\text{ mm}^3$) for its own blood supply to support further expansion, a stressful, hypoxic and acidic microenvironment develops within the tumour, providing a strong selection pressure for more aggressive cancer cells and the generation of signals necessary for the growth of new blood vessels (angiogenesis) (Thornton *et al.*, 2006; Boehm-Viswanathan, 2000; Adams, 2005). In fact, neovasculature is critical to the growth and spread of malignant tumours (Ferrara, 2004a), the generation of tumour mass having been shown to be impossible without endothelial cell proliferation (Folkman, 2006a). Hence, both tumour cell proliferation and angiogenesis together are vital to turn a small solid tumour into a life-threatening neoplasm (Folkman, 2006a; Rahman & Toi, 2003; Zhong & Bowen, 2006).

In 1971 Folkman proposed his then provocative hypothesis that arresting the growth of a tumour may be achieved by attacking its blood supply (Folkman, 1971; Verhoef *et al.*, 2006). However, it was not until recently with the identification of molecular targets and cellular pathways of angiogenesis that antiangiogenic chemotherapy came of age as a viable approach to combating the growth of solid tumours (Thornton *et al.*, 2006). Many angiogenesis inhibitors have now been approved for clinical use by the FDA in the USA and elsewhere in the world (Folkman, 2006a; Rosen, 2001; Rahman & Toi, 2003). Antiangiogenic therapy may also be useful in preventing metastasis or tumour recurrence (Rahman & Toi, 2003) and in the fight against multi drug resistance (MDR), a common feature of cancers (Rahman *et al.*, 2010).

Tumour angiogenesis is a multistep cascade that involves secretion or activation of angiogenic factors by tumour cells, activation of proteases, and proliferation, migration and differentiation of endothelial cells into new vasculature (Rahman & Toi, 2003). In normal tissues, angiogenesis is regulated via a complex balance of the actions of endogenous angiogenic inhibitors (e.g. endostatin) and proangiogenic factors (e.g. VEGF) (Ferrara, 2004b; Zhong & Bowen, 2006; Nieder *et al.*, 2006). When proangiogenic factors outweigh antiangiogenic factors, the “angiogenic switch” is turned on and endothelial cells become activated from their normal dormant state (Thornton *et al.*, 2006). Many factors, including vascular endothelial growth factor (VEGF) RTKs, p53, tubulin and cyclooxygenase-2 (COX-2), are known to regulate the equilibrium between angiogenic stimulants and inhibitors, hence these factors are good targets for antiangiogenesis drug design (Rahman & Toi, 2003; Zhong & Bowen, 2006).

Antiangiogenesis drugs can either inhibit proangiogenic factors or promote or mimic the actions of antiangiogenic factors. Currently, pharmacologically targeting angiogenesis means inhibiting growth factors that promote the growth of vascular endothelial cells or blocking their receptors (Rahman & Toi, 2003).

B2.6.1 Vascular targeting agents

The triggering of the angiogenic switch leads to the breakdown of basement membranes and the extracellular matrix, mainly because of the increased activity of matrix metalloproteases. These alterations to the matrix promote endothelial migration into the extravascular space where they proliferate. The new capillary network is formed as the endothelial cells arrange themselves into tubes and lumens. Pericytes are then recruited which attach to and stabilise the new mature vessels. Until this time, VEGF is necessary for the survival and integrity of the endothelial cells (Thornton *et al.*, 2006). However, although composed of normal endothelial cells, tumour blood vessels are morphologically very different to blood vessels formed in non-

diseased tissues and these differences make the vascular lining a valid target for therapy (Thornton *et al.*, 2006). For example, by exploiting this knowledge Professor Dharmarajan and colleagues at the Anatomy and Human Biology Department of UWA have discovered a compound, “Ang001”, that inhibits blood vessel formation *in vitro* and have applied for a provisional patent to further develop this compound. The tubulin inhibitors, ANG453 and combretastatin A4 phosphate, have been shown to induce apoptosis of preexisting tumour-associated endothelial cells and shut down blood flow leading to tumour necrosis and, in the case of combretastatin, completely eradicated one tumour (Yano *et al.*, 2006). Other antivasculature agents that show potential in the laboratory include the antibiotic borrelidin, which works by suppressing new capillary tube formation (Kawamura *et al.*, 2003; Moss *et al.*, 2006) and inhibitors of bone marrow precursor cells or matrix metalloproteases (Ferrara, 2004b).

B2.6.2 *Inhibitors of proangiogenic factors*

Neovascularisation is mediated through the secretion of proangiogenic growth factors, particularly basic fibroblast growth factor (bFGF) and VEGF. Approximately 60% of the 200 different types of human cancers express VEGF (Folkman, 2006a) and, at least in some tumours, overexpression of VEGF corresponds to a poor prognosis (Ferrara, 2004a). VEGF is also thought to be responsible for the hyperpermeability of tumour blood vessels to plasma and plasma proteins that characterises pathological angiogenesis (Satchi-Fainaro *et al.*, 2005). It is also associated with increased invasiveness and recurrent disease and foetal mice with only a single allele of VEGF-A die before birth (Thornton *et al.*, 2006). According to Ferrara (2004a; 2004b), tumour-expressed VEGF is a very attractive target for anticancer therapy because of its central role in promoting many cancers and because its activity is at the endothelial cell level, meaning tumour penetration is not so important for VEGF inhibitors compared to the conventional, generally more nonselectively toxic, agents that target the tumour cells themselves. Penetration is a major problem for conventional anticancer therapies because of the

elevated interstitial pressure of most tumours due to leakage of plasma proteins from the vasculature and the convoluted nature of the tumour vasculature itself, which can be difficult for blood to navigate (Ferrara, 2004a; Thornton *et al.*, 2006). Furthermore, when used in conjunction with conventional anticancer treatments, agents that inhibit tumour angiogenesis enhance the efficacy of chemotherapy and radiotherapy. This is partly because, not only does VEGF exacerbate the high tumour interstitial pressure, but it also protects tumour cells against the apoptosis that is normally induced by conventional therapies (Ferrara, 2004a). Therefore, it might be expected that maximal punch could be achieved by combining standard anticancer treatments with drugs that target VEGF or its receptors (Ferrara, 2004a). Understanding the role of VEGF in tumour angiogenesis has led to the development of agents that specifically disrupt angiogenesis. Three antiVEGF inhibitors FDA approved for treatment of solid tumours are the humanised monoclonal antibody bevacizumab, which binds VEGFR (Avastin[®]) (Ferrara, 2004a, 2004b; Thornton *et al.*, 2006), and the small molecule RTK inhibitors sunitinib and sorafenib (Herbst, 2006; Vokes *et al.*, 2006; Sledge *et al.*, 2006). In addition, according to Folkman (2006), erlotinib (Tarceva[®]) may be an EGF-specific RTK, but its main anticancer activity is to block VEGF and two other angiogenic proteins (bFGF and TGF- α).

Caplostatin, a nontoxic synthetic analogue of fumagillin (derived from the fungus *Aspergillus fumigatus fresnius*) conjugated to a water-soluble N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer, is the most broad-spectrum angiogenesis inhibitor known (Folkman, 2006a). It has been shown that caplostatin (and also fumagillin and angiostatin) antagonizes angiogenesis by inhibiting the vascular hyperpermeability effects of VEGF, histamine and platelet-activating factor (PAF) as well as VEGF-induced endothelial cell migration. It does so partly by inhibiting VEGF-induced phosphorylation of VEGFR(-2), calcium influx and activation of the RhoA protein (part of the Ras superfamily of proteins involved in the

regulation and timing of cell division) in endothelial cells. Furthermore, while caplostatin interferes with endothelial function, it does not affect their structure, nor does it inhibit the proliferation of any other cell type, including tumour cells (Satchi-Fainaro *et al.*, 2005). Such complete specificity is a useful characteristic for any drug candidate.

B2.6.3 *Antiangiogenic factors*

It is thought that primary tumours sometimes synthesise and release antiangiogenic proteins to suppress the growth of remote metastases, resulting in so-called “tumour dormancy”.

This might help explain why when certain primary tumours are removed, rapid progression of metastases often occurs (O'Reilly *et al.*, 1994; Beecken *et al.*, 2006). Endogenous angiogenesis inhibitors, therefore, act as tumour repressor proteins, much like p53, except that endogenous angiogenesis inhibitors generally do not affect tumour cell proliferation (Folkman, 2006a). Such endogenous angiogenesis inhibitors represent a potential new class of specific anticancer agents, having a lower risk of drug resistance and relatively less toxicity than conventional chemotherapeutics (Beecken *et al.*, 2006). So far, numerous endogenous inhibitors of angiogenesis have been identified, including the human plasma protein β_2 -glycoprotein-I (β_2 gpI, apolipoprotein H) (Beecken *et al.*, 2006), the serpin antithrombin (O'Reilly *et al.*, 1999) and angiostatin (O'Reilly *et al.*, 1994) and endostatin (O'Reilly *et al.*, 1997). Several of these endogenous angiogenesis inhibitors, including endostatin, interferon- β , 2-methoxyestradiol, tetrahydrocortisol and thrombospondin-1 (and -2) have made it to Phase II or III clinical trials, but none have been FDA-approved for public use although endostatin has been approved for use in China since 2005 (Folkman, 2006a; Folkman, 2006b; Hee Sun *et al.*, 2010).

However, the consequences of antiangiogenic therapy seem to be short-lived, as withdrawal from the treatment induces rapid regrowth of tumour vessels and a subsequent relapse of

tumour growth (Loges *et al.*, 2010). Hence, antiangiogenesis therapy is currently very fashionable, but due to the complex mechanisms that regulate tumour angiogenesis, monotherapy with most antivascular agents will probably not be enough to control cancer long-term. However, when combined with another anticancer strategy, such as targeting the tumour with other drugs or surgery, preventing angiogenesis or attacking the existing blood supply of a tumour represents a powerful new weapon against cancer (Yano *et al.*, 2006) that has now become routine oncological practice (Nieder *et al.*, 2006).

B2.7 Drugs that target DNA repair mechanisms

A major problem with cytotoxic drugs that target DNA synthesis (see section B1.1) or integrity (see section B1.2) (as well as ionising radiotherapy) is that they have been shown to induce secondary cancers several years after initial exposure. This is due to their potential to induce DNA damage in normal cells, which do not have the same ability to recognise damage and initiate DNA repair that cancer cells generally have. Research into the possibility of using inhibitors of DNA repair mechanisms in combination with chemotherapy to selectively target tumours and enhance the efficacy of current therapies is consequently undermined by the enhanced risk of inducing secondary cancers. While convincing evidence exists supporting the validity of DNA repair proteins as viable drug targets, as yet no anticancer drugs have been developed using this idea (Madhusudan & Hickson, 2005; Sánchez-Pérez, 2006).

As many mutations are necessary to give rise to a tumour, it is probably not surprising that almost all cancer cells are abnormally mutable, having acquired one or more defects in their metabolism of DNA. This genetic instability means the cell acquires the complex set of changes necessary to become cancerous more quickly than it would otherwise do so. Besides its action promoting cell proliferation, differentiation and survival, the *bcr-abl* fused protein of

the Philadelphia chromosome also inhibits DNA repair, leading to genomic instability (Laurent *et al.*, 2001). Therefore, it might be expected that imatinib could help manage this potential for additional carcinogenesis. However, patients who had already progressed into acute myeloid leukemia (where genetic instability had already set in) showed a response, but then relapsed- the cancer cells were able to evolve a resistance to the drug (Alberts, 2002).

Appendix C. MTT-formazan Solubilisation

The solubilisation of MTT-formazan crystals is a key step contributing to the efficiency of the MTT assay. Thus, it was important to ensure solubilisation was complete. Others (e.g. Abe & Matsuki, 2000; Wang *et al.*, 2006) have used different extraction buffers and this was the first parameter to be studied, albeit only in MDA-MB-468 and MCF7 cells. The extraction buffers tested were:

- A. 10% SDS, 50% isobutanol in 0.01M HCl;
- B. 10% SDS, 50% isobutanol in 0.1M HCl;
- C. 12% SDS, 40% DMF (dimethyl formamide), pH 4.7;
- D. 100% DMSO (dimethyl sulfoxide);
- E. 90% DMSO, 10% glycine buffer (0.1M glycine, 0.1M NaCl, pH 10.5); and
- F. 0.1M HCl in anhydrous isopropanol.

After incubation with MTT, 200 μ L of one of these buffers was added and plates incubated for 1 h and 2 h before absorbances were read at 595nm. From these data an extraction buffer was chosen for use in subsequent experiments. The effectiveness of various extraction buffers in solubilising formazan crystals was compared for MDA-MB-468 cells and MCF7 cells after 1 h and 2 h incubation. Qualitative assessment was combined with OD₅₉₅ readings to ascertain successful extraction of formazan. Figure C1 depicts the absorbance levels obtained after formazan crystals were solubilised with various extraction buffers.

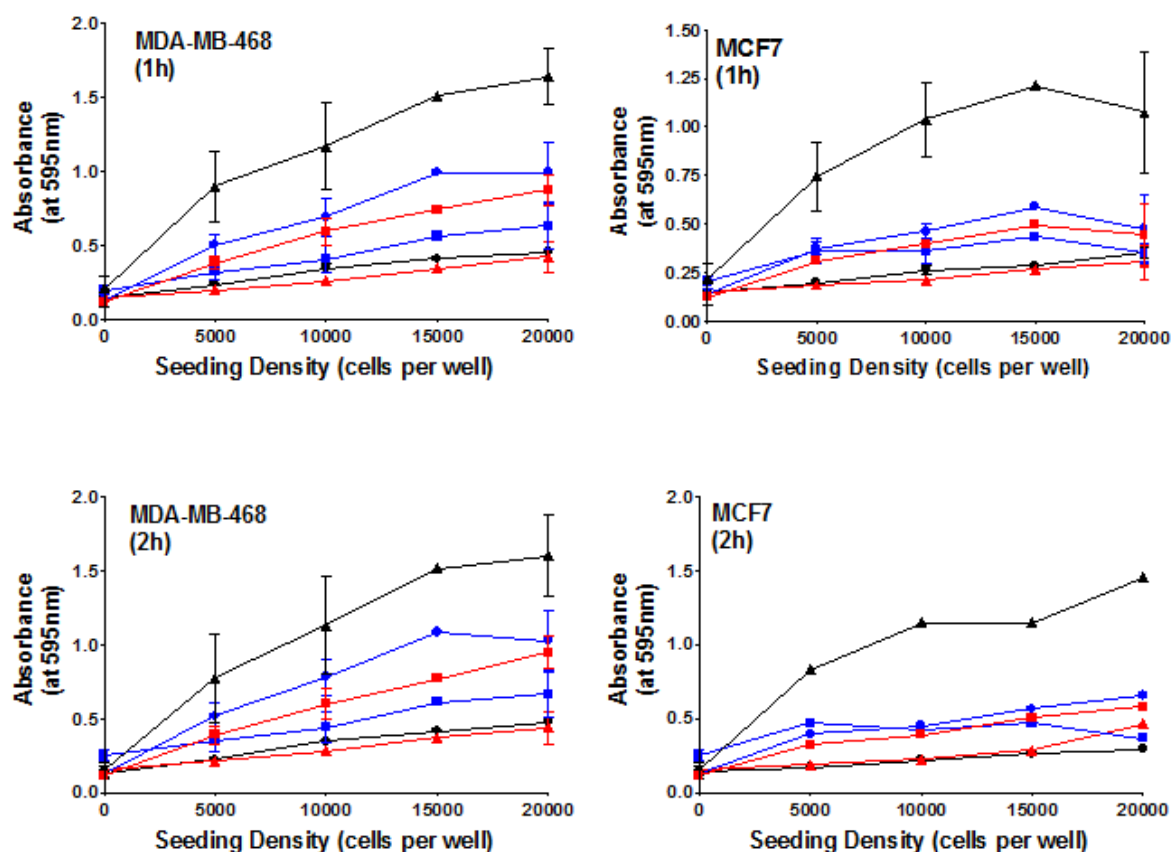


Figure C1 Effect of different MTT extraction buffers on absorbance.

Breast cancer cells were seeded at various densities and incubated under standard conditions for 72 h. After 2 h incubation with 10 μ L of a 5 mg/mL MTT solution, 200 μ L of an extraction buffer A (■), B (▲), C (●), D (■), E (▲) or F (●) was added. Cells were further incubated for 1 or 2 h before the absorbance at 595nm was read. The results represent the means ($\pm$ SE where applicable) of 2 or 3 separate experiments, each containing quadruplicate wells.

As can be seen from Figure C.1, for both cell lines tested, the highest absorbance values were constantly obtained by using extraction buffer B (10% SDS, 50% isobutanol in 0.1M HCl). Based on a seeding density of 10,000 cells per well, there was no significant difference between absorbance values obtained using this extraction buffer to solubilise the formazan product when incubated for 1 h compared to 2 h for either cell line tested. Using this extraction buffer, a seeding density of 10,000 cells per well was shown to result in absorbance values of around 1.0 after 72 h incubation for both cell lines.

Various extraction buffers were compared for their effectiveness to solubilise formazan crystals (Fig. C.1). It was found that the original extraction buffer used in this laboratory, B: 10% SDS, 50% isobutanol in 0.1M HCl, (Kicic *et al.*, 2002) was the best under the conditions used, as determined by the least turbidity, the “neatest” curves and the highest absorbance values. Extraction buffer B was also initially chosen as it was cheaper than DMSO.

However, when the MTT assay was re-examined later in the project, it was found that the method of Fox *et al.* (2005) was more efficient than that of Kicic *et al.* (2002) (see Section 3.2.2). One of the changes involved in the improved method was a switch from extraction buffer B to DMSO. Significantly, after assessment of many formazan solvent systems, DMSO was also the solubilising agent adopted by the NCI anticancer drug screening programme (Alley *et al.*, 1988) and others after optimisation experiments showed formazan crystals were completely dissolved in DMSO within 2 min (Li & Song, 2007).

Appendix D. Spectra of Selected Plant Extract Solutions

Plant extracts were dissolved at 100 mg/mL in DMSO and diluted to 1 mg/mL in DMEM (=1% DMSO).

These 1 mg/mL samples were spectrophotometrically scanned over wavelengths ranging from 800 to 200 nm. These spectral scans, along with those of appropriate controls, are presented in Figures D1 to D9.

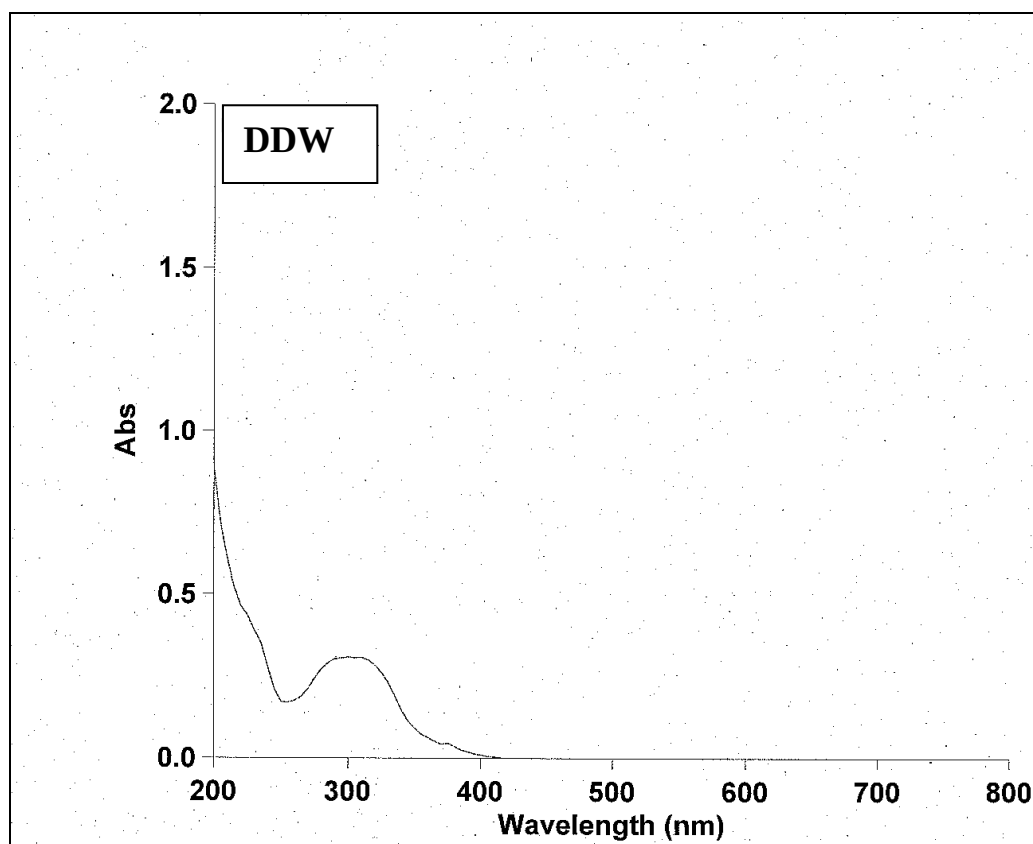


Figure D1 Spectral scan of DDW.

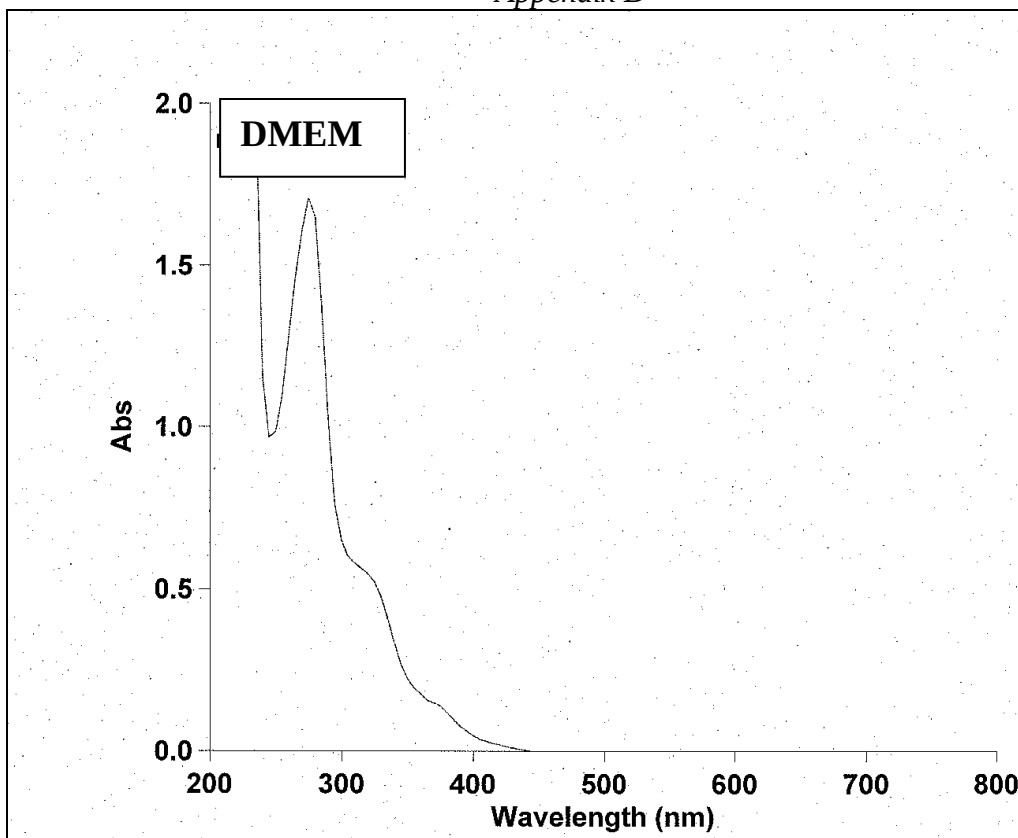


Figure D2 Spectral scan of DMEM.

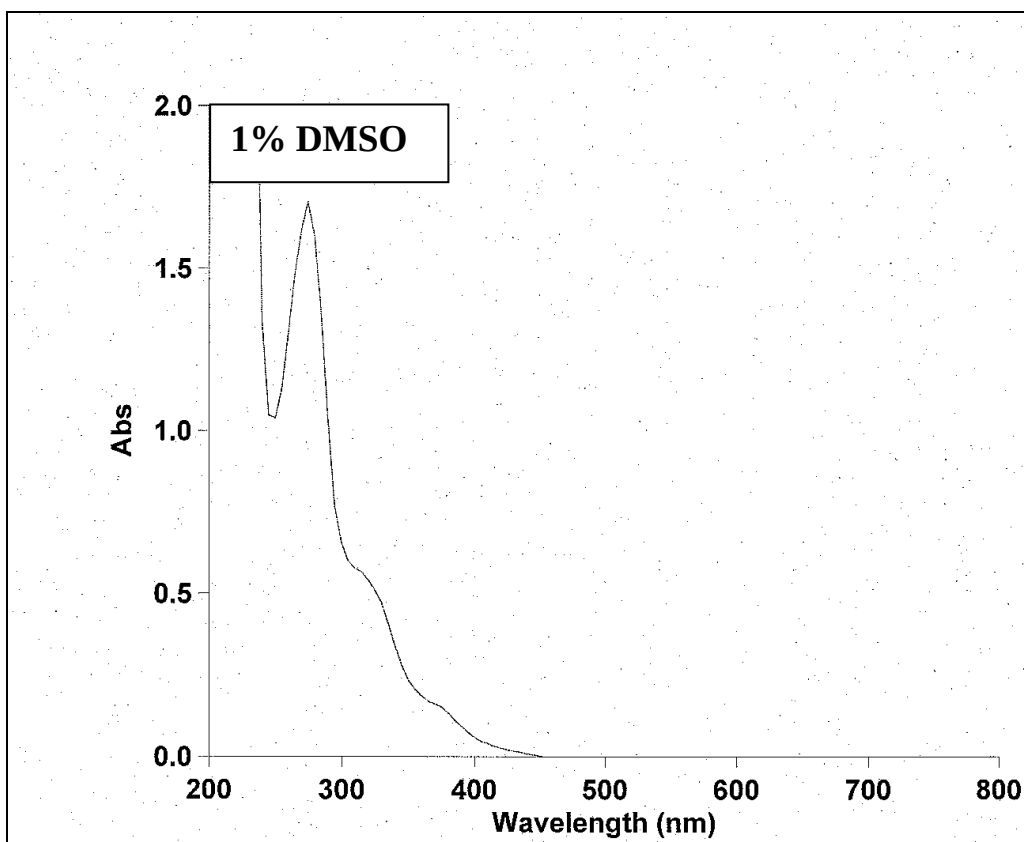


Figure D3 Spectral scan of 1% DMSO.

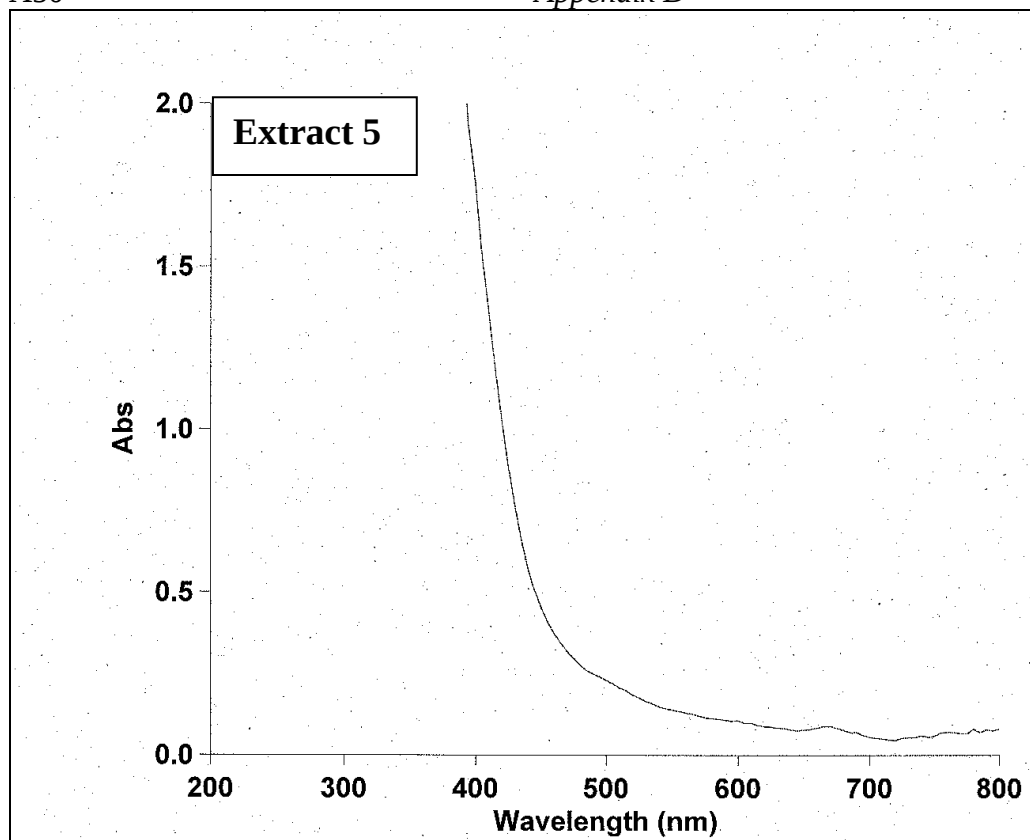


Figure D4 Spectral scan of Extract 5.

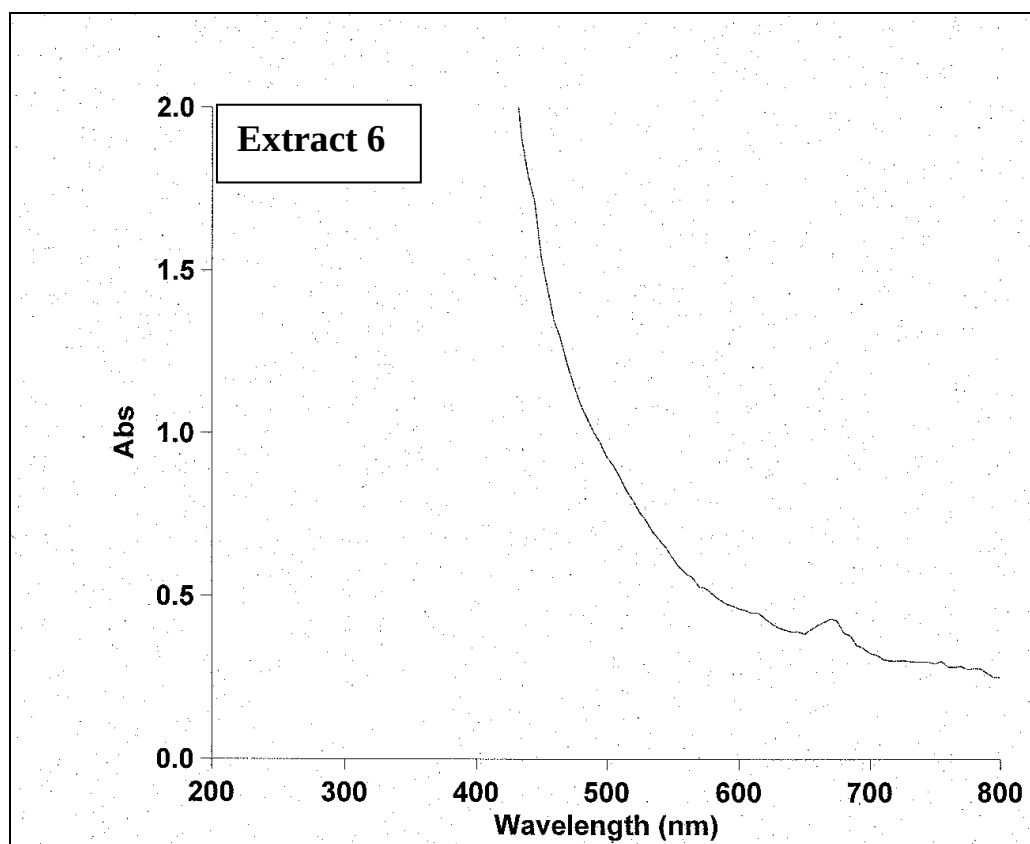


Figure D5 Spectral scan of Extract 6.

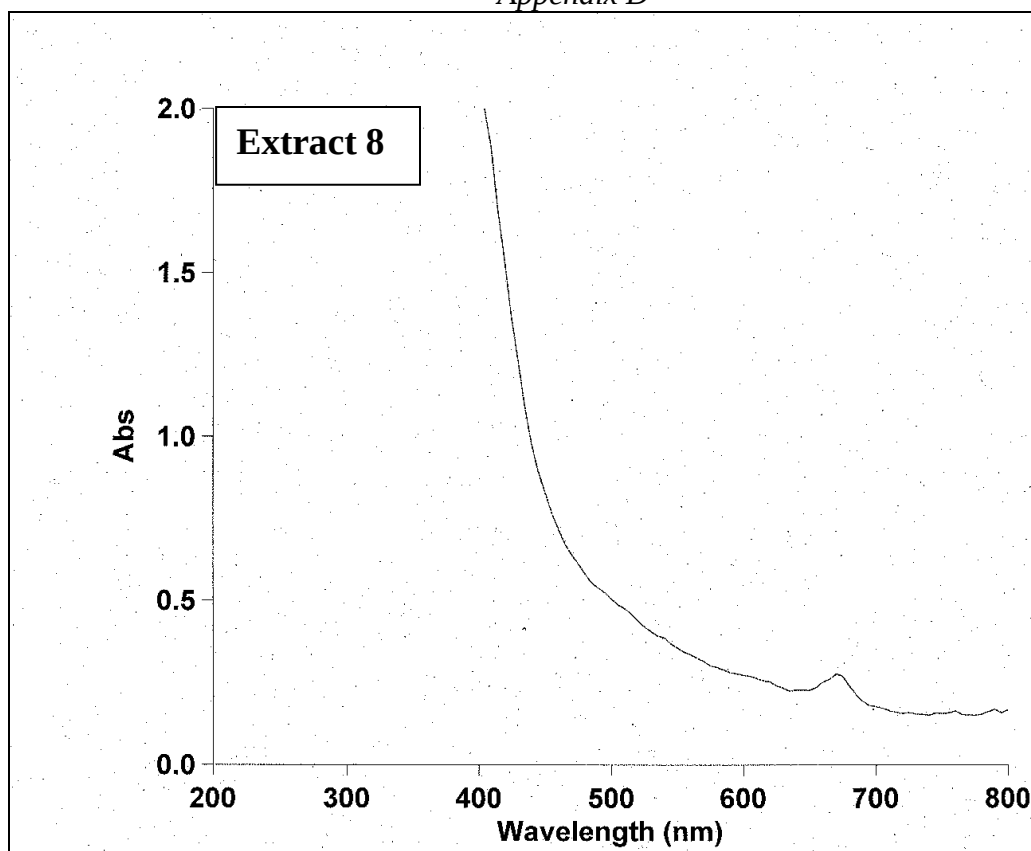


Figure D6 Spectral scan of Extract 8.

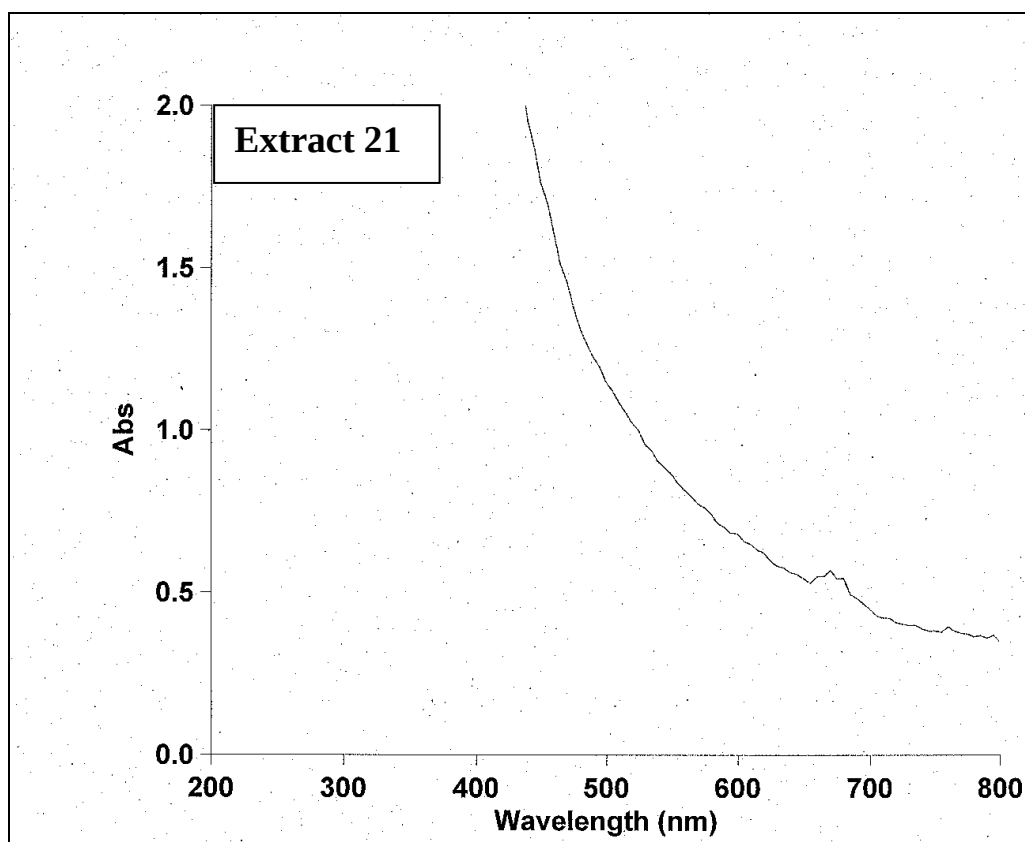


Figure D7 Spectral scan of Extract 21.

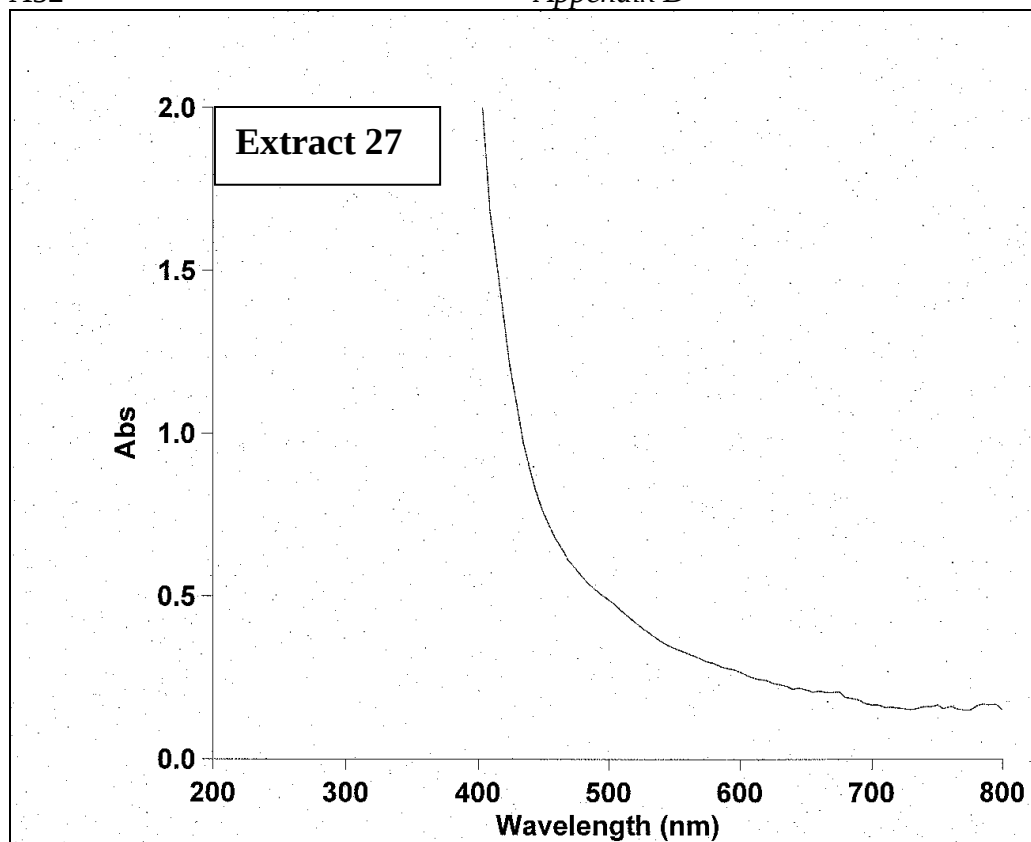


Figure D8 Spectral scan of Extract 27.

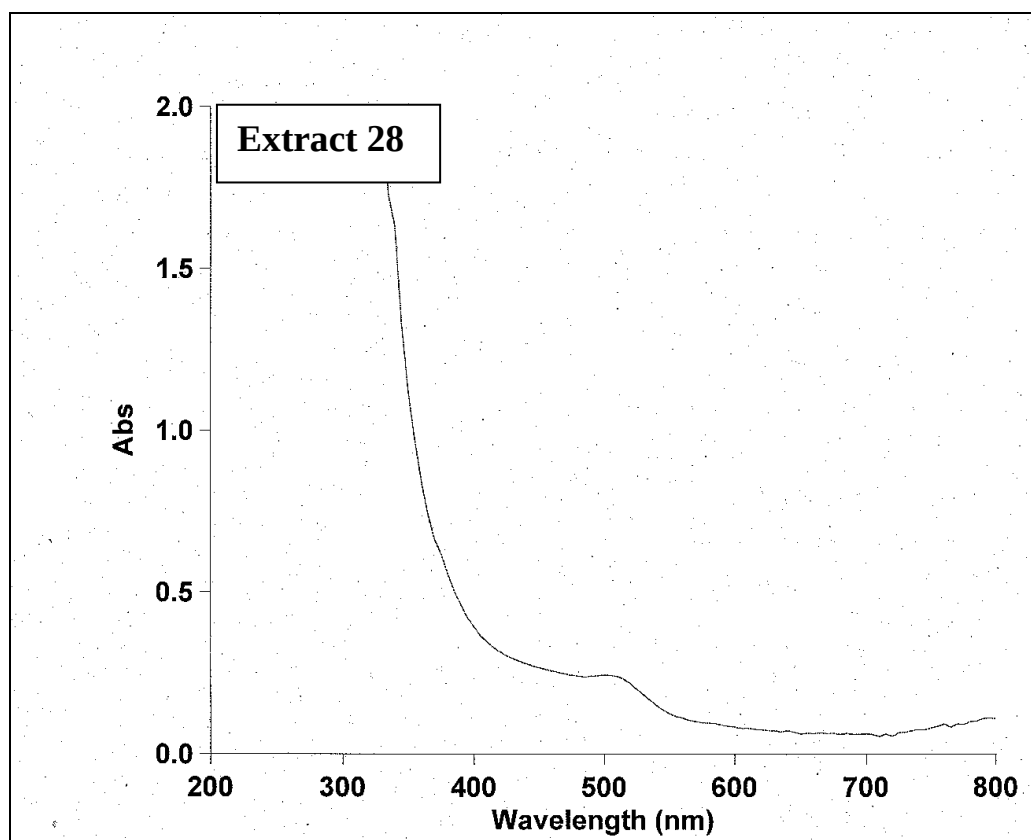


Figure D9 Spectral scan of Extract 28.

Appendix E. Data for Typical Dose-Response Curves

Figures E1-E3 are the nonlinear regression curves fitted to dose-response data of the most promising crude methanolic plant extracts as described in Chapter 4 (Figs. 4.5-4.7). The associated IC_{50} and r^2 values are presented in Table E1.

Table E1 IC_{50} Values Calculated for Cell Line-Extract Pairs (4P Model).
Caco-2

Extract Code	IC_{50} ($\mu g/mL$)	IC_{50} Range (95% CI) ($\mu g/mL$)	Hillslope	r^2
1	489.7	6.29 - 38129	-7.65	0.972
2	622.1	-	-9.36	0.977
3	201.1	137.2 - 294.8	-3.34	0.984
5	218.1	10^{-9} - 10^{12}	-12.0	1.000
6	465.8	235.2 - 922.2	-9.17	0.981
8	482.3	-	-13.8	0.994
11	1166	-	-1.83	0.700
12	48.3	1.04 to 225	-0.985	0.776
19	168.1	158.0 - 178.7	-7.79	0.995
21	131.5	122.6 - 141.1	-8.48	0.990
27	353.1	282.2 - 441.8	-5.01	0.986
28	10^6	-	-0.326	0.998
A	550.0	359.5 - 841.5	-8.53	0.955
B	153758	153758	-1.00	0.863
C	1962	$0.027 - 10^8$	-0.718	0.987
D	646.4	296.3 to 1411	-2.29	0.998

MM253

Extract Code	IC_{50} ($\mu g/mL$)	IC_{50} Range (95% CI) ($\mu g/mL$)	Hillslope	r^2
1	901.8	-	35.0	0.910
2	398.1	53.9 - 2939	4.68	0.994
3	45.4	41.9 - 49.2	-3.84	0.999
5	435.7	-	-13.3	0.987
6	168.8	80.6 - 353.3	-1.84	0.978
8	565.0	1.09 - 292824	-2.73	0.983
11	548.1	-	-10.5	0.960
12	612.5	398.5 - 941.5	-4.70	0.980
19	210.7	177.0 - 250.8	-3.86	0.992
21	2042	$10^5 - 10^{11}$	-0.763	0.970
27	263.5	162.9 - 426.1	-4.11	0.970
28	125.4	97.3 - 161.7	-3.88	0.956
A	265.8	121.6 - 581.0	-4.90	0.979
B	325.9	325.9	-4.90	0.988
C	94373	-	-1.01	0.984
D	515.3	$0.05 - 10^6$	-24.0	0.932

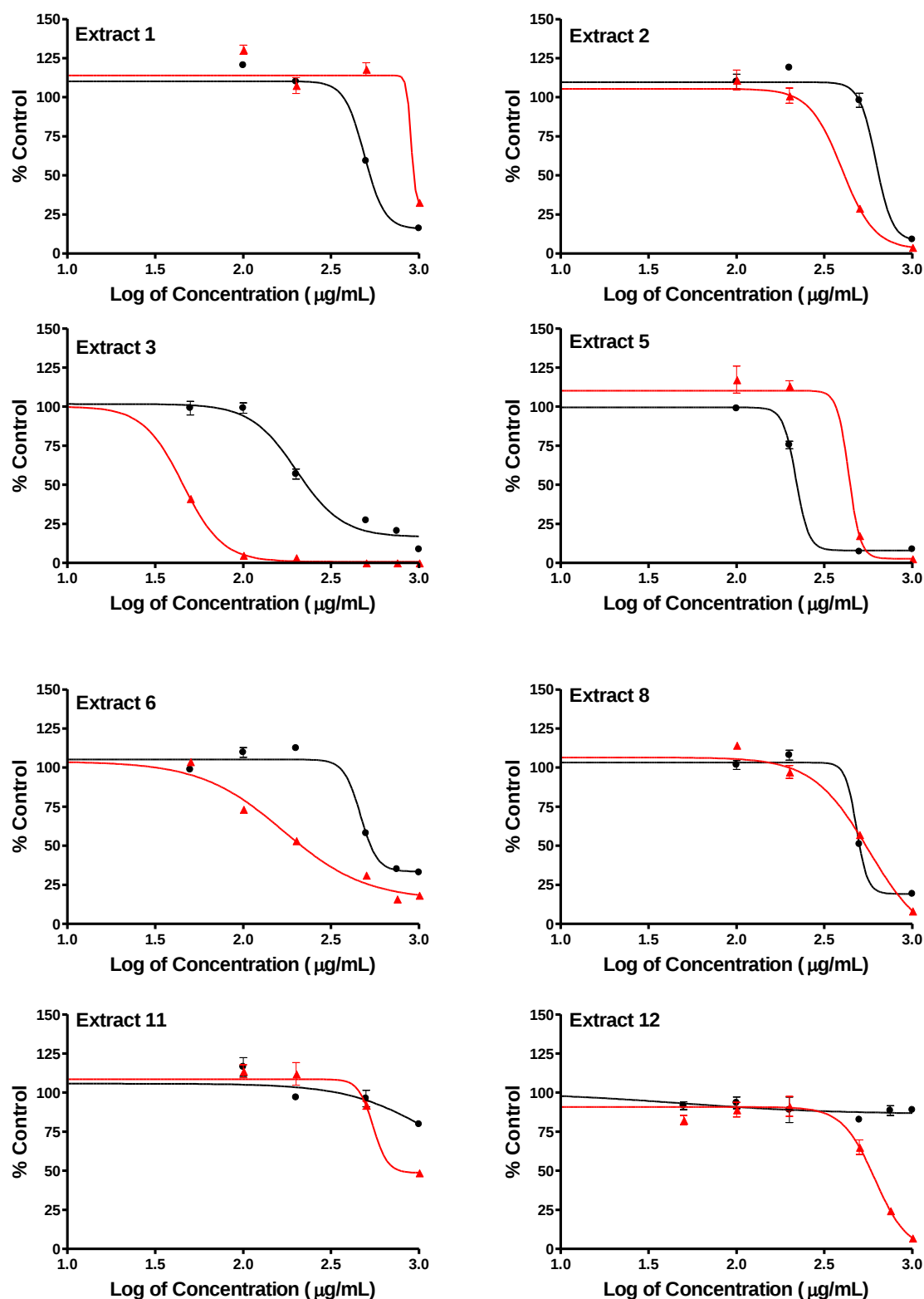


Figure E1 Nonlinear regressions of promising Batch 1 extracts.

Caco-2 (●) and MM253 (▲) cells were exposed to extracts over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values of representative curves (Fig. 4.5) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) according to the 4P method for each extract-cell line combination.

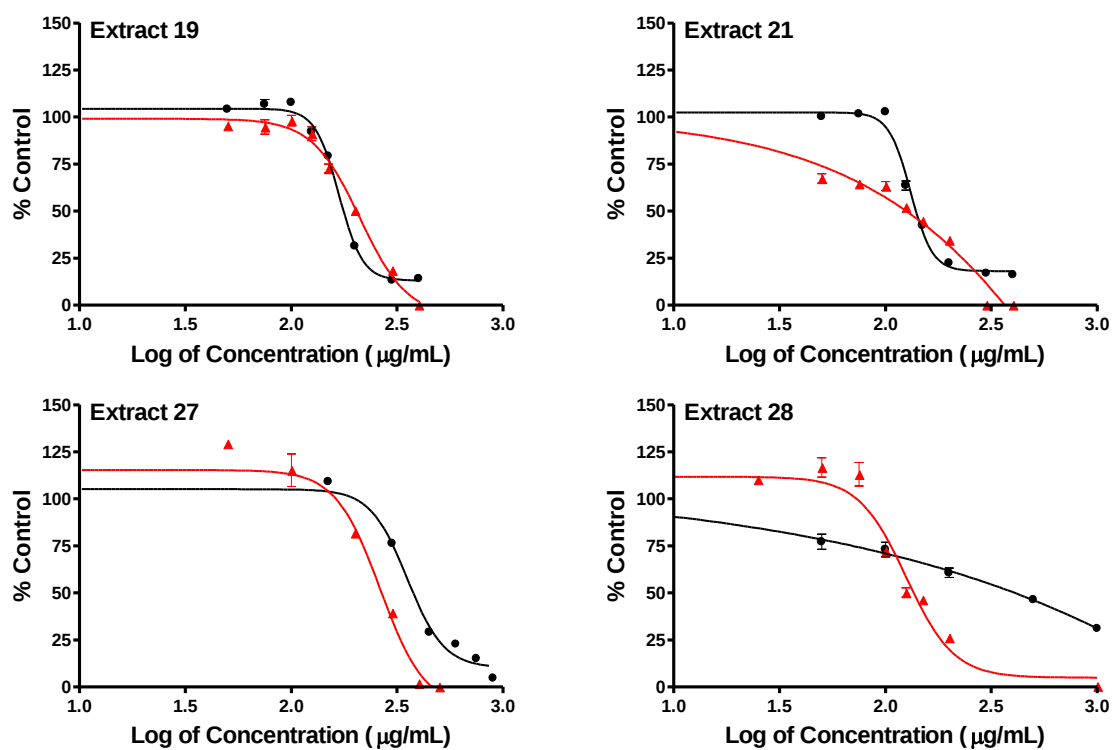


Figure E2 Nonlinear regressions of promising Batch 2 extracts.

Caco-2 (●) and MM253 (▲) cells were exposed to extracts over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values of representative curves (Fig. 4.6) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) according to the 4P method for each extract-cell line combination.

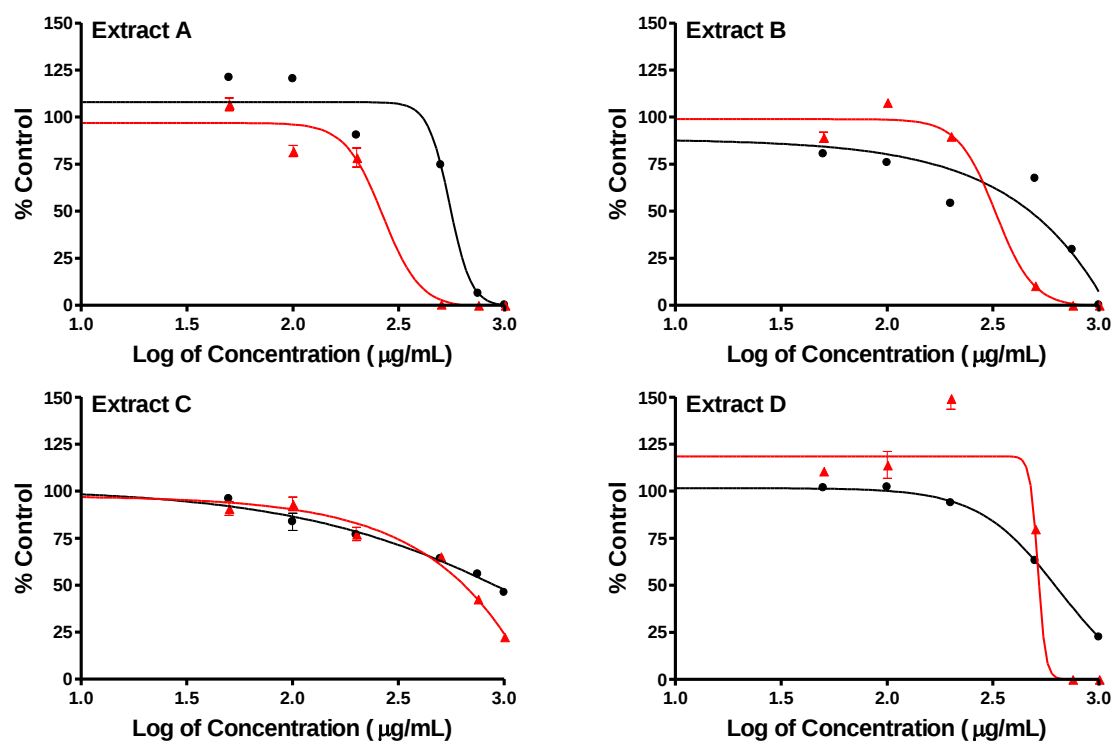


Figure E3 Nonlinear regressions of promising Batch 3 extracts.

Caco-2 (●) and MM253 (▲) cells were exposed to extracts over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values of representative curves (Fig. 4.7) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) according to the 4P method for each extract-cell line combination.

Appendix F. Nonlinear Regressions of Figure 4.8.

Figures F1-4 are the nonlinear regression curves fitted to dose-response data of the six most active crude methanolic plant extracts as described in Chapter 4 (Fig. 4.8). The curve fits of the presented figures are dependent upon which model was used to set the top and bottom parameters.

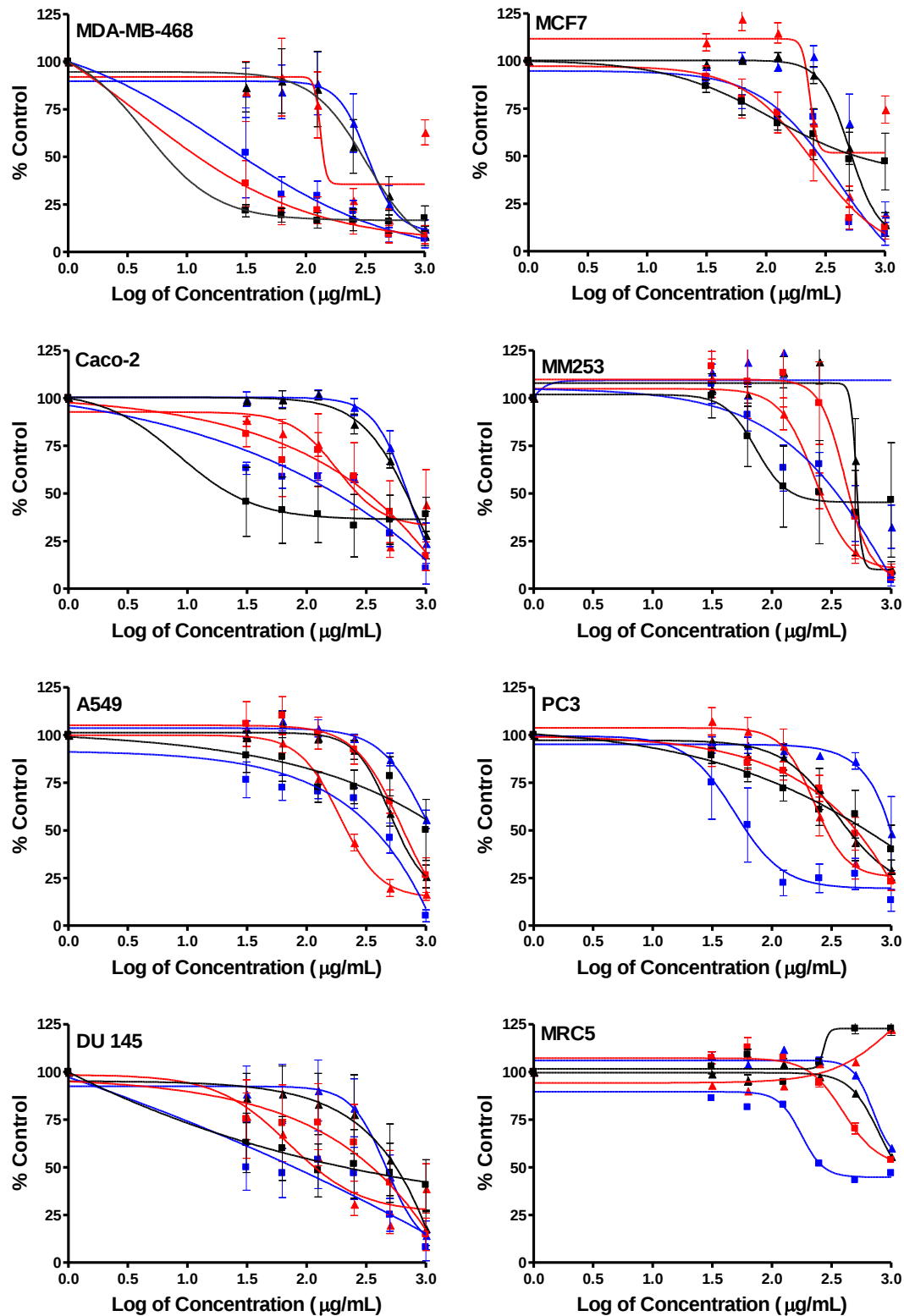


Figure F1 Nonlinear regressions of 6 most active extracts using the 4Parameter (4P) model.

Cell lines were exposed to 6 different extracts, 5 ($\blacktriangle$), 6 ($\blacktriangle$), 8 ($\blacktriangle$), 21 ($\blacksquare$), 27 ($\blacksquare$), 28 ($\blacksquare$), over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values (Fig 4.8) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) with no constraints set.

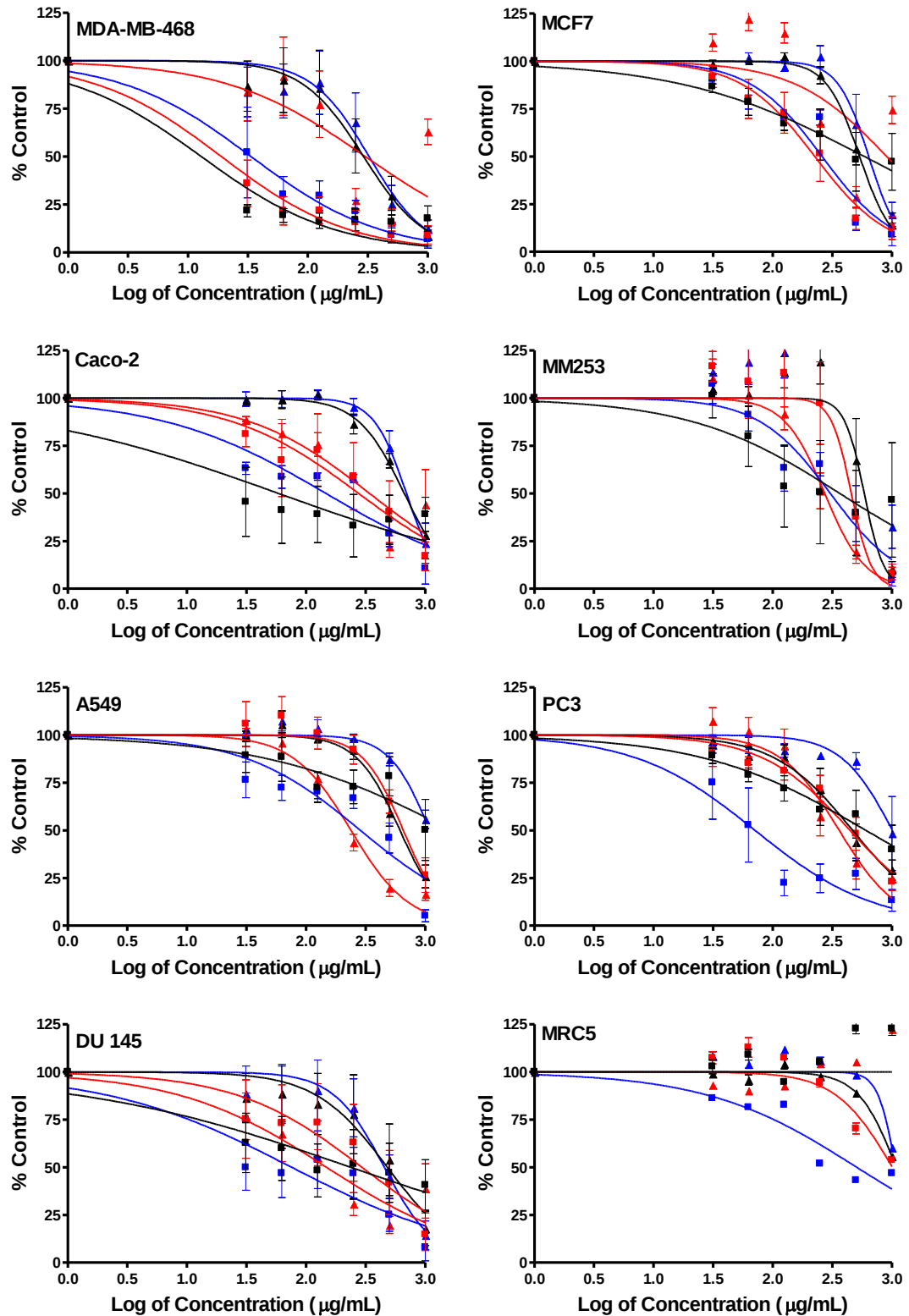


Figure F2 Nonlinear regressions of 6 most active extracts using the Top- and Bottom-fixed (TB) model.

Cell lines were exposed to 6 different extracts, 5 (▲), 6 (▲), 8 (▲), 21 (■), 27 (■), 28 (■), over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values (Fig 4.8) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) with the top parameter set at 100% and the bottom parameter fixed at 0%.

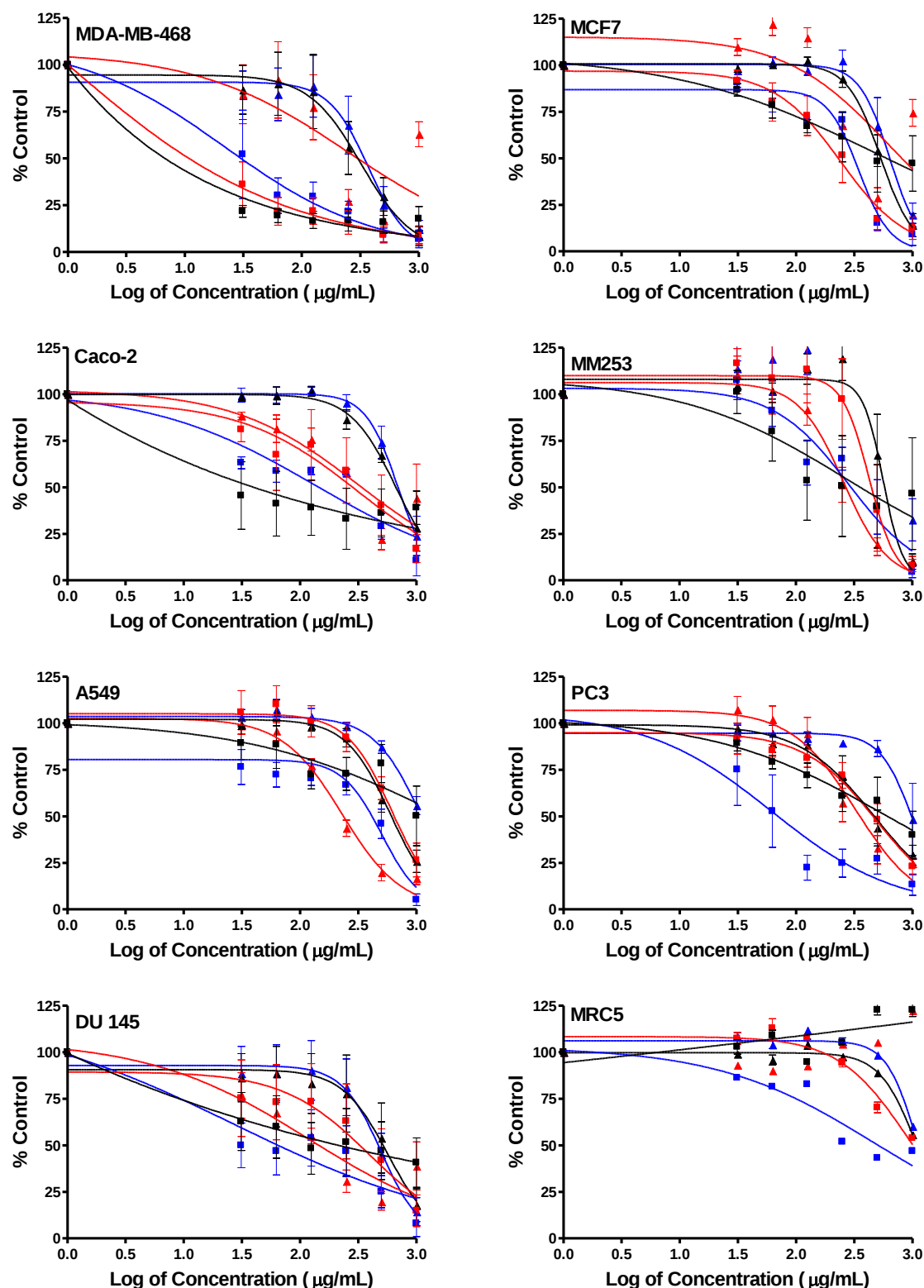


Figure F3 Nonlinear regressions of 6 most active extracts using the Bottom-fixed (B) model.

Cell lines were exposed to 6 different extracts, 5 ($\blacktriangle$), 6 ($\blacktriangle$), 8 ($\blacktriangle$), 21 ($\blacksquare$), 27 ($\blacksquare$), 28 ($\blacksquare$), over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values (Fig 4.8) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) with the bottom parameter set at 0%.

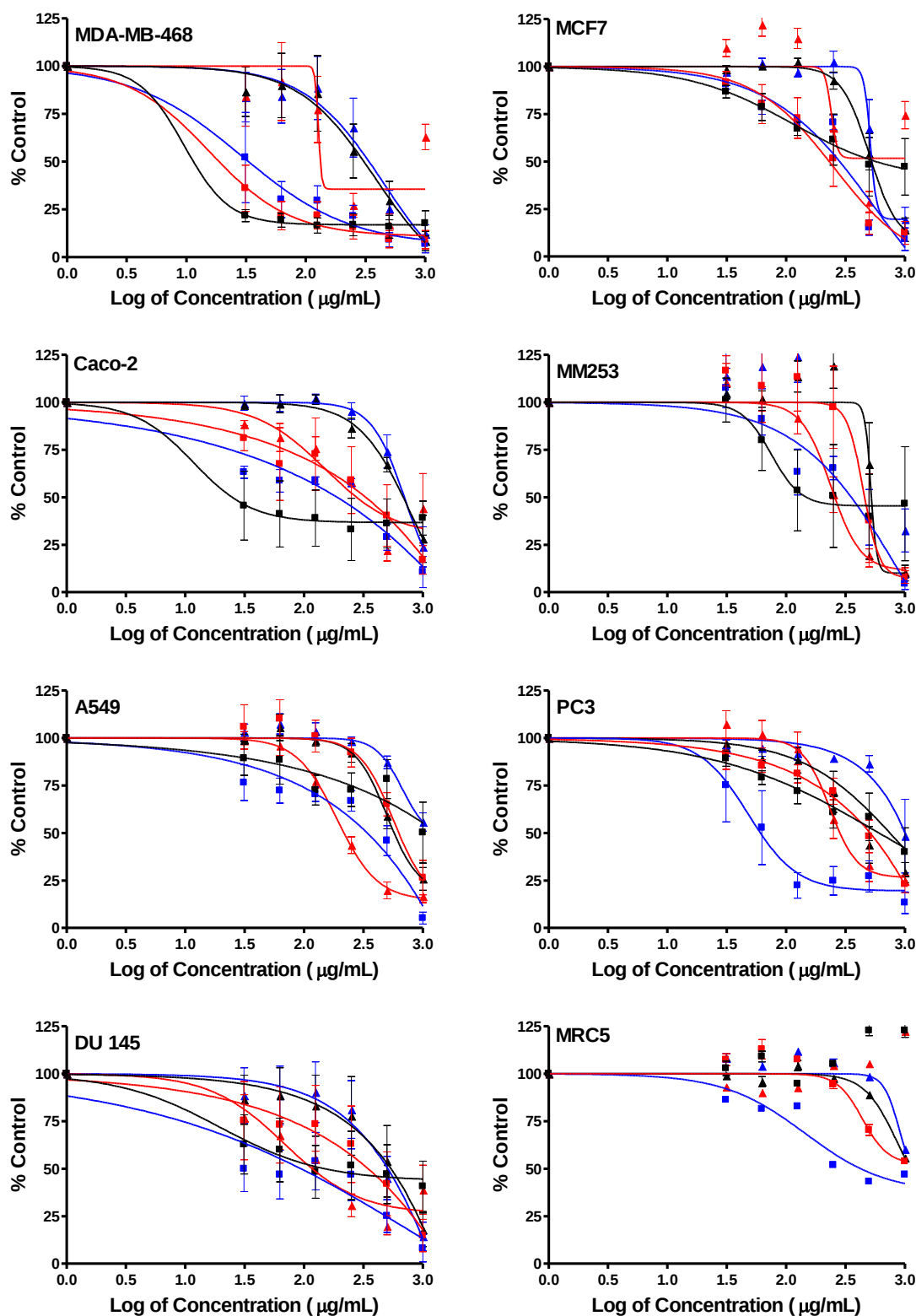


Figure F4 Nonlinear regressions of 6 most active extracts using the Top-fixed (T) model.

Cell lines were exposed to 6 different extracts, 5 ($\blacktriangle$), 6 ($\blacktriangle$), 8 ($\blacktriangle$), 21 ($\blacksquare$), 27 ($\blacksquare$), 28 ($\blacksquare$), over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values (Fig 4.8) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) with the top parameter set at 100%.

Appendix G. HPLC-UV Data

HPLC-UV chromatograms are provided for each methanol and ethyl acetate sample. The first page of a representative report (for EA3 at 350 nm) is presented as Figure G1. For brevity's sake, the remainder of the table listing the retention times, heights and areas of major chromatographic peaks are not presented. To keep it simple, the UV spectra are not shown either. It must be noted that the named compounds are incorrect for every chromatogram, due to an operator error.

To facilitate comparisons between samples, the chromatograms for the methanol fractions of each sample are presented in Figures G2 (254 nm) and G3 (350 nm). Similarly, chromatograms for ethyl acetate fractions of each sample are shown in Figures G4 (254 nm) and G5 (350 nm). UV detection at a wavelength of 254 nm was used for all phenolic compounds, while 350 nm was used for flavonoids.

To highlight the main differences between samples, the major peaks for these chromatograms are summarised in Tables G1 (methanol) and G2 (ethyl acetate). Values are colour-coded as in Chapter 5 to indicate samples that are from the same plant species.

Thus,

Sample 1 is from *Euphorbia drummondii*;

Sample 2 is from *Eremophila sturtii*;

Samples 3 and 4 are from *Eremophila duttonii*; and

Samples 5 and 6 are from *Acacia tetragonophylla*.



SFW Individual Report

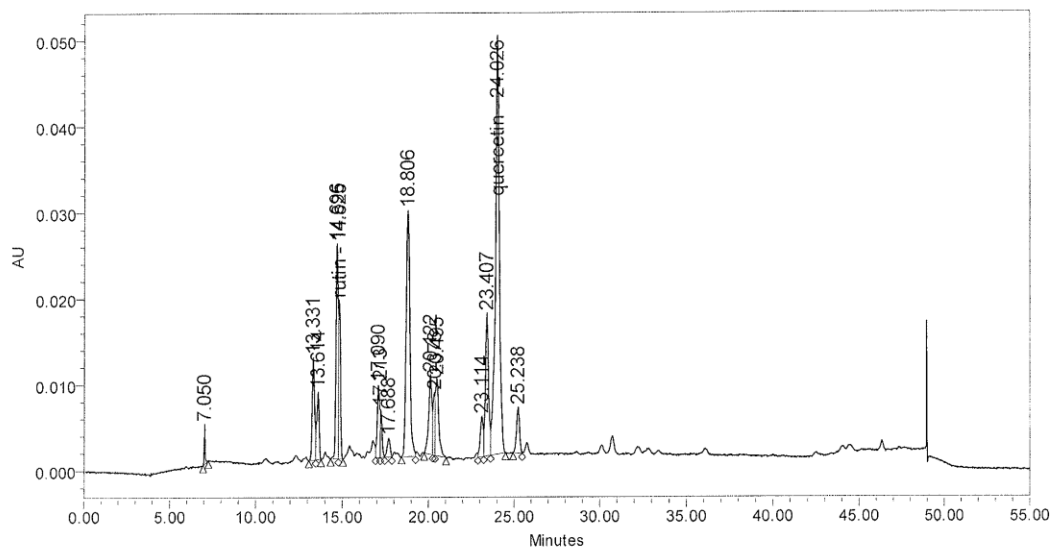
Reported by User: SFWANG (SFWANG)

Project Name: SFWRI

SAMPLE INFORMATION

Sample Name: S3 EtOAc
 Sample Type: Unknown
 Vial: 14
 Injection #: 1
 Injection Volume: 10.00 ul
 Run Time: 55.0 Minutes
 Sample Set Name: SFW100808

Acquired By: SFWANG
 Date Acquired: 11-Jul-08 4:49:10 AM
 Acq. Method Set: SFWFLA_1
 Date Processed: 11-Jul-08 10:00:04 AM
 Processing Method: flavon_quan
 Channel Name: 254
 Proc. Chnl. Descr.: PDA 350.0 nm



	Peak Name	RT	Area	% Area	Height
1		7.050	18873	0.74	4463
2		13.331	117308	4.58	11945
3		13.614	75344	2.94	7877
4		14.696	219510	8.57	25008
5	rutin	14.825	130915	5.11	18545
6		17.090	83831	3.27	8441
7		17.213	38132	1.49	5562
8		17.688	27169	1.06	2201
9		18.806	429620	16.77	28628

Report Method: SFW Individual Report

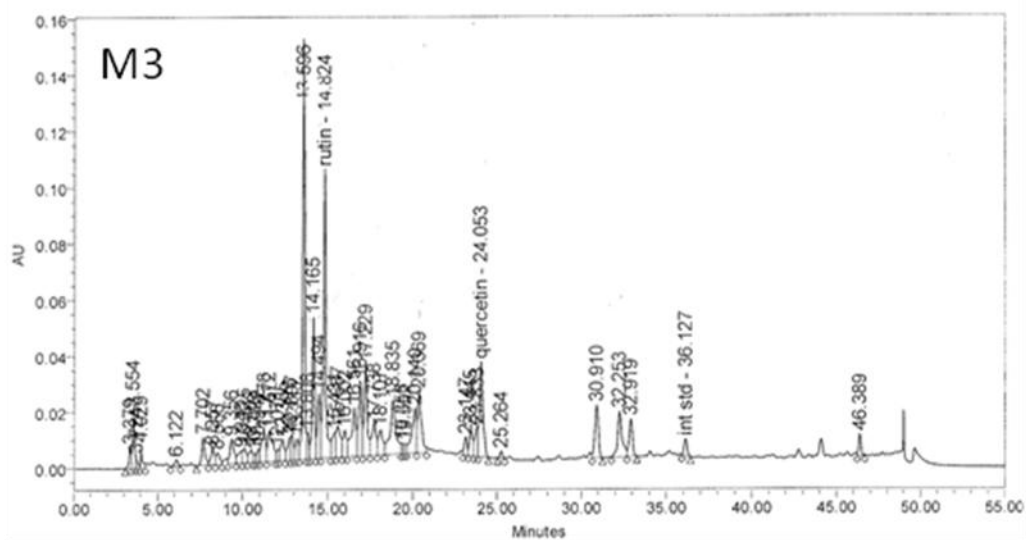
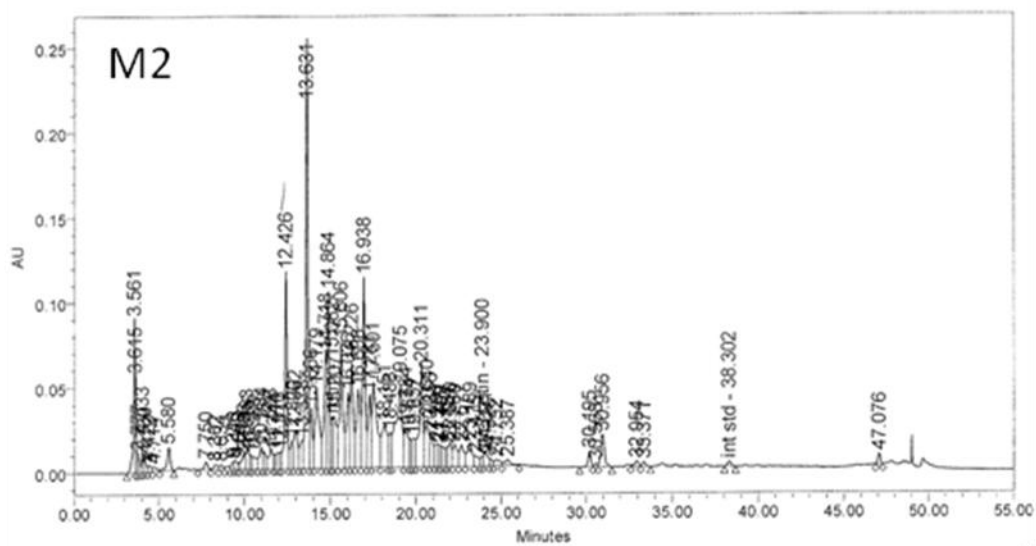
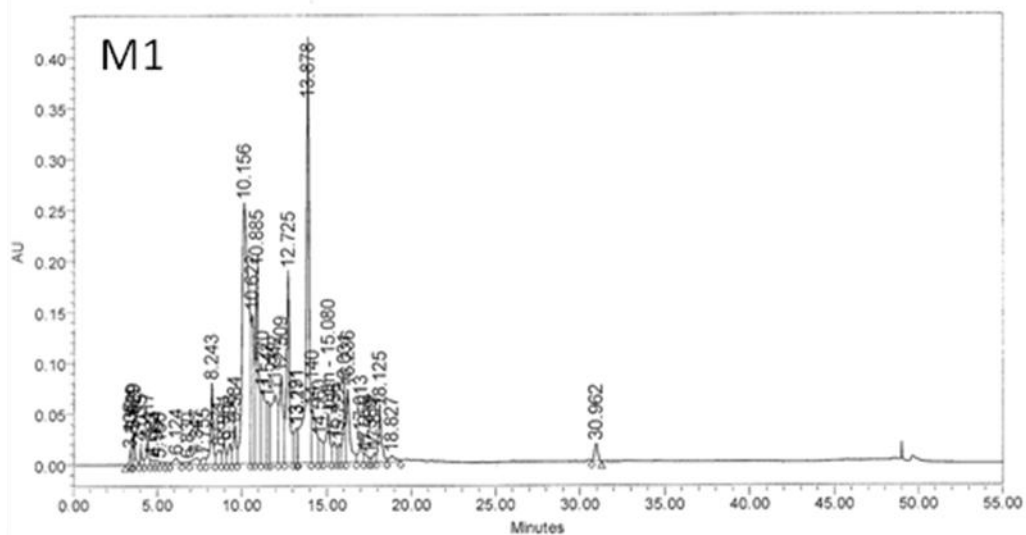
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Page: 1 of 3

Figure G1 Representative HPLC-UV chromatogram report.

The first page of the HPLC-UV chromatogram report for Sample 3 (EA3) at 350 nm.



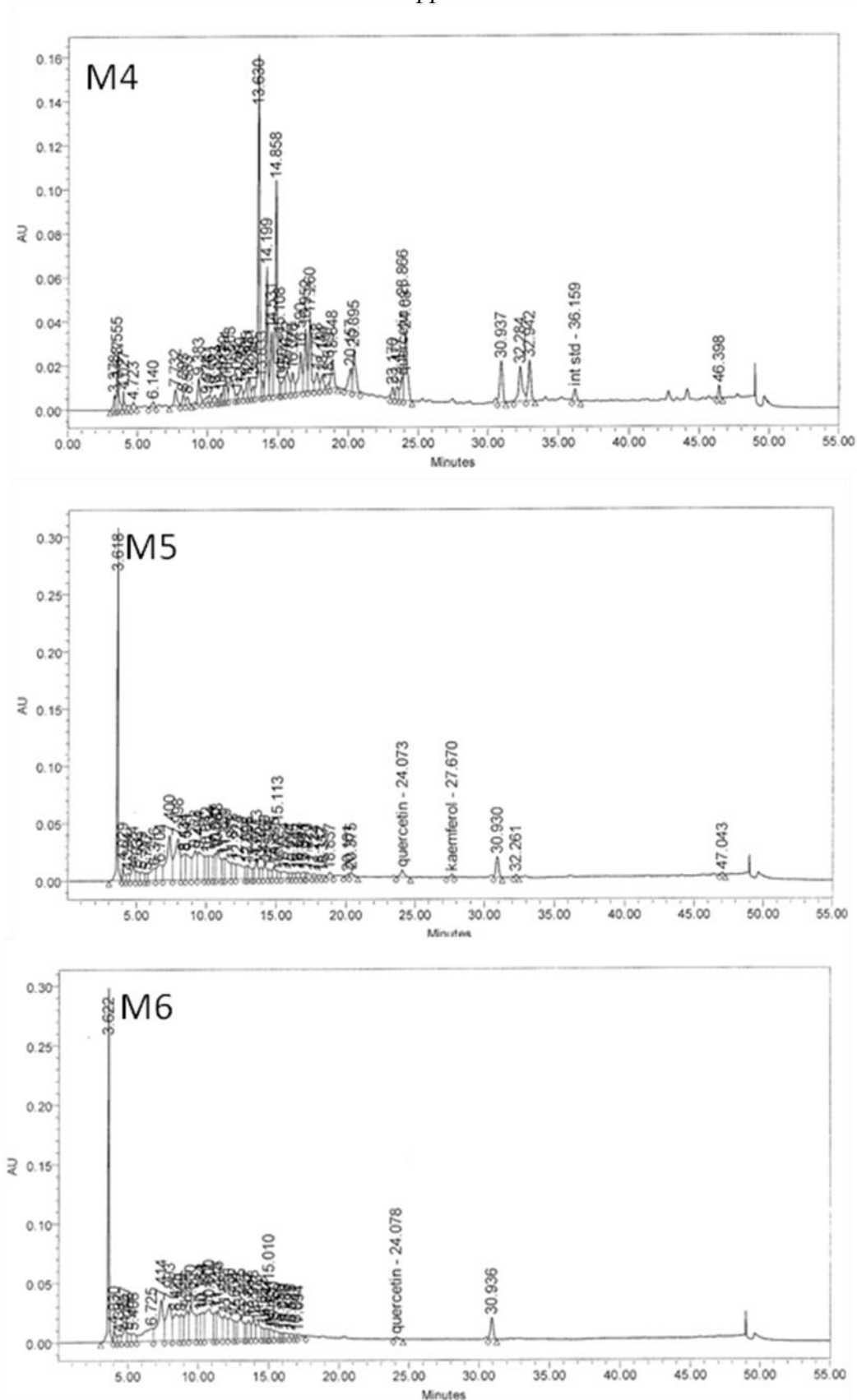
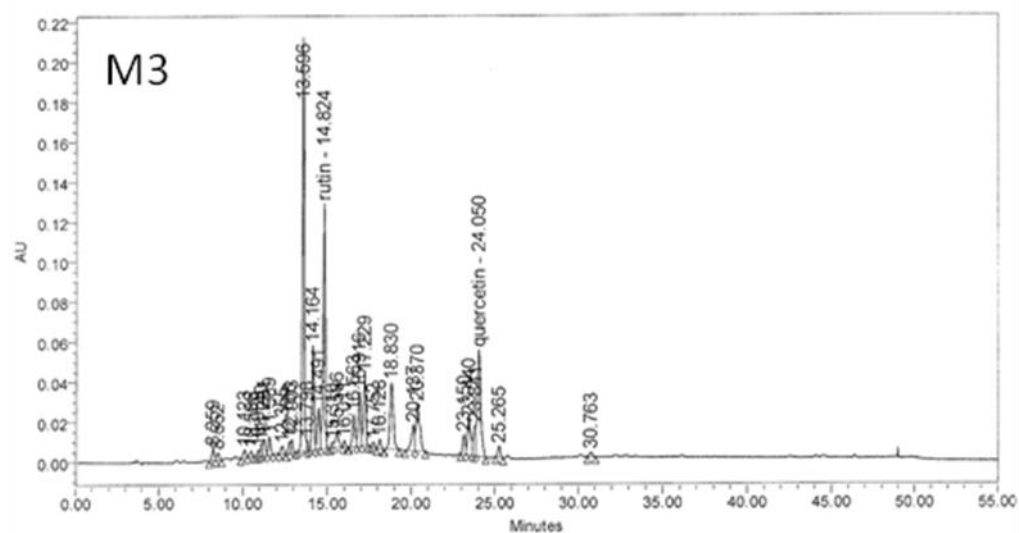
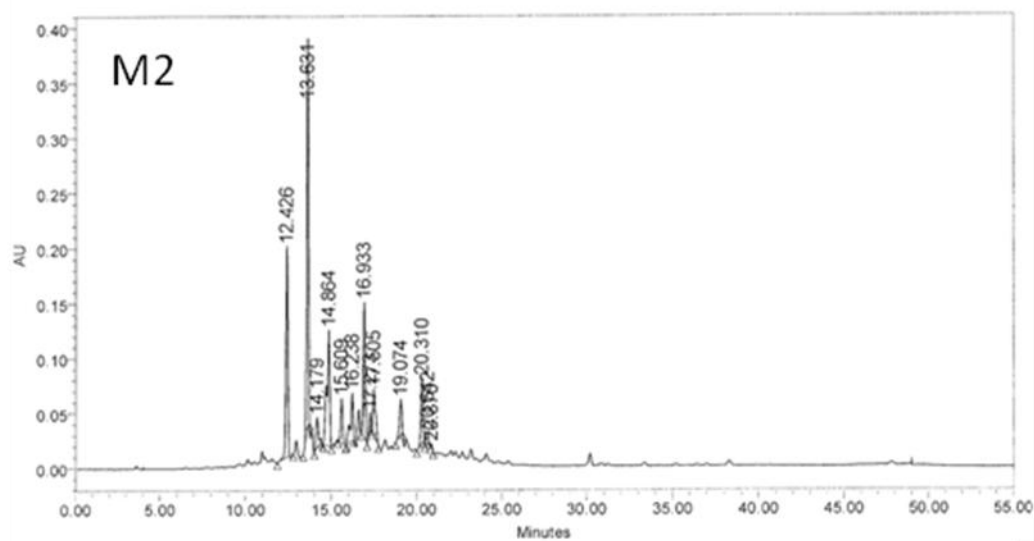
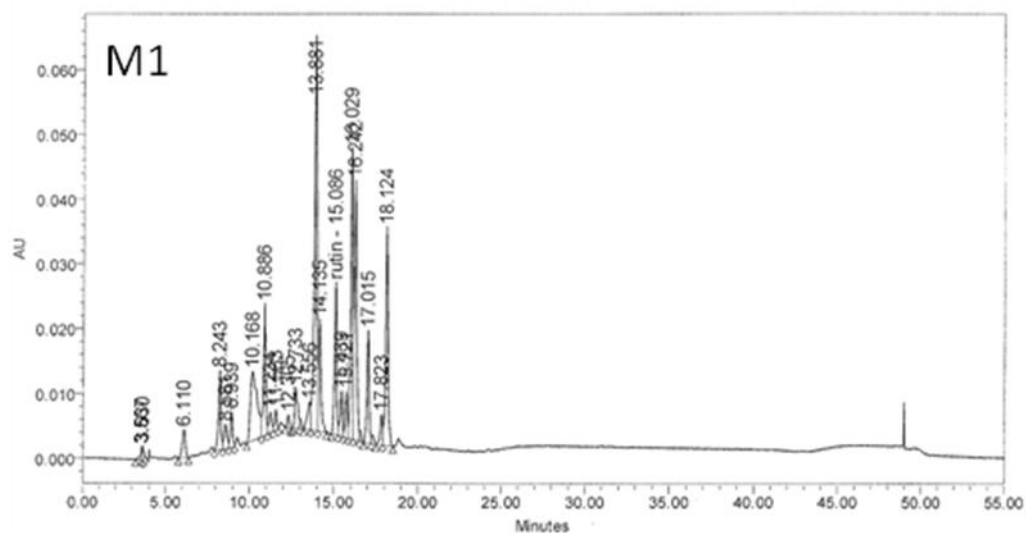


Figure G2 HPLC-UV chromatograms for methanol samples (254 nm).



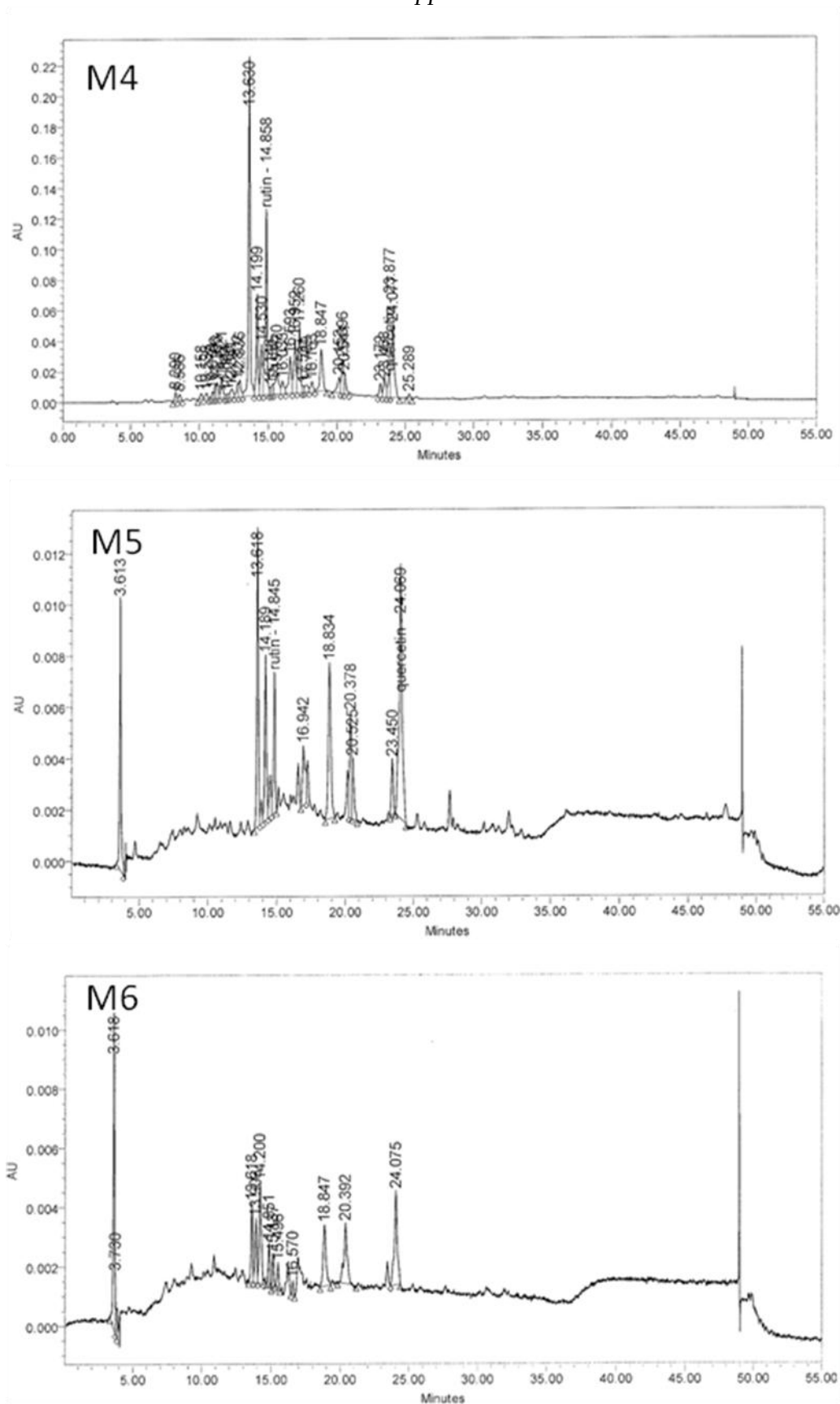
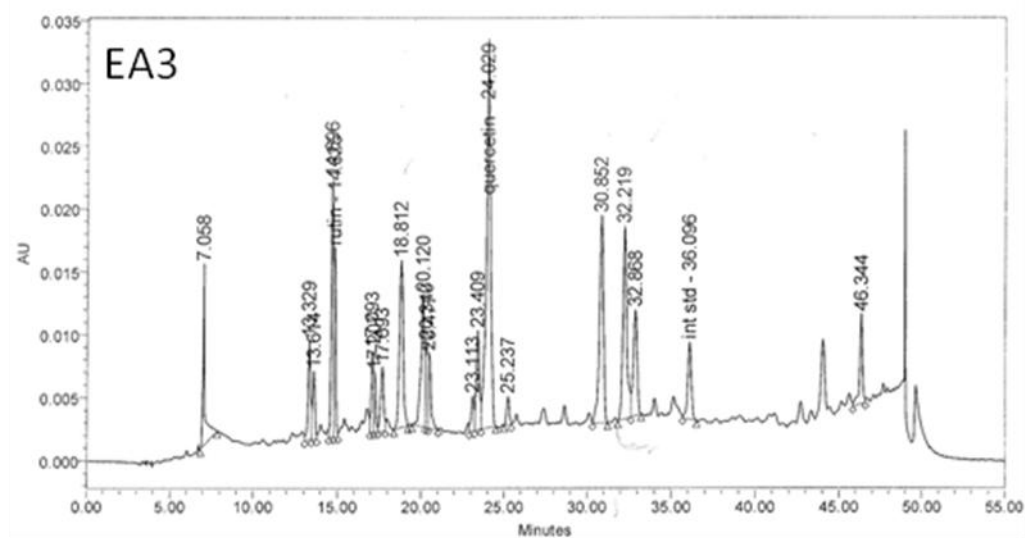
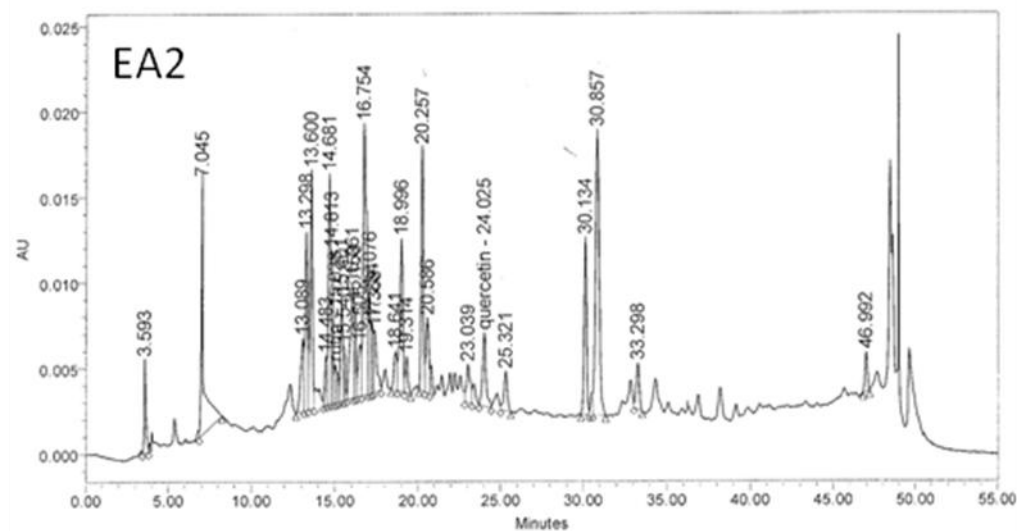
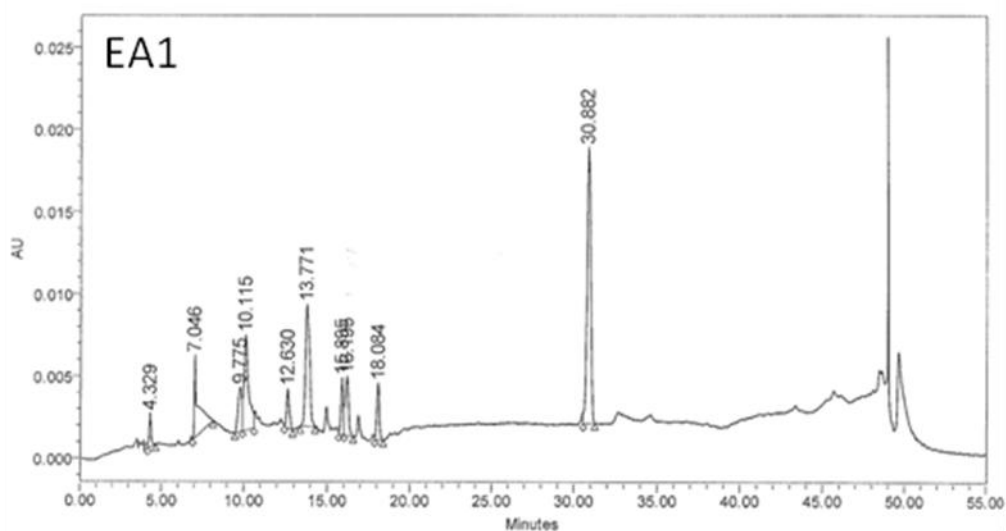


Figure G3 HPLC-UV chromatograms for methanol samples (350 nm).



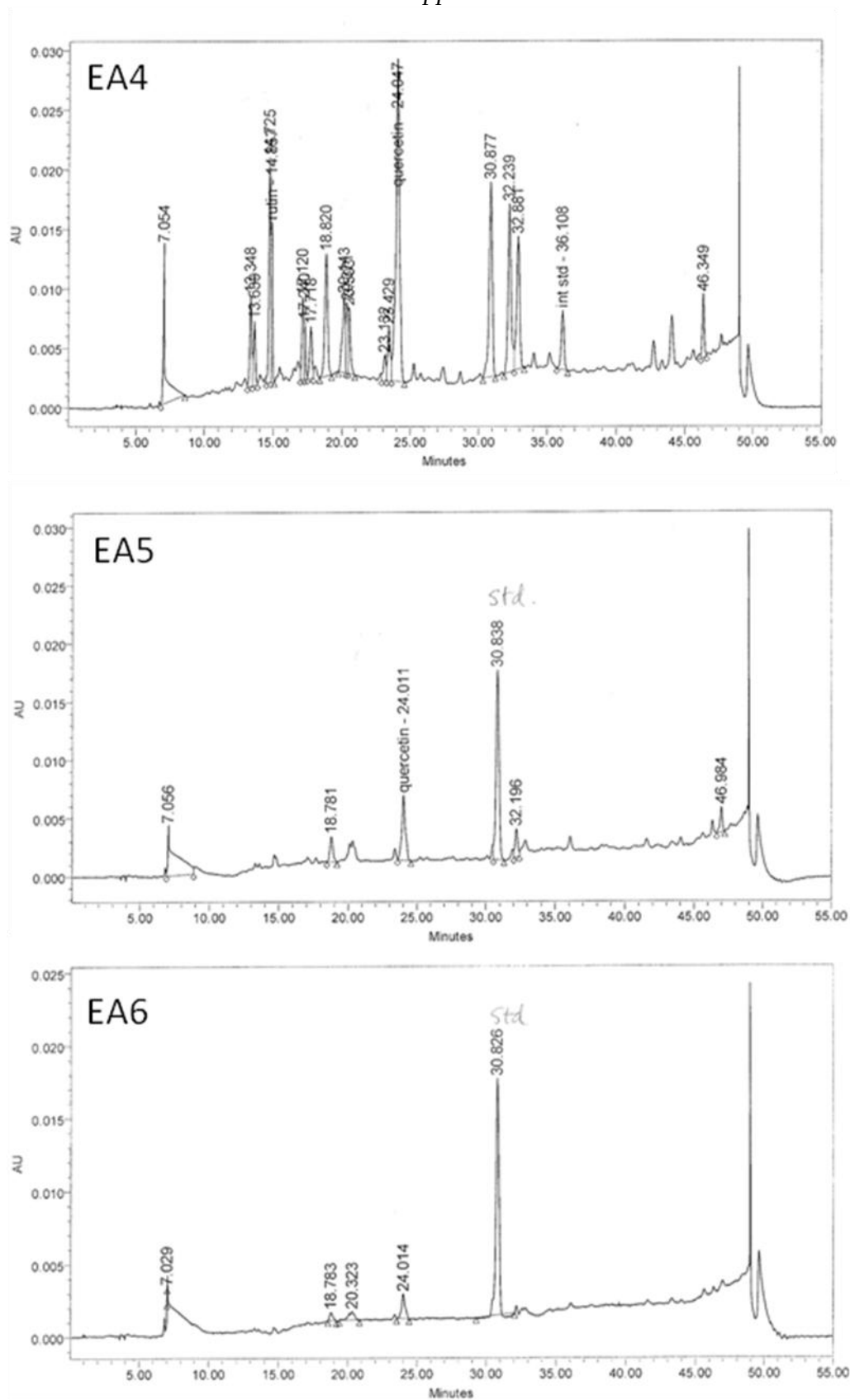
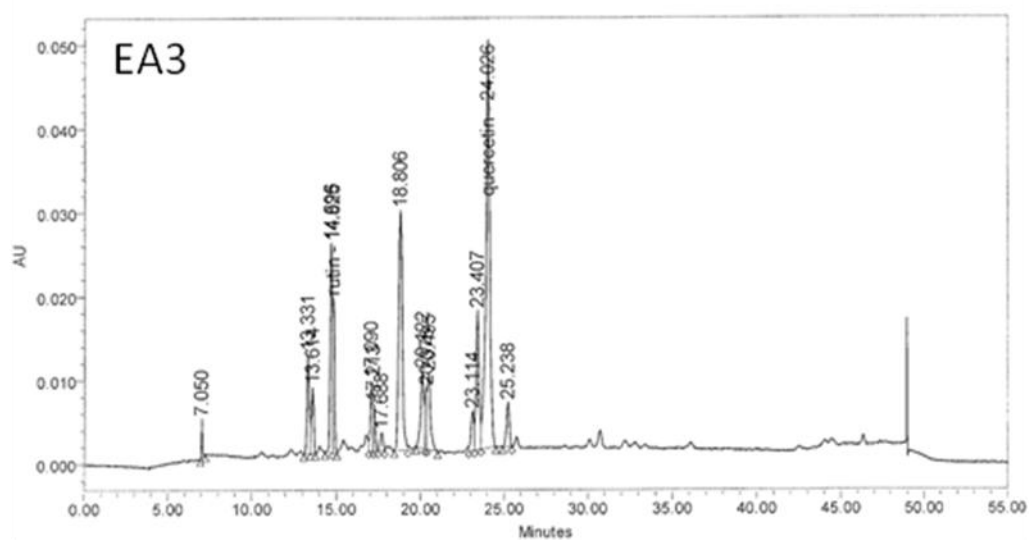
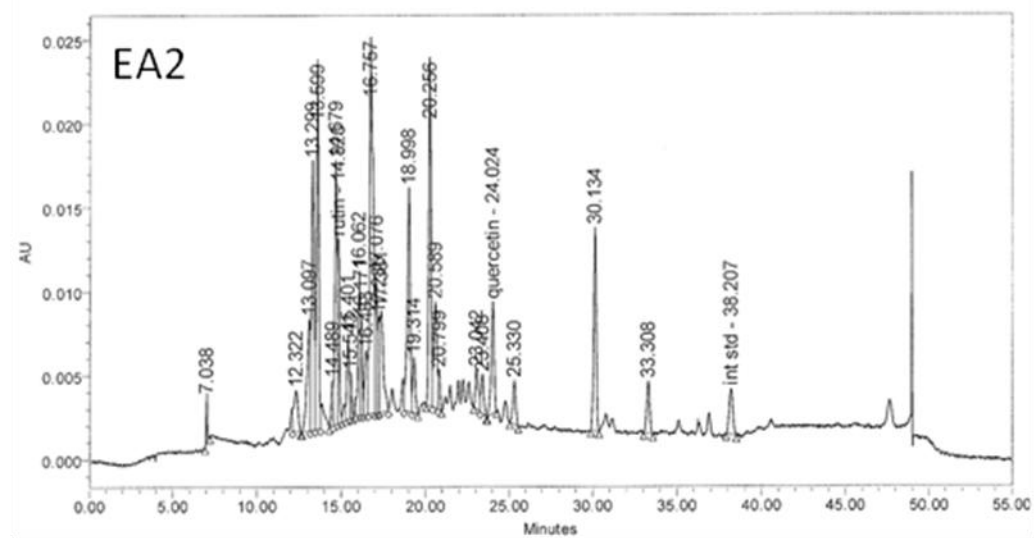
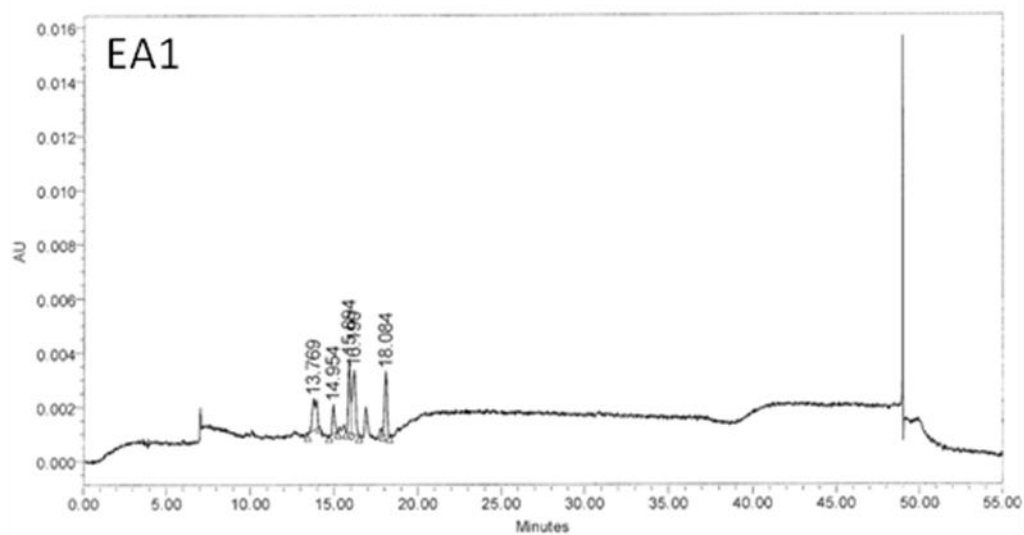


Figure G4 HPLC-UV chromatograms for ethyl acetate samples (254 nm).



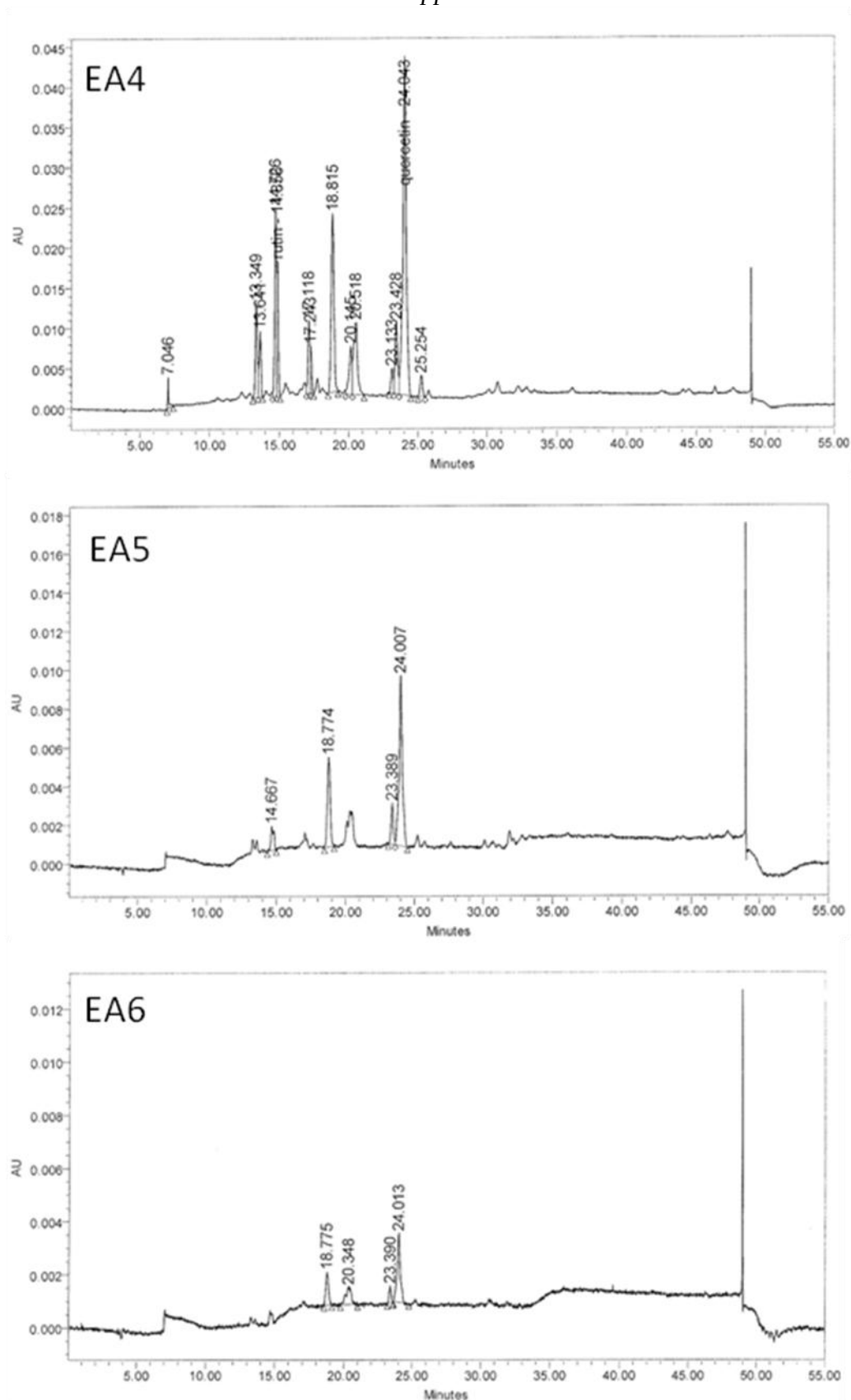


Figure G5 HPLC-UV chromatograms for ethyl acetate samples (350 nm).

Table G1 Major Peaks for Methanol Fractions of Samples 1-6.

MeOH at 254 nm																			
RT (min)	3.62	10.2	12.4	12.7	13.6	13.9	14.2	14.9	16	16.2	16.9	16.9	18.1	18.8	19.1	20.3	24.1	32.3	32.9
	% area																		
S1		20		6.91		15.1													0.69
S2			4.79				3.58	4.15				5.35							1.23
S3					12.9		5.07	10.6			3.39						4.88	3.05	1.87
S4					14.7		6.32	10.9			3.95						5.24	3.33	2.97
S5	14.5																		1.94
S6	13.5																		1.94
correction for standard																			
S1		20.14		6.96		15.20													
S2			4.85				3.62	4.20				5.42							
S3					13.21		5.21	10.85			3.48						5.02	3.13	1.92
S4					15.12		6.52	11.28			4.07						5.40	3.43	3.06
S5	14.79																		
S6	13.75																		
MeOH at 350 nm																			
S1						18.1	4.98		11.2	11.8			9.79						
S2			18.4		30.2			14.8				7.04			3.4	5.55			
S3					22.7		2.92	14.2			3.8			6.43			10		
S4					21.6		6.94	12.9			4.59			4.34			7.58		
S5					14.9		9.42	7.65			5.03			13.2			24.5		
S6					7.21		12.4	3.11						10.3			16.2		

Table G2 Major Peaks for Ethyl Acetate Fractions of Samples 1-6.

EtOAc at 254 nm																						
RT (min)	7.05	9.78	10.1	13.3	13.6	14.7	14.8	15.9	16.2	18.1	16.8	17.4	19	20.1	20.5	20.3	23.4	24	30.1	32.2	36.1	% area
S1	10.9	5.38	14.4	4.79	16.2			4.04	6.57	4.73	10		5.03			5.83		2.71	4.94			32.2
S2	6.91			4.79	6.07	5.81	2.8				10		5.03			5.83		2.71	4.94			11.7
S3	4.26			3	2.04	6.68	4						7.44					19.4		9.09	3.32	10.1
S4	6.32			3.21	2.21	6.82	4.03						6.28					19.8		9.23	2.89	10.9
S5	27.5					7.06							5.49					16.2		5.29		41.2
S6	30.6												2.23				18.9	6.28				60.9
correction for standard																						
S1	16.03	7.93	21.23	7.06	23.87			5.96	9.69	6.97	14.77		7.42			8.60		4.00	7.28			
S2	7.82			5.42	6.87	6.58	3.17				11.35		5.70			6.60		3.07	5.59			
S3	4.74			3.34	2.27	7.43	4.45						8.28					21.61		10.11	3.69	
S4	7.10			3.60	2.48	7.66	4.53						7.05					22.19		10.36	3.25	
S5	46.73					12.01							9.34					27.57		9.00		
S6	78.26												5.71				48.44	16.08				
EtOAc at 350 nm																						
S1					19		9.09	21.9	28.7	21.3												
S2				7.27	8.72	6.34					13.4	3.71	6.77			8.03	1.16	3.38	5.43			
S3				4.58	2.94	8.57	5.11						16.8	4.76	6.87		8.36	30.7				
S4				5.47	3.73	9.56	5.77						14.7	4.08	8.13		5.44	32.9				
S5						5.57							21.4			17.6	7.82	47.7				
S6*													24.1		18.9			57				

*: Ethyl acetate of S6 contains very low flavonoids. The peaks recorded here are very small.

Appendix H. GC-MS Report

07D450
S Wang
(08) 9222 3040

4th Sept. 2008

Faculty of Physics and Life Science
The University of WA
Nedlands, WA 6009

Attention to Ms Donna Savigni

REPORT ON THE ANALYSIS OF FOUR PLANT SAMPLES RECEIVED ON JUNE 2008.

Sample History:

Four plant samples of plant were received for preparation of extract, GC-MS analysis for volatiles, HPLC for chemical profilic analysis and LC-MS for identification of non-volatiles.

Test Method:

In house GC-MS method

Result of Examination:

Samples were extracted with a solvent mixture (hexane: ether 1:1). After filtration of samples, organic solution was concentrated under nitrogen to a small volume of solution. Hexane was added to dissolve residue and the solution was analysed using GC-MS. Volatiles were identified based on their mass spectrometry comparison to standard compounds in GC-MS library.

The results were shown in Table 1, 2 and 3. There are a few points to be noted.

- 1) The further confirmation for big peaks will be done by co-injections of standards with samples once the standards have been purchased.
- 2) The percentages of sample 4 are expressed as area percentage. The area percent for other samples were **accidentally deleted** during processing of gc-ms computer.

- 3) Chemical profiles of volatiles from samples 3 and 4 are very similar. A number of mono-terpene and sesquiterpene were found in volatiles. The major compounds are α -pinene and guaiol. In addition, other monoterpene including camphene, β -pinene and m-cymol were detected in the samples. Sesquiterpene such as bulnesol, α -caryophyllene, β -caryophyllene, α -eudesmol, β -eudesmol and spathulenol were major constituents of volatiles.
- 4) The major volatile in sample 2 is elemol greater than 80%. In addition, a few sesquiterpenes were found in this sample, including caryophyllene, elemol, γ -eudesmol, α -eudesmol and β -eudesmol.
- 5) Major volatile constituents found in sample 1 were elemol, phytol, caryophyllene, and a group of compounds, which have similar mass spectrometry.
- 6) Some suggestions:
- a) These compounds were presented in dichloromethane extracts, if the dichloromethane extracts show activity, it would be testing the following compounds: α -pinene, β -pinene, guaiol, elemol, α -caryophyllene and β -caryophyllene.
- b) In addition, it may be worthwhile to test these volatiles including monoterpene camphene, and m-cymol and sesquiterpene such as bulnesol, α -, α -eudesmol, β -eudesmol and spathulenol if you have time.

Please see table for more analytical details.

This report relates specifically to the sample as received.

N E ROTHNIE
CHIEF
FOOD AND BIOLOGICAL CHEMISTRY

S F Wang
Senior Chemist and Research Officer

Note. Tables H1 to H3 have not been modified from the originals, except to reduce the column widths to fit on the page and to prefix the table name with an “H” and rename them so that their order of presentation is in keeping with the numbering system of the samples. Nothing else was adjusted from the original report, including spelling mistakes. The highlighting in the report was not explained by the authors and could not be removed. GC-MS was not performed on Samples 5 and 6.

Table H1 Identification of volatiles of sample 1 using GC-MS analysis

Sample 1					
RT (min)	Name	Formula	Mt	peak	
7.344	3,4-dimethyl-2-pentane ?	C ₇ H ₁₄ O	114?	72	
7.455*	unknown		170?	84	
7.546*	unknown			84	
8.134*	unknown			84	
8.233*	unknown			84	
8.476	unknown			58	
8.719*	unknown			84	
8.87*	unknown			84	
9.24	unknown		140	85, 57	
9.94**				73	
10.61	3-methyl-3-hexanol	C ₇ H ₁₆ O	116	73	
12.296**	unknown			73	
14.25*	unknown		184?	84	
17.703	(E,E),2,4-hexadienal		96	81	
18.98	acetic acid		60	60	
23.64	caryophyllene	C ₁₅ H ₂₄	204	204	
24.09	unknown		204	88	
25.07	unknown				
36.15	elemol	C ₁₅ H ₂₆ O		93.59	
44.96	phytol	C ₂₀ H ₄₀ O	296	71	
49.18	unknown				

*: mass spectroemtry patter similar;
 **: Mass spectrometry patter similar.

Table H2 Identification of Volatiles of Sample 2 Using GC-MS

Sample 2					
RT (min)	Name	Formula	Mt	peak	
10.855	β-terpinyl acetate	C ₁₂ H ₂₀ O ₂	196	68	
23.55	carpophyllene	C ₁₅ H ₂₄	204	93,134	
25.63	α-carpophyllene	C ₁₅ H ₂₄	204	93	
26.73	unknown				
35.18	elemol	C ₁₅ H ₂₆ O	222		
36.84	elemol	C ₁₅ H ₂₆ O	222		
37.841	γ-eudesmol	C ₁₅ H ₂₆ O	222		
38.504	bulnesol ?	C ₁₅ H ₂₆ O	222		
38.668	α-eudesmol	C ₁₅ H ₂₆ O	222		
38.804	β-eudesmol	C ₁₅ H ₂₆ O	222		
40.73	unknown				
40.94	unknown		220		
45.95	unknown		204		

Table H3 Identification of volatiles of Sample 3 and 4 using GC-MS.

RT (min)	Name	Formula	Mt	Sample 3		Sample 4
				peak	%	%
5.4	unknown	C ₁₀ H ₁₆	136	93		1.02
5.8	α -pinene	C ₁₀ H ₁₆	136	93		14.2
6.6		C ₁₀ H ₁₆	136	57		<0.1
6.8	camphene	C ₁₀ H ₁₆	136	93		0.3
7.35	3-methyl,2-hexanone	C ₇ H ₁₄ O	114	43		0.2
7.497*	unknown			84		<0.1
7.57*	unknown			84		<0.1
7.95	β -pinene	C ₁₀ H ₁₆	136	93		<0.1
8.53	cis-verbenol	C ₁₀ H ₁₆ O	152			0.1
9.275	δ -carene	C ₁₀ H ₁₆	136			<0.1
9.789	unknown	C ₁₀ H ₁₄	136	73		0.1
10.855	δ -terpinyl acetate	C ₁₂ H ₂₀ O ₂	196	68		0.5
12.168	unknown			73		2.1
12.765	cis-ocimene	C ₁₀ H ₁₆	136	93		0.1
13.28	m-cymol	C ₁₀ H ₁₄	134	119		0.3
18.778	isopropenyl toluene	C ₁₀ H ₁₂	132	117		<0.1
19.17	acetic acid					2.4
20.305	copaene	C ₁₅ H ₂₄	204			<0.1
21.28	unknown		204	162		0.1
21.473	α -gurijunene	C ₁₅ H ₂₄	204	204		<0.1
22.375	unknown					<0.1
22.638	unknown		204	105, 56		<0.1
23.39	unknown		204	93		<0.1
23.51	caryophyllene	C ₁₅ H ₂₄	204	133, 93		0.7
23.67	1H-cyclopropa[a]naphthalene	C ₁₅ H ₂₄	204	161,		<0.1
23.89	valencene	C ₁₅ H ₂₄	204	107		<0.1
24.03	eremophilene	C ₁₅ H ₂₄	204	161,		<0.1
24.461		C ₁₅ H ₂₄	204	73		<0.1
25.573	α -caryophyllene	C ₁₅ H ₂₄	204	93		0.8
25.76	β -caryophyllene	C ₁₅ H ₂₄	204	93		0.4
26.005	related to m-cymol		152	119		0.3
26.35		C ₁₅ H ₂₆ O ₂	238			<0.1
26.466	unknown					0.1
26.721	unknown	C ₁₅ H ₂₄	204	161,		<0.1
27.06	unknown	C ₁₅ H ₂₄ O	220	149		0.3
27.41	γ -elemene	C ₁₅ H ₂₄	204	121		0.06
28.145	δ -cadinene	C ₁₅ H ₂₄	204	161		0.08

Table H3 (continued) Identification of volatiles of sample 3 and 4 using GC-MS.

RT (min)	Name	Formula	Mt	peak	Sample 3	sample 4
					%	%
28.383						0.1
29.342	unknown		220	94		0.1
29.914	unknown		222	189, 207		<0.1
30.124	calamenene		202	159		0.07
	ethanone,1-(5,6,7,8-tetrahydro- 2,8,9-trimethyl-4H-					
31.075	cyclohepta[b]furan-5-yl	C ₁₄ H ₂₀ O ₂	220	205		<0.1
32.97	dendrolasin	C ₁₅ H ₂₂ O	218	69,81		
33.86	β-caryophyllene	C ₁₅ H ₂₄ O	220	79		0.3
34.199	unknown	C ₁₅ H ₂₄ O	220	93		
34.466	unknown					0.3
	12-oxabicyclo[9.1.0]dodeca-3,7- dine,1,5,5,8-tetramethyl-[1R- (1R*,3E,7E,11R*)]					
35.112		C ₁₅ H ₂₄ O		109		<0.1
36.1	unknown	C ₁₅ H ₂₄	204	93		0.1
36.39	Guaiol	C ₁₅ H ₂₆ O	222	161		2.1
36.97	spathulenol	C ₁₅ H ₂₄ O	220	205		0.16
38.088	tau-muurorol	C ₁₅ H ₂₆ O	222	161,95		0.1
38.446	bulnesol	C ₁₅ H ₂₆ O	222	107		1
38.668	α-eudesmol	C ₁₅ H ₂₆ O	222	59		0.4
38.751	β-eudesmol	C ₁₅ H ₂₆ O	222	149, 59		0.63
42.179	bohlmann k3089	C ₁₅ H ₂₀ O ₃	248	83		1.5
	unknown	C ₁₅ H ₂₀ O ₃	248	83		2
	Long chain					
44.28	carbohydratetetratetracontane	C ₄₄ H ₉₀	620	57		44.8
46.61	Unknown		282	151		2.6
46.31	unknown		282	151		2.41
48.295	unknown					3.9
49.01	unknown					8.65

Appendix I. Nonlinear regression of Figure 5.3

Figures G1-4 are the nonlinear regression curves fitted to dose-response data of the eight most active plant extracts as described in Chapter 5 (Fig. 5.3). The curve fits of the presented figures are dependent upon which model was used to set the top and bottom parameters.

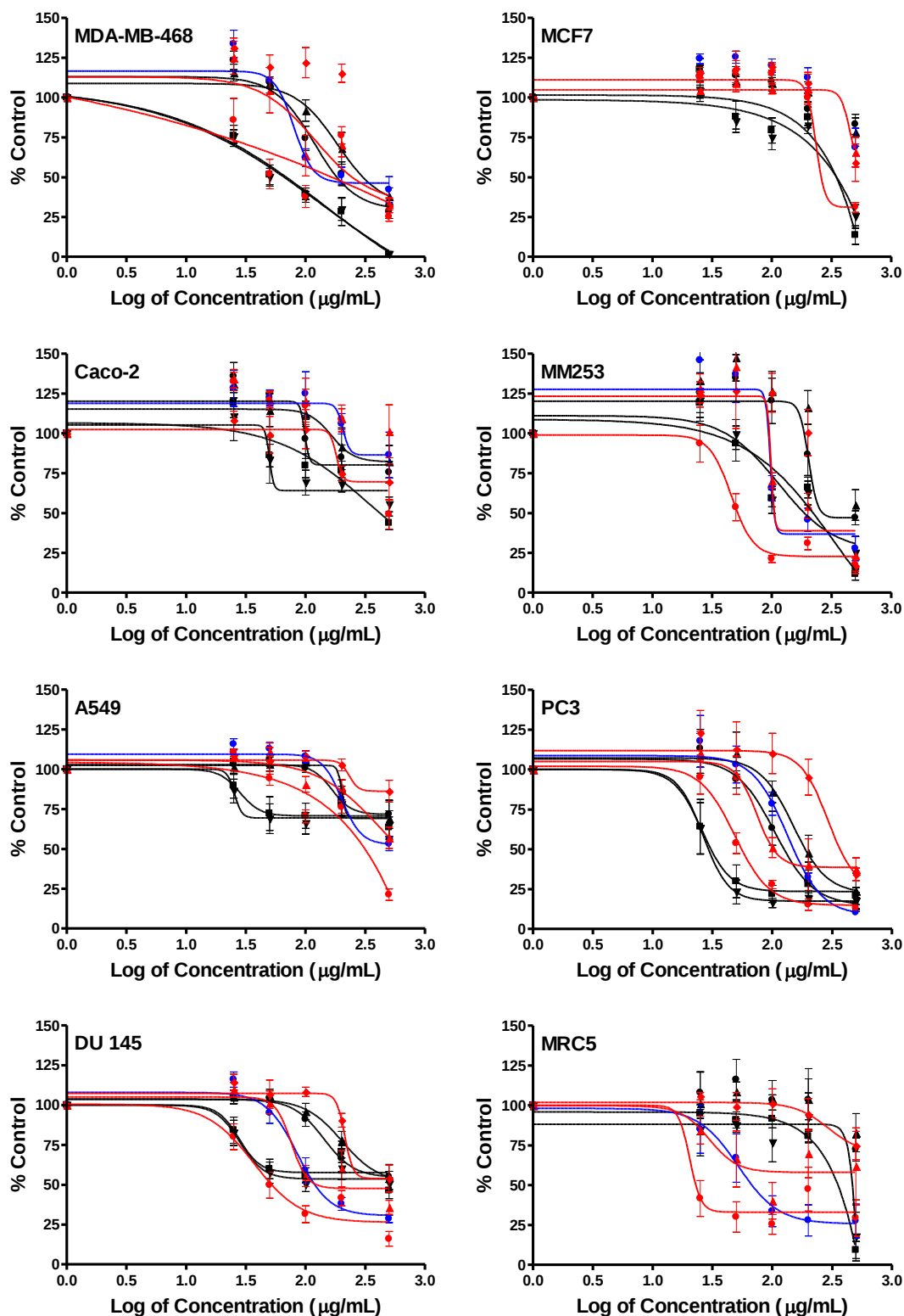


Figure I1 Nonlinear regression of the 8 most active extracts using the 4 Parameter (4P) model.

Cell lines were exposed to 8 different extracts, EA3 (■), EA4 (▼), EA5 (●), EA6 (▲), A5 (●), M1 (◆), M5 (●), M6 (▲), over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values (Fig 5.3) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) with no constraints set.

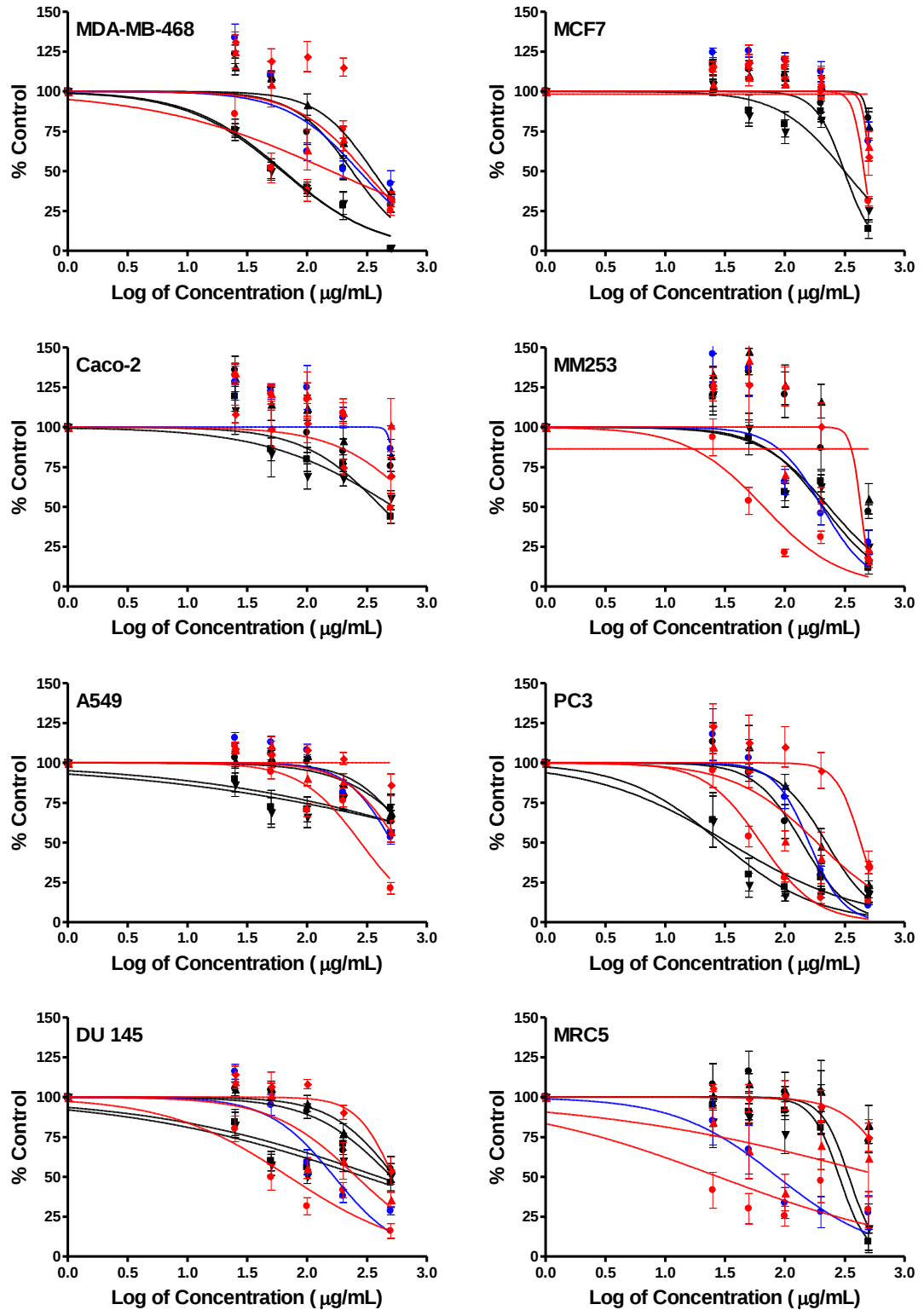


Figure I2 Nonlinear regression of the 8 most active extracts using the Top- and Bottom-fixed (TB) model.

Cell lines were exposed to 8 different extracts, EA3 (■), EA4 (▼), EA5 (●), EA6 (▲), A5 (●), M1 (◆), M5 (●), M6 (▲), over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values (Fig 5.3) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) with the top parameter set at 100% and the bottom parameter fixed at 0%.

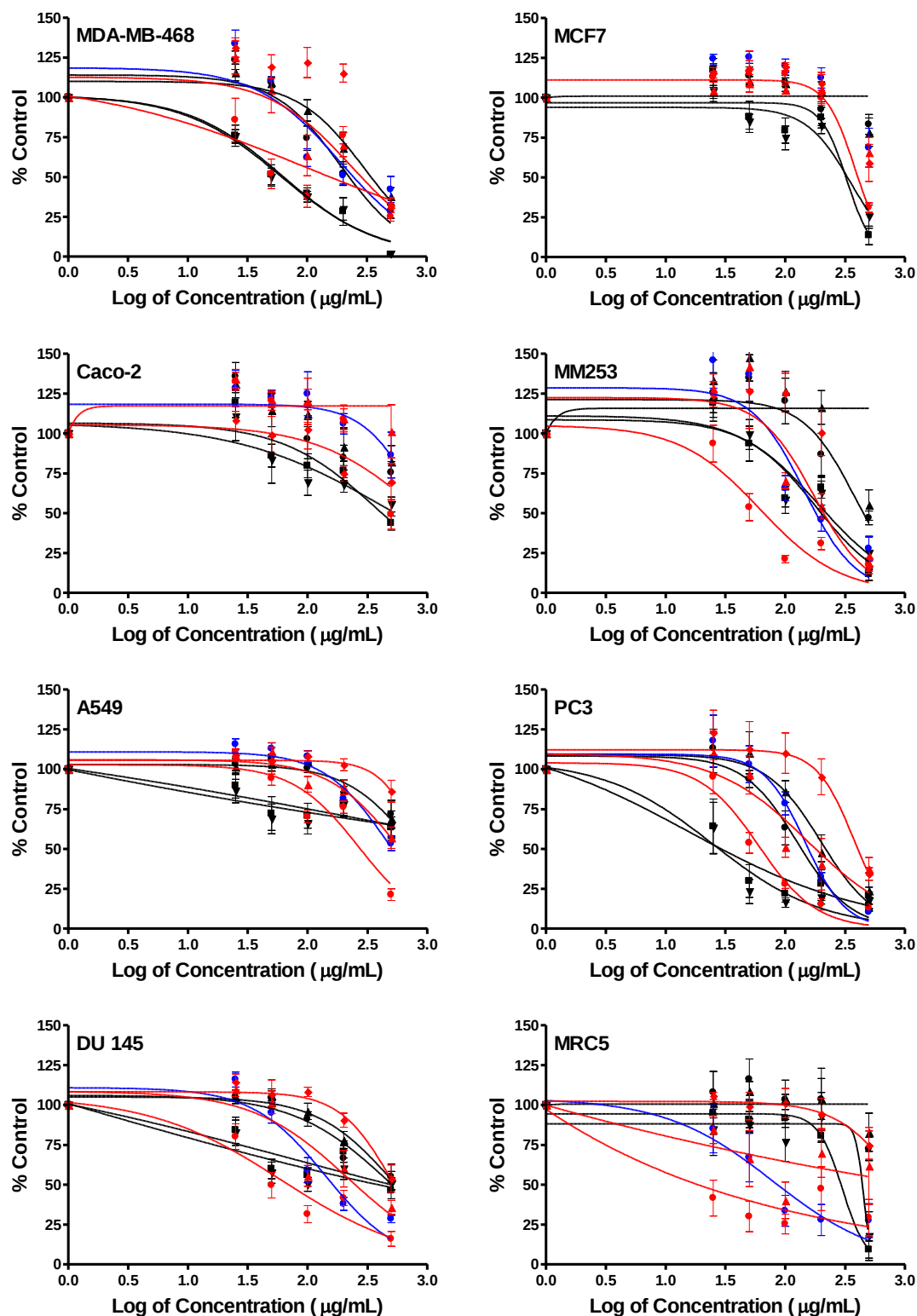


Figure I3 Nonlinear regression of the 8 most active extracts using the Bottom-fixed (B) model.

Cell lines were exposed to 8 different extracts, EA3 (■), EA4 (▼), EA5 (●), EA6 (▲), A5 (●), M1 (◆), M5 (●), M6 (▲), over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values (Fig 5.3) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) with the bottom parameter set at 0%

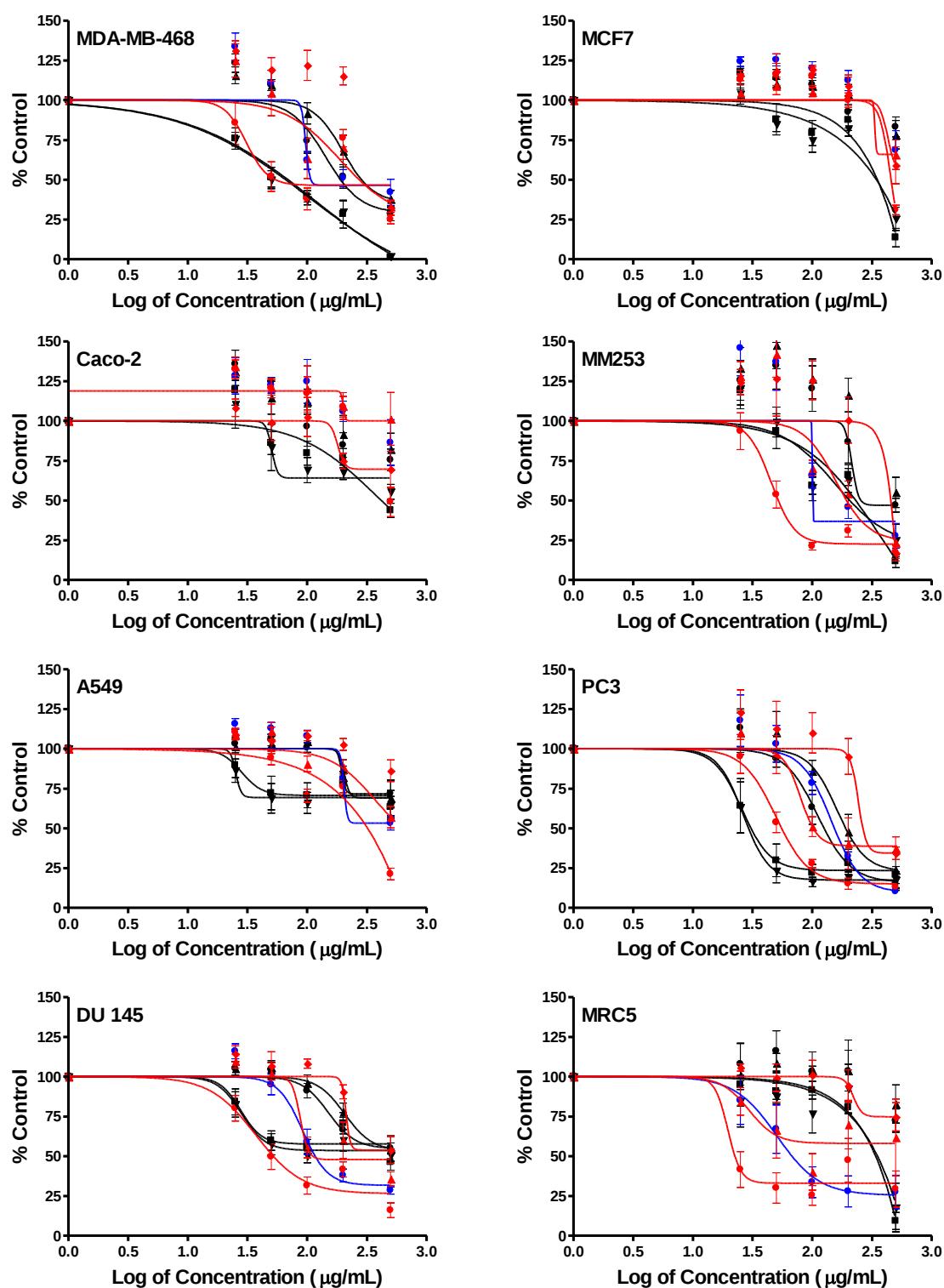


Figure I4 Nonlinear regression of the 8 most active extracts using the Top-fixed (T) model.

Cell lines were exposed to 8 different extracts, EA3 (■), EA4 (▼), EA5 (●), EA6 (▲), A5 (●), M1 (◆), M5 (●), M6 (▲), over a range of concentrations for 48 h before being subjected to the MTT assay. Dose values (Fig 5.3) were transformed into $\log_{10}$ values and curves were fitted using GraphPad Prism nonlinear regression dose-response (variable slope) with the top parameter set at 100%.

Appendix J. Ca^{2+} Signalling

Underlying the speed and effectiveness of Ca^{2+} signalling is the more than 20,000-fold difference in concentration between intracellular Ca^{2+} ($\sim 100 \text{ nM}$) and extracellular Ca^{2+} (2 mM) levels (Clapham, 2007). The concentration of Ca^{2+} inside the ER can be up to 10 mM (Meldolesi & Pozzan, 1998), while mitochondria can contain up to 0.5 mM (Clapham, 2007). Cells maintain this steep Ca^{2+} gradient by actively pumping any excess Ca^{2+} out of the cell or into the intracellular storage organelles (Campbell, 1990). Thus, even a minor increase in the permeability of the plasma membrane or a small release of Ca^{2+} from an intracellular store will cause a very large rise in $[\text{Ca}^{2+}]_i$ and either activate signalling processes within the cell or injure it if $[\text{Ca}^{2+}]_i$ remains too high for too long (Campbell, 1990). Cytosolic Ca^{2+} buffering also significantly impacts on Ca^{2+} signalling, but this is not discussed here as Ca^{2+} bound to proteins is not detectable using the Fura-2 ratiometric method employed.

Several mechanisms exist by which small transient increases in Ca^{2+} are introduced into the cytosol for signal transduction purposes. Ca^{2+} enters the cell from the two largest sinks, the extracellular space and the ER, via specialised ion channels (Clapham, 2007). The two primary types of channels involved are voltage-operated Ca^{2+} channels (VOCs), in which the gating depends on voltage, and ligand-gated, or receptor-operated Ca^{2+} channels (ROCs) (Carafoli *et al.*, 2001). When a cell is activated, these channels open and approximately 10^6 ions/s/channel flow down the intracellular Ca^{2+} gradient into the cytosol (Clapham, 2007). Ca^{2+} release from intracellular stores occurs primarily via two ROCs, the ryanodine receptor (RyR) and the inositol 1,4,5-triphosphate (InsP_3) receptor (Strigrow & Ehrlich, 1996). These two channels are responsible for the initial rise in $[\text{Ca}^{2+}]_i$ that occurs

following cell stimulation (Striggow & Ehrlich, 1996). In nonexcitable cells, such as epithelia like the PC3 cells used in these experiments, the slower InsP_3 -mediated pathway is the most common (Clapham, 2007). RyRs are also sensitive to Ca^{2+} itself, causing Ca^{2+} -induced Ca^{2+} release (CICR) in excitable cells, but this mechanism has not yet been shown for the InsP_3 receptor (Yao *et al.*, 2004).

In nonexcitable cells, Ca^{2+} influx from the extracellular fluid is via hyperpolarisation. Open K^+ channels force the membrane potential to more negative potentials, which draws Ca^{2+} across the plasma membrane more rapidly (Clapham, 2007). Among the ROCs associated with the plasma membrane are the store-operated Ca^{2+} channels (SOCs), the best studied of which are the calcium release activated channels (CRACs) (Mori *et al.*, 2002). This type of entry is often termed capacitative Ca^{2+} entry (CCE) as these channels are activated only after Ca^{2+} has been discharged from intracellular stores (Berridge, 1995; Mori *et al.*, 2002). In order to refill the internal stores, SOCs maintain prolonged elevations in $[\text{Ca}^{2+}]_i$ (Striggow & Ehrlich, 1996). CICR has also been shown to be associated with some SOCs in nonexcitable cells (Yao *et al.*, 2004). Other ways Ca^{2+} can enter the cytosol include via the transient receptor potential (TRP) family of non-selective monovalent cation channels that mediate capacitative entry (Mori *et al.*, 2002; Birnbaumer, 2009) and the sodium calcium exchanger (NCX) working in reverse mode (Blaustein & Lederer, 1999).

Excess intracellular Ca^{2+} is mainly extruded from the cytoplasm into the extracellular fluid or into the internal storage organelles via the NCX in forward mode (Blaustein & Lederer, 1999) and/or actively pumped into the extracellular fluid via the plasma membrane Ca^{2+} -ATPase (PMCA) (Carafoli, 1992). There is also a Ca^{2+} pump in the ER membrane, known as the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA), which is responsible for Ca^{2+}

re-sequestration by the ER (Clapham, 2007). Mitochondria are take up Ca^{2+} via the mitochondrial calcium uniporter (MCU), a high affinity Ca^{2+} -selective ion channel located in the organelle's inner membrane that is driven by an electrochemical gradient rather than ATP hydrolysis (Kirichok *et al.*, 2004).

Appendix K. Flavonoids

Flavonoids can be categorised according to the presence of an oxygen group at position 4, a double bond between carbon atoms 2 and 3, or a hydroxyl group in position 3 of the C (middle) ring (Middleton *et al.*, 2000). Six subclasses of flavonoids are commonly recognised: the flavones, flavonols, flavanones, flavanols (or catechins), anthocyanidins and isoflavones (Beecher, 2003; Hirpara *et al.*, 2009). The general structures of these most common subclasses are shown in Figure K1.

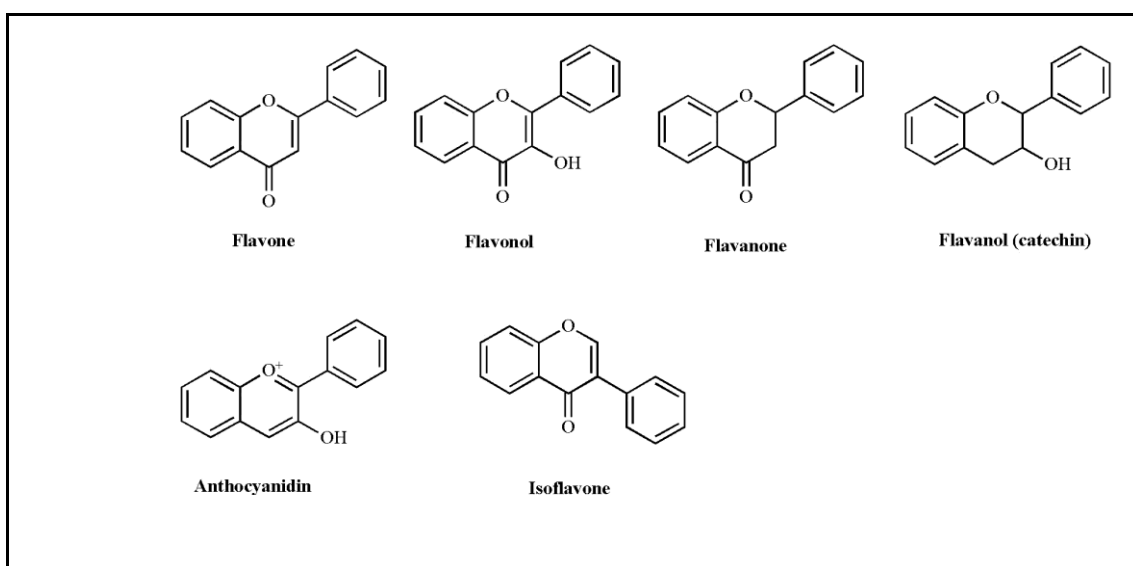


Figure K1 Chemical structures of the six most common flavonoid subclasses.

Note. From “Quercetin and its derivatives: synthesis, pharmacological uses with special emphasis on anti-tumor properties and prodrug with enhanced bio-availability.” by K.V. Hirpara, P. Aggarwal, A.J. Mukherjee, N. Joshi and A.C. Burman, 2009, *Anticancer Agents in Medicinal Chemistry*, 9(2), p. 138.

Slight structural differences, including distinct patterns of hydroxylation, methylation, and conjugation with various mono- and disaccharides distinguish individual flavonoids (Graf *et al.*, 2005). Luteolin and apigenin are both examples of prominent flavones, naringenin is a flavanone and quercetin is the main representative of the flavonol subclass (Beecher, 2003; Jeong *et al.*, 2009). Rutin (or quercetin-3-rutinoside) is the glycoside of quercetin, the form that quercetin occurs most frequently as in plants (Erlund *et al.*, 2000). The close

similarity between the structures of the aglycone, quercetin, and its glycoside, rutin, can be seen in Figure K2. The differing functional group attached to carbon 3, rutinose, is a disaccharide with the molecular formula $C_{12}H_{22}O_{10}$. Figure K2 also illustrates that the hydroxyl group at carbon 3 in the quercetin molecule is the only difference from the luteolin structure.

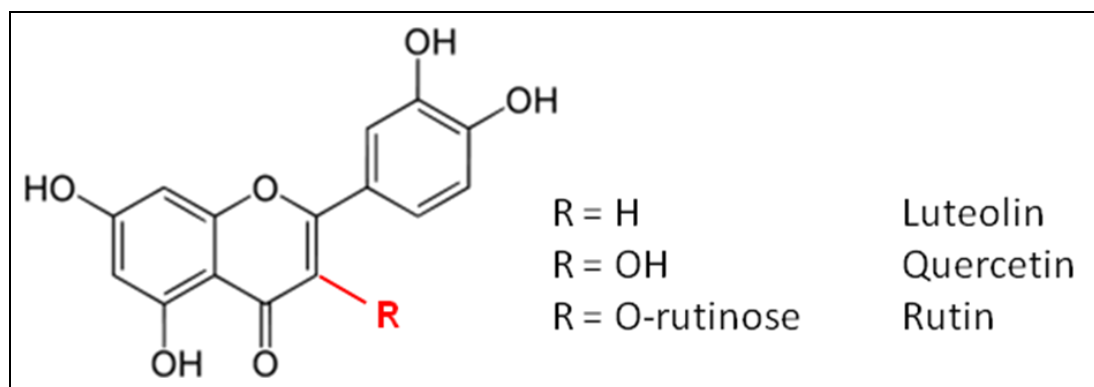


Figure K2 Chemical structures of luteolin, quercetin and rutin.

Note. From “Structure-activity relationships for antioxidant activities of a series of flavonoids in a liposomal system.” by A. Arora, M.G. Nair and G.M. Strasburg, 1998, *Free Radical Biology and Medicine* 24(9), p. 1356.

Appendix L. Extraction of Sponges

The following procedures were performed by Ms Jessie Moniodis, an Honours student within the Discipline of Pharmacy (UWA). The methods described herein were taken from her thesis with permission.

Freeze-dried sponge material was manually divided into small pieces and extracted (3X) with methanol at room temperature for 24 h. The methanol solutions were combined and filtered to remove insoluble particles, after which the samples were dried under reduced vacuum. The sponge sample afforded a dark brown precipitate.

The crude extract was prefractionated on a Diaion HP20ss resin (45 g; Sigma Aldrich). The resin was prepared by sequential rinsing with 30 mL of acetone, methanol followed by a 1:1 methanol:water solution, water and lastly, methanol. The washed resin was then stored under methanol until required.

A portion of the resin slurry was transferred into a 50 mL polypropylene syringe column (125 mm length x 28 mm diameter; Grace Discovery) ensuring that 2 to 3 cm of solvent remained above the resin to prevent drying and allow easier application of the crude extract. The dried crude extracts (3.05 g) was redissolved in 80 mL of 1:3 methanol/water solution. A small volume (0.5 mL) was withdrawn and labelled crude sample (1-28Crude). The remainder was carefully applied to the column in order to minimize disruption of the resin surface. The sample and mobile phase were allowed to run through the column at one drop per second and the eluent was collected in a pre-weighed round bottomed flask labelled A (Fraction 1-28A). A stepwise gradient elution from water to acetone was then

performed (25% steps, 10 mL), followed by final rinsing with 40 mL of acetone and 50 mL of ethyl acetate. The eluent from each step was collected individually resulting in 6 additional fractions: 1-28B (1:3 v/v acetone:water); 1-28C (1:1 v/v acetone: water); 1-28D (3:1 v/v acetone:water); 1-28E (acetone); 1-28F (acetone); 1-28G (ethyl acetate). Fractions A to G and the crude sample were dried under reduced vacuum and masses recorded before submission for bioassay.

A common way of dealing with such samples is to chromatograph an unknown extract using a mobile phase of wide ranging polarity i.e. using a default gradient elution, adequately separating the mixture and eluting all components. Following prefractionation, samples were further separated using reversed phase HPLC (detection at 254 nm). Fractions were selected as a result of their bioactivity towards the brine shrimp.

Each sample was dissolved in a suitable solvent and filtered (Acrodisc® Syringe Filter 0.2 µm) before application to a C18 column (Apollo; 5 µm; 250 x 10 mm; Grace Discovery). The sample was subjected to a gradient using a water:acetonitrile mobile phase (95:5 H₂O:CH₃CN to 100:CH₃CN over 60 min; flow rate 2.5 mL/min) and fractions were collected at 10 min intervals. The column was then washed with 100% acetonitrile for an additional 10 min and this eluate was collected separately. All prefractions were dried under reduced vacuum and submitted for bioassay in the brine shrimp and MTT assays.

A summary of the separation procedures is provided in the following flowchart (Fig. L1).

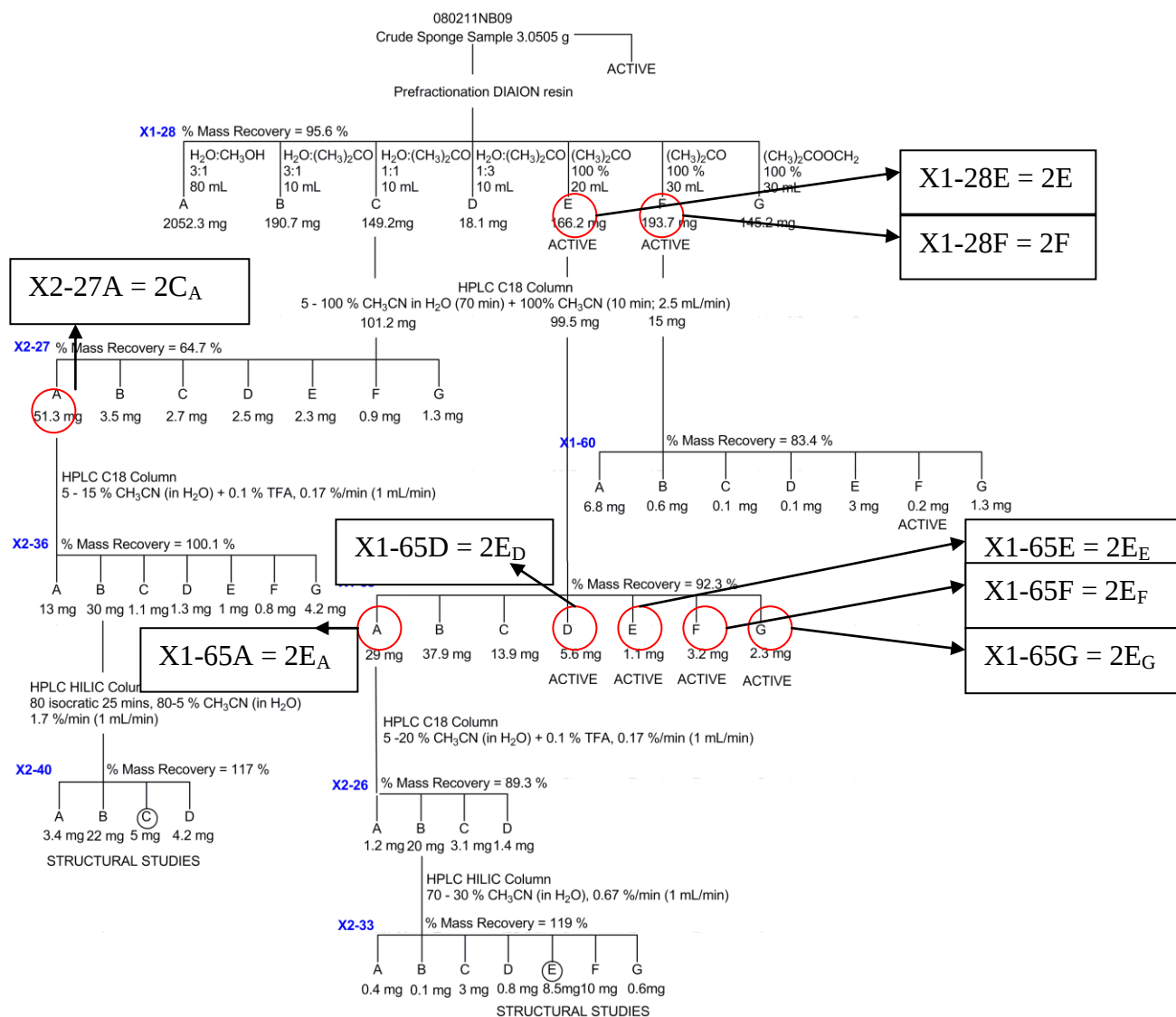


Figure L1 Flow diagram of separation method.

For simplicity, these identifier codes were modified for my experiments such that:

- | | | |
|---|---|-----------------|
| 1 | = | 2F |
| 2 | = | 2C _A |
| 3 | = | 2E _D |
| 4 | = | 2E _E |
| 5 | = | 2E _F |
| 6 | = | 2E _G |
| 7 | = | 2E |
| 8 | = | 2E _A |

Appendix M. Isolation of Paclitaxel

Although first collected as part of the NCI anticancer drug screen from the bark of the Pacific yew tree (*Taxus brevifolia*) in 1962, it was not until two years later that the crude extract was processed and shown to have modest anticancer activity (Goodman & Walsh, 2001). Initially named taxol, the pure compound was isolated in 1966 and its structure finally published four years later (Wani *et al.*, 1971; Goodman & Walsh, 2001). However, the Pacific yew tree is very rare and slow growing and harvesting the bark is fatal to the tree. Moreover, paclitaxel is only present in miniscule quantities in *T. brevifolia* so it was imperative that an alternative source be found if paclitaxel was ever to become a useful anticancer drug. Hence, the presence of the active compound was investigated in other *Taxus* species.

While all *Taxus* species contain paclitaxel or related taxanes (Jennewein & Croteau, 2001), initial relatively low and highly variable yields meant it was not until the mid 1990s that clinically useful quantities were finally isolated from these sources. Concurrently, methods to chemically synthesise paclitaxel were proving difficult and there were solubility issues. However, the unique mode of action of paclitaxel (Schiff *et al.*, 1979) provided a motivating force to overcome these serious impediments to drug development (Jennewein & Croteau, 2001). In 1988 Potier and colleagues published a semi-synthetic route to paclitaxel via other more accessible precursors, baccatin molecules, taxanes present in a related species, the European yew, *Taxus baccata* (Denis *et al.*, 1988). *T. baccata* is not only a more common species, being widely planted as an ornamental tree, but baccatin molecules are present in the needles (leaves) and twigs of the yews, meaning the source is renewable (Dewick, 2009). A year later, Holton patented a more practical approach with

twice the yield of the Potier process via structurally related baccatin molecules (Holton *et al.*, 1995).

While total synthesis of paclitaxel has been achieved (Holton *et al.*, 1994; Nicolaou *et al.*, 1994), these methods have proven economically non-viable (Jennewein & Croteau, 2001). After many variations and improvements on the protocols involving intermediary pathways funded by Bristol-Myers Squibbs, the drug company that commercialised Taxol[®], most of today's paclitaxel is produced via plant cell fermentation technology. This involves propagating a specific *Taxus* cell line in aqueous medium in large fermentation tanks and directly extracting the paclitaxel, purifying it by chromatography and isolating it by crystallisation. Not only does this method save trees, but it requires less hazardous chemicals and considerably less energy than the semi-synthetic route (Suffness & Wall, 1995; Kingston, 2001; Dewick, 2009).

Appendix N. Hormesis

Traditionally, the fundamental nature of the dose-response has been universally recognised as being sigmoidal in shape and displaying a threshold response in the low-dose zone (Calabrese *et al.*, 2006a). This sigmoidally-based threshold model has been the overwhelmingly dominant paradigm in essentially all disciplines dealing with dose-response relationships (Calabrese & Baldwin, 2003b). It has been applied to essentially all biological models, all endpoints measured and all chemical substances tested, regardless of chemical class (Calabrese *et al.*, 2006a). In this conventional model, toxicologists assume a linear relationship between dose and effect, which holds true up (or down) to a certain threshold, beyond which no more effects are observed. In this monotonic dose-response relationship, as the dose increases, so does the effect and *vice versa*. A variation of this model is the more cautious linear non-threshold (LNT) model used for carcinogens (Hadley, 2003). However, substantial evidence indicates that the threshold model is not as universal as believed and is, in fact, less commonly observed than the hormetic model (Calabrese *et al.*, 2006a). The hormetic model, rejects the standard assumption that effects at low doses can be extrapolated from data obtained from high doses, and instead describes the relationship as a U- or an inverted U- (or J-) shaped curve, depending on whether the substance causes an increased (Fig. N1a) or decreased (Fig. N1b) effect (Calabrese & Baldwin, 2003a; Hadley, 2003).

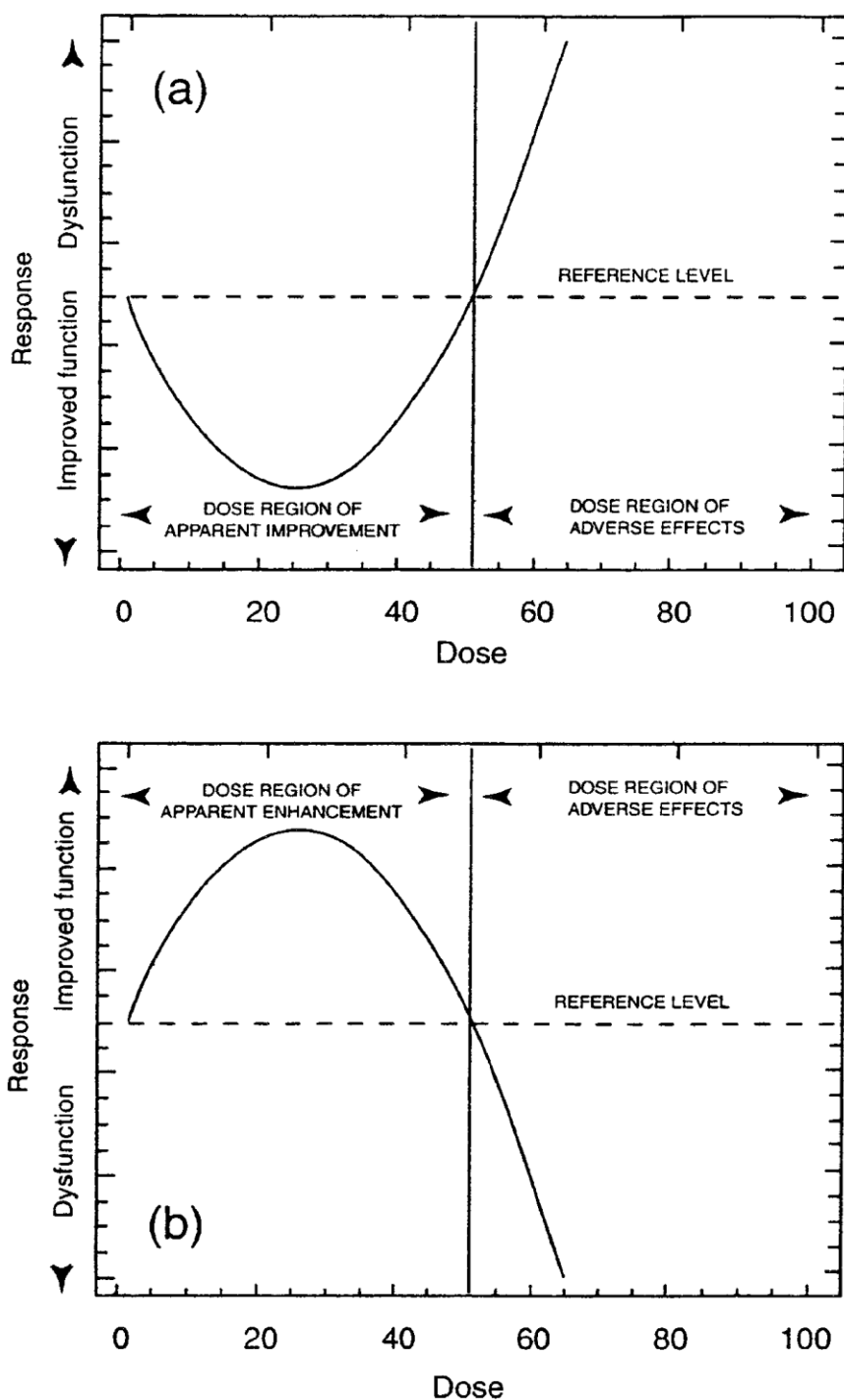


Figure N1 Hormetic dose-response relationships.

General representations of dose-response curves based on the (a) the U- or J-shaped hormetic model depicting low-dose reduction and high-dose enhancement of adverse effects and (b) the inverted U-shaped hormetic model depicting low-dose stimulatory and high-dose inhibitory responses.

Note. From “Hormesis: the dose-response revolution.” by E.J. Calabrese and L.A. Baldwin, 2003a, *Annual Review of Pharmacology and Toxicology*, 43(1), p. 178.

More than 15% of the 400-plus papers with hormesis in the title that appear on the PubMed list are by the same author, Edward Calabrese. While not the first modern scientist to propose hormesis as a valid biological hypothesis, Calabrese and his group have almost single-handedly revived the debate concerning the existence of hormesis (Stebbing, 1982; Calabrese & Baldwin, 1998). They have shown that the hormetic model is actually the most fundamental dose response, significantly outcompeting other leading dose-response models in large-scale, head-to-head evaluations with a frequency of at least 40% in the toxicology literature (Calabrese & Baldwin, 2003b; Calabrese *et al.*, 2006b; Calabrese *et al.*, 2008; Calabrese, 2005a, 2005c, 2005d, 2008b). These authors contend that early key leaders of the biomedical sciences made a profound error when deciding on the fundamental nature of the dose-response. This conceptual misunderstanding was consolidated by the scientific community in the 1930s when they adopted the threshold model as the default dose-response paradigm (Calabrese, 2009a). This has meant that, historically, hormesis has been a little discussed phenomenon, and although evident in many published articles on dose-response studies, it is rarely acknowledged by the authors. If not completely ignored, it is usually dismissed by the investigators as an artefact (Calabrese *et al.*, 1999; Hadley, 2003). The extreme marginalisation of the hormetic theory was due to many contributing factors which have been extensively reviewed (Calabrese & Baldwin, 1999; Calabrese, 2005d, 2009a). What follows is a brief history summarising just how and why the toxicological community missed what Calabrese (2004, p127) called “the basic reality of the hormetic dose response” in favour of the conventional threshold model.

The term *hormesis* was not coined until 1943 when Southam and Ehrlich noted an antibiotic they were working with apparently stimulated growth of otherwise sensitive organisms at certain concentrations (Randall *et al.*, 1947). However, the concept of

hormesis has been around since at least the 1500s when Paracelsus, regarded as the father of toxicology, famously observed “all things are poison and nothing is without poison, only the dose permits something not to be poisonous” (Goldstein & Gallo, 2001; Mattson, 2008). This is often paraphrased to the dictum “only the dose makes the poison” (Bagchi, 2006; Stumpf, 2006; Jonas & Ives, 2008). In 1854 Virchow described how low concentrations of NaOH and KOH caused an increase in the beating activity of the ciliae of tracheal epithelia mucosa, but a concentration-dependent decrease to arrest at higher concentrations (Henschler, 2006). The notion gained momentum in the late 1800s when Hugo Schulz reported the occurrence of stimulation from very low concentrations of toxic substances based on his research on various disinfectants on yeast metabolism (Calabrese & Baldwin, 2000a). Collaborations with the practising homeopath, Rudolph Arndt, eventually led to the Arndt-Schulz Law which stated “for every substance, small doses stimulate, moderate doses inhibit, large doses kill” (Calabrese & Baldwin, 2000a; Calabrese, 2009a). Independent research on chemical stimulation of bacterial growth by a contemporary, Ferdinand Hueppe, the protégé of Robert Koch and himself one of the founders of bacteriology, supported the perceived truism which became better known as Hueppe’s Rule due to his distinguished reputation (Calabrese & Baldwin, 2000a, 2000b; Calabrese, 2009a). Despite being widely referred to in the pharmacological literature for over 30 years, it fell into disuse because there was no explanation to account for it (Stebbing, 1998; Calabrese *et al.*, 1999; Lindsay, 2005). Instead, the principle was adopted by the homeopathy community in support of their ideas (Calabrese, 2009a; Kendig *et al.*, 2010). The defining principle of the highly controversial medical practice of homeopathy is the “law of similars” or “like cures like” (Ernst, 2005). It holds that ultra-low dilutions of a substance that causes a particular adverse effect in a healthy person can be used to treat a patient suffering from those same symptoms (Ernst & Pittler, 1998; Jonas *et al.*, 2003).

Examples include the use of *Apis mellifera*, the common honey bee, to treat symptoms of localized swelling, soreness, and inflammation, the same symptoms caused by bee stings and using onions to cure the attendant symptoms of colds and allergies (Tedesco & Cicchetti, 2001). In layman's terms, it is the notion that you can be cured by "the hair of the dog that bit you" or that a little poison can be good for you (Jonas & Ives, 2008). The close and unfortunate association with homeopathy appears to be the principal reason why hormesis as a concept was rejected by the wider scientific community of the day (Calabrese, 2005c, 2005d, 2009a).

In addition to having no strong advocates for hormesis, toxicologists have been traditionally more interested in the upper end of the dose-response curves, where dose and risk are at their highest. As hormetic effects are characteristically modest and are therefore difficult to measure and quantify without extensive studies using many animals, they were not often seen in experiments primarily designed to assess high-dose inhibition (Hadley, 2003; Calabrese, 2005b). This only reinforced the initial bias against hormesis as a concept (Calabrese & Baldwin, 2003a).

On the other hand, unlike the toxicology community, molecular pharmacologists have studied various dose responses and identified over 30 receptor systems that show hormesis (Calabrese & Baldwin, 2003a). The difference between the two fields was explained by Hadley (2003), who noted their differing approaches to dose-response relationships: while toxicology is concerned with the toxic effects of substances, which are commonly displayed at high doses, pharmacology is more interested in the therapeutic value of substances which are frequently seen at low doses. According to the same author, molecular biologists generally go another step further than both toxicologists and

pharmacologists, not only identifying a biological effect, but also investigating its fundamental causes. However, because hormesis has been observed for so many different species, substances and endpoints, it has been proposed that there is a generalised mechanistic strategy, but no single molecular mechanism to explain its existence. This high generalizability suggests that the hormetic dose-response strategy has been selected for and is adaptive in nature (Stebbing, 1998). It is seen, for example, in the context of adaptive/preconditioning responses where a prior exposure to a low dose of a toxic agent up-regulates adaptive mechanisms that protect against subsequent exposures to similar toxic agents (Calabrese, 2008a). Thus, hormesis represents an overcompensation to an alteration in homeostasis. When the dose progressively increases, the system's capacity to compensate becomes overwhelmed, the toxicity threshold is exceeded, and toxic effects are seen (Calabrese *et al.*, 1999). In other words, the mechanism is perceived to be a disruption of cellular homeostasis, followed by a modest overcompensation by the cell, and the re-establishment of homeostasis, but in an adapted state (Lindsay, 2005). The first adaptive response, was for *E. coli*, where a reduced mutagenic effect of the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was seen if the bacteria had previously been exposed to a low dose of the same compound (Jeggo *et al.*, 1977). Since then, many other adaptive responses have been characterized for various agents in both bacteria and mammalian cells (Hoffmann, 2009).

Calabrese (2008a) believes the theories of Szabadi (1977) on agonists and opposing receptor subtypes, which are supported by experimental examples, may help account for numerous cases of hormetic-like biphasic dose-responses. In this model, a single agonist may bind to two receptor subtypes, one activating a stimulatory pathway and the other an inhibitory one. The receptor subtype with greatest agonist affinity would typically have

fewer receptors, hence a lower capacity, and its pathway activation effects would dominate at lower doses. Conversely, the other receptor subtype would have lower agonist affinity, more receptors and greater capacity, becoming dominant at higher concentrations (Calabrese, 2008a). In summary, “the hormetic process represents a common strategy for resource allocation when systems need to respond to low-level metabolic perturbations” (Calabrese & Baldwin, 2003a, p183).

However, other authors are not so convinced of the prevalence of hormesis, although they acknowledge its existence (Crump, 2007; Douglas, 2008; Mushak, 2009). Additionally, several authors have expressed concern that *in vitro* hormetic effects may not correlate with the *in vivo* situation (Anderson, 2005; van der Woude *et al.*, 2005), but many studies have confirmed *in vivo* hormesis (Lindsay, 2005; Ergüven *et al.*, 2007; Bruchey & Gonzalez-Lima, 2008; Gocke & Wall, 2009; Fosslien, 2009). Most authors agree further debate is required before hormesis becomes the default model of dose-response as advocated by Calabrese (Hadley, 2003; Callahan, 2005; Calabrese *et al.*, 2006b). Questions to resolve include whether all biphasic dose-response curves should be considered representative for hormesis and just what might be clinical significance of hormetic effects or their consequences for risk assessment or (Anderson, 2005; van der Woude *et al.*, 2005). It is also desirable to design future experiments to describe hormetic phenomena with respect to a time factor, allowing for detection of the specific type of hormesis (Calabrese & Baldwin, 2002; Carelli & Iavicoli, 2002). As the meaning of the operational term hormesis and its close synonyms have been obscured by inconsistent and confusing usage, an interdisciplinary team has proposed new definitions that distinguish between direct stimulus and overcompensation stimulus (Calabrese *et al.*, 2007). Thus, *conditioning hormesis*, in which prior exposure to a low dose of a substance protects against a larger

dose of the same (or similar) stressor substance, and *postexposure conditioning hormesis*, which denotes phenomena in which the toxicity of a high stressor dose is ameliorated by subsequent exposure to a low dose of the same stressor (Calabrese *et al.*, 2007; Agutter, 2008). Regardless, the concept of hormesis is now becoming increasingly recognised by the scientific community as evidenced by the meteoric rise in journal articles from 15 citations per year in the 1980s to more than 2,400 in 2009 alone (Calabrese, 2010). Moreover, all recent textbooks on toxicology now contain sections on hormesis, giving it a clear standing in the field (Calabrese, 2009b). Additionally, as hormesis provides a framework for the study and assessment of chemical mixtures, incorporating the concept of additivity and synergism (see section 8.2.8), it is expected that it will become even more significant within toxicological evaluation in the future (Calabrese, 2008b).

Appendix O. Other General Cytotoxicity Assays

Alternative methods of determining any losses in membrane integrity include measuring the release of enzymes from cells in culture into the incubation media and assessing the cells' abilities to take up or release a radioisotope tracer. However, neither of these methods were suitable for the conditions of the current study as outlined below.

Many unsuccessful attempts were made to assess compromised membrane integrity of all plant extracts using a commercially available kit (Sigma DG158K) to measure the activity of aspartate aminotransferase (AST). This enzyme is found in large quantities in pulmonary, cardiac and hepatic cells, and so the measurement of serum AST activity has long been used in the differential diagnosis of diseases of these organs (Amador & Wacker, 1962). However, AST is actually present in all cells. The AST colourimetric assay is based on a coupled REDOX reaction such that the rate of decrease in absorbance at 340 nm is directly proportional to AST activity. However, as this assay was time-dependent and the spectrophotometer available to measure samples in cuvettes drifted over the course of the assay, the data (not shown) were possibly invalid. Furthermore, most of the extracts themselves absorbed at the wavelength used (see Appendix D), so little confidence was placed on resultant data and these experiments were eventually abandoned.

Another way of identifying cell damage is to measure the amount of leakage of another ubiquitous cytosolic enzyme, such as lactate dehydrogenase (LDH), adenylate kinase (AK), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). However, as all these enzymatic release assays have several practical limitations, including multiple reagent additions, scalability, low sensitivity, poor linearity, or wash steps and medium exchanges (Cho *et al.*,

2008), it was decided not to attempt to measure the activity of any of these enzymes.

Additionally, the LDH assay is also performed at 340 nm and, as with the AST assay, samples are run serially (Korzeniewski & Callewaert, 1983), hence, it was thought similar problems to the AST assay would ensue. Moreover, while LDH is considered relatively stable in culture with a half-life of approximately 9-10 h (Riss *et al.*, 2006), it is less stable than AST, which has a half-life of 12.5-22 h (Lin *et al.*, 2000). Thus, the changing levels of LDH over time must be taken into account when interpreting results from experiments where cells are exposed to damaging agents for longer periods of time. For example, in this study, if all the cells were killed at the beginning of the 48 h exposure period, there would be little activity remaining at the end-point of the experiment when the measurements were made. Not only that, but endogenous LDH present in the serum of the growth media can cause high background absorbance levels, limiting the sensitivity of this assay (Riss *et al.*, 2006).

Compromised cell membrane integrity can also be detected by measuring another cytosolic enzyme, glucose-6-phosphate dehydrogenase (G6PD). While the original method described by Lee and Wood (1982) entailed following G6PD activity at the same unsuitable wavelength of 340 nm, Batchelor and Zhou (2004) recently updated the procedure. In the modified assay, the activity of extracellular G6PD is correlated to the reduction of resazurin (Alamar blue) to resorufin, via a coupled-enzyme reaction. An advantage over the LDH assay is that background noise from endogenous G6PD is minimal as serum contains 5-fold less G6PD than LDH (Batchelor & Zhou, 2004). However, at only 2 h, the half-life of G6PD in culture medium following cell lysis (Riss & Moravec, 2004) is much lower than that of LDH, meaning that measuring G6PD activity would be even less useful for the longer (48 h) exposure periods of these experiments. However, the main problem with the

G6PD assay is that the end-product of the coupled reaction is the highly fluorescent dye, resorufin (Batchelor & Zhou, 2004), and at the time of these experiments a fluorescence spectrophotometer was not available for use. For the same reason it was not possible to prelabel cells with carboxyfluorescein diacetate and measure its release fluorometrically (Suzuki *et al.*, 1991). Similarly, the use of bioluminescent cytotoxicity assays in which released intracellular markers (e.g. proteases) are measured (Cho *et al.*, 2008), was not feasible as access to the required instrument was not available.

It was also not possible to determine the viability of cells by assessing their uptake or release of a radioisotope tracer, such as ^{51}Cr (Brunner *et al.*, 1968; Neville, 1987) or ^{125}I -iododeoxyuridine (Cohen *et al.*, 1971), because of a lack of facilities, funding and time required for authorisation. Simpler methods of determining cell viability by measuring cell membrane impairment, such as through the use of vital dyes (e.g. trypan blue and neutral red) or fluorescent compounds (e.g. propidium iodide), were not contemplated due to their labour intensiveness restricting their utility for high-throughput screening.

Appendix P. Determination of Compound Interactions

Individual constituents of mixtures, such as plant extracts, may interact in the combination to produce greater than or less than responses than would be expected from their individual dose-response curves (Savelev *et al.*, 2003). Most often, the assessment of these synergistic or antagonistic interactions is made from experiments in which an effect level is chosen and doses (concentrations) of drug A alone, drug B alone and the combination (a, b) that give this effect are determined. Doses that produce the same effect are called isobols (Tallarida, 2002; Prakash *et al.*, 2009).

The *interaction index* is denoted by γ and calculated using the isobolar equation (1):

$$\gamma = a/A + b/B \quad (1)$$

where

- A is the dose of one drug alone,
- B is the dose of another drug alone,
- a is the dose of drug A in combination,
- b is the dose of drug B in combination.

The quantities in Eq. (1) are obtained from the dose response curves of drugs A, B, and the combination. If $\gamma = 1$, the interaction is additive; if $\gamma < 1$ it is super-additive (synergistic), and if $\gamma > 1$ it is sub-additive (antagonistic) (Montes *et al.*, 2000; Tallarida, 2002).

The same approach can be used for any number of compounds in combination such that:

$$\gamma = \sum_{i=1}^n (i/I) \quad (2)$$

where

n = a number of agents in a combination, with $i = 1, 2, 3, \dots, n$ (Savelev *et al.*, 2003).

The results of isobolographic analysis can be easily visualized using isobolograms (Fig. P1). An isobologram is a two-dimensional plot in which the concentrations of the two drugs (on an arithmetic scale) are the coordinates. An isobol is a curve that starts from a concentration of drug A on the x axis and ends at an isoeffective concentration of drug B on the y axis, connecting the concentrations of all combinations showing the same effect. An additive isobol, the graphical representation of Eq. 1, is a straight line from the x axis to the y axis, connecting the isoeffective concentrations of drugs A and B alone. When only one drug is active, the additive isobol (known as an indifferent isobol in this case) is parallel to the axis along which the concentrations of the inactive drug are plotted, starting from the concentration of the active drug that produces an effect. An isobol that deviates to the left or right from the indifferent isobol indicates synergy or antagonism, respectively (Stergiopoulou *et al.*, 2008).

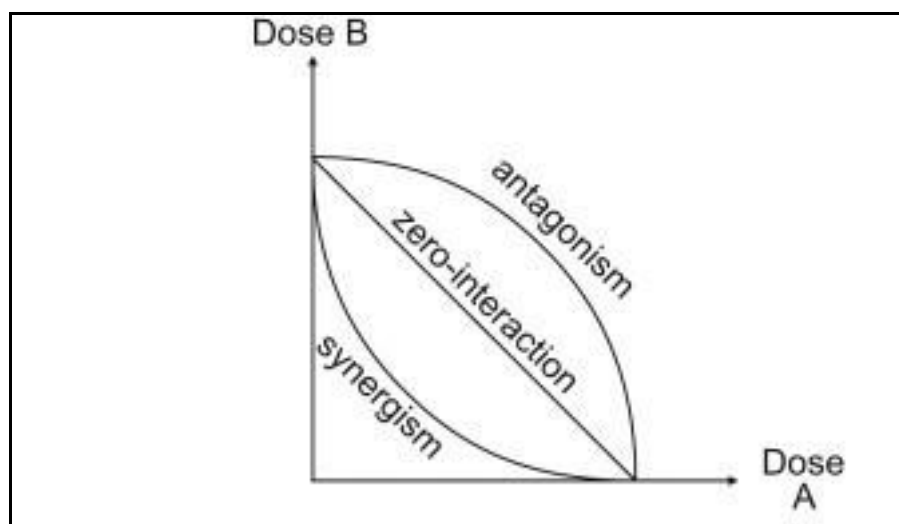


Figure P1 Isobols for zero-interaction, synergism and antagonism.

Note. From “Synergy research: approaching a new generation of phytopharmaceuticals.” by H. Wagner and G. Ulrich-Merzenich, 2009, *Phytomedicine*, 16(2-3), p. 98.

Thus, for a particular growth level (e.g., 15%, 50%, or 85% of the growth of the drug-free control), the total concentration of both drugs for a fixed ratio of each combination is compared with the isoeffective theoretical additive total concentration (Stergiopoulou *et al.*, 2008).

Others have used different cut-offs to define synergy, reasoning that the observed effect must deviate significantly from 1 to be meaningful. It has been recommended that synergy occurs when the γ is ≤ 0.5 , while antagonism is defined by a $\gamma > 4$ ("Antimicrobial Agents and Chemotherapy," 2007). The logic behind these definitions, based on inherent inaccuracies and biological relevance, was succinctly described by Johnson *et al.* (2004). Stergiopoulou *et al.* (2008) defined the interaction between two compounds as synergistic or antagonistic when two or more sequential fixed ratios of each growth level had significantly ($P < 0.05$) lower or higher interaction.