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ANTICANCER ACTIVITY OF CRUDE EXTRACT AND CAROTENOID PIGMENTS FROM FRUITS

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ABSTRACT

The human diet contains important micronutrients namely vitamins C and E, carotenoids and flavonoids which are essential for maintenance of human health. Multiple dietary sources of these compounds are present virtually in all plant material. The nutritional importance of foods is due to the presence of these functional food ingredients and antioxidant nutraceuticals or phytochemicals. Phytochemicals are present in edible fruits and vegetables and when eaten potentially modulate human metabolism in a favourable manner, thereby prevent chronic and degenerative diseases. Increase in fruits and vegetables consumption protects our body against degenerative pathologies such as cancer and therosclerosis. An epidemiological surveys has shown an inverse relationship between dietary flavonoid intake from citrus and cardiovascular diseases. Citrus fruits are the main source of important phytochemical nutrients and for long have been valued for their wholesome nutritious and antioxidant properties. Moreover, it is now appreciated that other biologically active, non-nutrient compounds found in citrus fruits such as phytochemical antioxidants, soluble and insoluble dietary fibres are known to be helpful in reducing the risk for cancers, many chronic diseases like arthritis, obesity and coronary heart diseases. The present study is aimed at studying the Anticancer activity Crude Extract and Carotenoid pigments of certain selected fruits.

KEYWORDS: Human Diet, Micronutrients, Phytochemicals, Fruits and Anticancer activity.

INTRODUCTION

Ananas comosus (L.) Merrill belonging to the family Bromeliaceae is an important tropical and subtropical plant widely cultivated in the tropical areas of the world. Its fruit is consumed fresh or canned as a commercial product in many countries. Pineapple has also been known for a number of beneficial biological activities such as antioxidative, anti-browning, anti-inflammatory and anti-platelet activities. The enzyme complex of *A. comosus* called bromelain is known for its clinical applications particularly modulation of tumor growth, blood coagulation and anti-inflammatory Effect **(Tripoli *et. al*, 2007)**.

Oranges as excellent source of vitamin C, contain powerful natural antioxidant, folate, dietary fibre and other bioactive components, like carotenoids and flavonoids that prevent cancer and degenerative diseases (**Ejaz *et al.*, 2006**). Consumption of foods rich in vitamin C improves body immunity against infectious agents and scavenging harmful, pro-inflammatory free radicals from the blood. Sweet orange contains a variety of phytochemicals like hesperetin and narigenin. Naringenin has a bioactive effect on human health as antioxidant, free radical scavenger, anti-inflammatory, and immune system modulator. Oranges also contain a very good amount of vitamin A, and other flavonoid antioxidants such as alpha and beta carotenes, beta-cryptoxanthin, zeaxanthin and lutein, compounds that have antioxidant properties. Vitamin A is necessary for maintaining healthy mucus membranes, skin and essential for vision. It is also a very good source of B-complex vitamins such as thiamin, pyridoxine and folates. These vitamins are essential as the body requires them from external sources to replenish. Orange fruit also contains a very good amount of minerals like potassium and calcium. Potassium in an important component of cell and body fluid that helps to control heart rate and blood pressure. Vitamin A is also required for maintaining healthy mucus membranes and skin and is also essential for vision. Consumption of natural fruits rich in flavonoids helps body to protect from lung and oral cervical cancers. The alkaline properties in the orange stimulate the digestive juices, thus, reliving constipation. Regular intake of orange juice reduces the chances in the formation of calcium oxalate which causes kidney stones. Polyphenols present in oranges prevents viral infections. Oranges protect the skin from damage caused by free radicals, thereby helping one to look young and keeps the skin fresh and glowing (**Tsuda *et. al.*, 2004**). Musa sapeintum (musaceae) is extensively cultivated throughout India, as one of the most popular fruit crop. Banana flowers are rich in vitamins like C and E, flavonoids, protein, minerals like potassium, calcium,

iron, copper, phosphorous, magnesium and dietary fibre. It is free from sodium. This flower has been used as a traditional medicine to treat bronchitis, constipation, menstrual cramps and ulcer problems (**Sarita Panigrahy *et al*, 2015).** Antifungal and antibiotic principles are found in the peel and pulp of fully ripe bananas. The antibiotic acts against Mycobacteria. Banana nutrition benefit people at risk of certain cancers. In a study of family history the past history of illness and dietary information of 279 patients who suffered from colorectal cancer were studied. A protective effect provided by banana and papaya on colorectal cancer was served. Dietary fiber was found to decrease the colorectal cancer risk. Thus banana is of great nutritional value. It has a rare combination of energy value, tissue-building elements, protein, vitamins and minerals. It is a good source of calories since it is rich in solids and low in water content as compared to any other fresh fruit **(Sampath Kumar *et al*, 2012).**

Lemon is an important medicinal plant of the family Rutaceae. It is cultivated mainly for its alkaloids, which are having anticancer activities and the antibacterial potential in crude extracts of different parts (viz., leaves, stem, root and flower) of Lemon against clinically significant bacterial strains has been reported (**Kawaii *et al*., 2000**). Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities (**Burt, 2004 and Ortuno *et al*., 2006**). Flavonoids can function as direct antioxidants and free radical scavengers, and have the capacity to modulate enzymatic activities and inhibit cell proliferation (**Duthie and Crozier, 2000**). In plants, they appear to play a defensive role against invading pathogens, including bacteria, fungi and viruses (**Sohn *et al*., 2004**). The peel of *Citrus* fruits is a rich source of flavonoid glycosides, coumarins, and volatile oils (**Shahnah *et al*., 2007**). Many polymethoxylated flavones have several important bioactivities, which are very rare in other plants (**Ahmad *et al*., 2006**). In addition the fiber of citrus fruit also contains bioactive compounds, such as polyphenols, the most important being vitamin C (or ascorbic acid), and they certainly prevent and cure vitamin C deficiency-the cause of scurvy (**Aronson, 2001**). The health benefits of lime include weight loss, skin care, good digestion, relief from constipation, eye care, and treatment of scurvy, piles, peptic ulcer, respiratory disorders, gout, gums, urinary disorders, etc. The first fruit that comes to our minds when it comes to medicinal uses is perhaps the good old lime. This sour citrus fruit can do what many specialist medicines cannot. Thus Lime bearing the scientific name *Citrus aurantifolia*, is being used for ages for treatment of various ailments.

Citrus flavonoids can prevent câncer through selective cytotoxicity, antiproliferative actions and apoptosis (**Elangovan *et. al*., 1994; Hirano *et. al*., 1994**). Flavonoids are antimutagenic, thus protects the DNA from damage by their ability to absorb ultraviolet light (**Stapleton and Walbot, 1994**). They neutralize free radicals that promote mutations when they are generated near DNA. This hás been shown in mice body irradiated with c-ray (**Shimoi *et. al*., 1994**). Flavonoids can also protect the DNA by interacting directly with the tumoral agents, as in the induced chromosomal aberrations by bleomycin (**Heo *et. al*., 1994**). The inhibitory effect of citrus flavonoids on tumoral development and cell proliferation by rat malignant cells, in cardiac and hepatic tissue of syngenetic rats have been reported (**Bracke *et. al*., 1989**). All these properties help to promote overall health (**Cha *et. al*., 2001**) (**Figure 1**).

Figure 1: Dried fruit samples

CAROTENOIDS

Carotenoids are an abundant group of naturally occurring pigments. They occur ubiquitously in all organism of conducting photosynthesis**.** They are found in photosynthetic membranes of phototropic bacteria and cyanobacteria. More than 600 different carotenoids from natural sources have been isolated and characterized **(www.upb.pitt.edu)**. Carotenoids consist of 40 carbon atoms (Tetraterpenes) with conjugated double bonds. They consist of 8 isoprenoid units joined in such a manner that the rearrangement of isoprenoid units is reversed at the centre of the molecule so that the two central methyl groups are in a 1, 6 position and the remaining non terminal methyl groups are in a 1,5 position relationship **(Joanna Fiedor and Kvetoslva Burda, 2014).** Carotenoid hydrocarbons are called carotenes and their derivatives containing oxygen are called xanthophylls. Because of the extensive double bond system in the carotenoid molecule, a carotenoid can exist in a large number of geometric isomers (cis/trans isomers). Most Carotenoids are, in fact, found to be in the all-trans form, but cis isomers do exist

(www.nature.com). The most obvious structural feature of a carotenoid molecule is the chromophore of conjugates double bonds which, in carotenoids of plant tissues, varies from 3 in the colourless phytoene to 13 in canthaxanthin, which is red. This double bond system also renders them susceptible to isomerization and oxidative degradation **(P.M.Dey and J.B.Haarborne, 1997).**

Carotenoids are important in human health. The most active role is protection against serious disorders such as cancer, heart diseases and degenerative eye diseases. It is an antioxidant and acts as regulators of the immune system. Carotenoids are a class of hydrocarbon (carotene) and their oxygenated derivatives (xanthophyls). In mammals, such as humans and monkeys, the most important metabolic products of carotenoids are the retinoids, including vitamin A and retinal. It was demonstrated that the formation of vitamin A from β-carotene could occur either by central or by eccentric cleavage of β carotene. α-carotene, β-carotene and β-cryptoxanthin can be converted to retinal or vitamin A in the intestine and liver by the enzyme 15-151 β- carotenoid dioxygenase. Such in vivo formation of retinal appears to be homeastatically controlled, such that conversion to retinol is limited in persons having adequate vitamin A. Age-related muscular degeneration (ARMD) associated with ageing can lead to a total blindness in healthy people **(D. E. Okwu, 2008).**

MATERIALS AND METHODOLOGY

SAMPLES USED IN THE PRESENT STUDY ARE AS FOLLOWS

Orange (*Citrus reticulate* Blanco)
Lemon (*Citrus limon* (L.)Brum.f.)
Pineapple (*Ananas comosus* (L.)Merr.)
Banana (*Musa acuminate* Colla.)

PREPARATION OF EXTRACTS

The Fruits were collected and dried in shade for few weeks. The dried samples were ground into powder. 5gm of the dried sample powder was weighed and immersed in 50 ml of the solvents – Ethanol, Ethyl acetate and Chloroform for 48 hours. After 48 hours, the extracts were filtered. The filtrates were used for further phytochemical analysis which includes Test for Carbohydrates, Proteins, Glycosides, Tannins, Alkaloids, Flavonoids, Terpenoids, Saponins, Resins, Quinones, Cardiac Glycosides, Coumarins, Ssteroids, Phytosteroids, Phenols, Anthraquinones and Phlobotannins following standard protocols. The carotenoid pigments were isolated using Column Chromatography and was quantified using the formula

Total carotenoid content (µg/g) = A x V (ml) x 10^4 / $A^{1\%}$cm x W (g)

Where A is the absorbance of the carotenoid pigment at 450 nm, V is the total extract volume, $A^{1\%}$cm is the absorption coefficient of β carotene in hexane (2600), W is the sample weight. The samples were further subjected to Thin Layer Chromatography. The Anticancer activity of Fruits was carried out using the MTT Assay methodology.

ANTICANCER ACTIVITY OF THE EXTRACTS - MTT ASSAY

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

Requirements

Cancer cell lines (MCF7)**,** 96 well plate**,** Dulbecco's Modified Eagle Medium, Foetal Bovine Serum, Antibiotics**,** MTT Reagent and Dimethylsulphoxide.

Procedure

Cancer cell lines were purchased from Cancer Institute, Chennai. The cells were grown in a 96 well plate in Dulbecco's Modified Eagle Medium, supplemented with 10% Foetal Bovine Serum and antibiotics (Penicillin-G). About 200µl of the cell suspension was seeded in each well and incubated at 37^{o}C for 48 hours with 5% CO_2 for the formation of confluent monolayer. The monolayer of cells in the plate was exposed to various concentrations of the Fruit extracts and their carotenoid extracts and incubated for 24hours. The cytotoxicity was measured using MTT (5mg/ml). After incubation at 37^{o}C in a CO_2 incubator for four hours, the medium was discarded and 200µl of DMSO was added to dissolve the formazon crystals. The absorbance was read in a micro plate reader at 570nm.

Cyto toxicity was calculated by the following formula:
Viability % = (Test OD/Control OD) x 100
Cell toxicity % = 100 – Viability %

RESULTS AND DISCUSSIONS

ISOLATION OF CAROTENOID PIGMENTS BY COLUMN CHROMATOGRAPHY

Carotenoid pigments were effectively separated from the sample extracts separately in a silica gel column with 100% hexane. The yellow colour band which gets separated when eluted with 100% hexane is identified to be carotenoid pigments **(Figure 2)**. The carotenoid pigments eluted with hexane was collected and stored in vials at -20ºC.

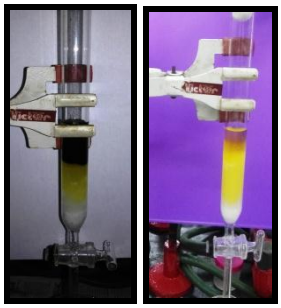

Figure 2: Isolation of Carotenoid pigment

QUANTIFICATION OF CAROTENOIDS

The total carotenoid content quantified is as follows

Total carotenoid content in orange = $0.245x10x10^4$ / 2600x10 = **0.94 µg/g.**

Total carotenoid content in lemon = $0.220x10x10^4$ / 2600x10 = **0.84 µg/g.**

Total carotenoid content in pineapple = $0.251x10x10^4$ / 2600x10 = **0.96 µg/g.**

Total carotenoid content in banana = $0.254x10x10^4$ / 2600x10 = **0.97 µg/g.**

THIN LAYER CHROMATOGRAPHY

The crude extracts and the purified carotenoid pigments and the standard were subjected to thin layer chromatography. The standard used was beta carotene. The mobile phase used was hexane and acetone in the ratio 6:4. The respective Rf values for the fruits (Orange, Lemon, Pineapple and Banana) were calculated **(Table 1).**

ANTICANCER ACTIVITY OF THE EXTRACTS - MTT ASSAY

TABLE 1 : Rf VALUES OF CRUDE EXTRACT AND CAROTENOID

SAMPLE	ETHANOL CRUDE	ETHYL ACETATE CRUDE	CHLOROFORM CRUDE	CAROTENOIDE PIGMENT
ORANGE	0.95	0.94	0.94	0.94
LEMON	0.92	0.92	0.92	0.92
PINEAPPLE	0.92	0.92	0.92	0.92
BANANA	0.94	0.91	0.95	0.94

The cytotoxicity of the crude extracts of Ethanol, Ethyl acetate, Chloroform and the purified carotenoid pigments of each sample was analysed against human breast cancer cell lines, MCF 7 using MTT assay. It is an colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2 -yl) 2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase in the live cells. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. Cells were treated with 100µg and 150µg of the crude extract and carotenoid extracts **(Figures 3 - 5).**

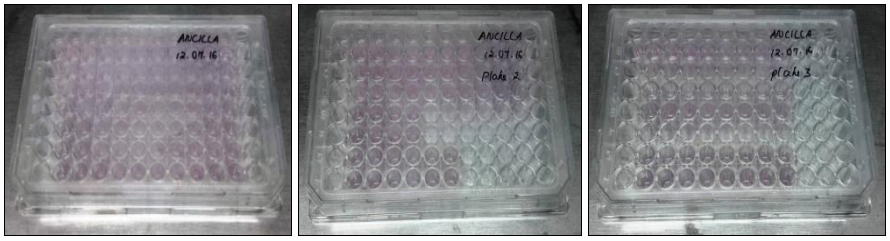

Figure 3: Cells before treatment of the extracts

Figure 4: Cells after adding the extracts

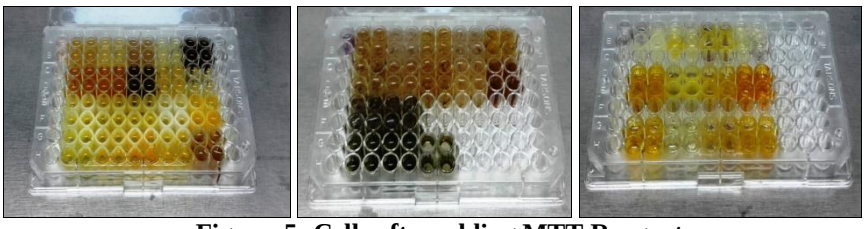

Figure 5: Cells after adding MTT Reagent

The Ethyl acetate crude extracts of Orange, Pineapple, Banana and Lemon showed increased cytotoxicity when compared to other two solvents. The crude and their respective isolated carotenoid pigment showed higher cytotoxicity than the ethyl acetate crude. Over all **Orange** and **Banana** gave the best results in anticancer activity among the fruits **(Table 2 and Figures 6 -7).**

TABLE 2 : ANTICANCER ACTIVITY OF FRUITS									
SAMPLE	CONC	ETHANOL		EHTYL ACETATE		CHLOROFORM		CAROTENOID	
		CELL VIABILITY %	CELL TOXICITY %	CELL VIABILITY %	CELL TOXICITY %	CELL VIABILITY %	CELL TOXICITY %	CELL VIABILITY %	CELL TOXICITY %
ORANGE	100	73.36	26.64	55.4	44.6	67.44	32.56	51.22	48.78
	150	70.61	29.39	51.02	48.98	65.61	34.39	50.3	49.7
PINEAPPLE	100	64.79	35.21	63.36	36.64	74.08	25.92	59.08	40.92
	150	63.46	36.54	63.16	36.84	63.77	36.23	47.14	52.86
BANANA	100	73.06	26.94	54.69	45.31	62.04	37.96	54.38	45.62
	150	63.26	36.74	54.08	45.92	61.02	38.98	48.97	51.03
LEMON	100	68.36	31.64	61.02	38.98	73.67	26.33	51.22	48.78
	150	67.85	32.15	60	40	64.89	35.11	49.79	50.21

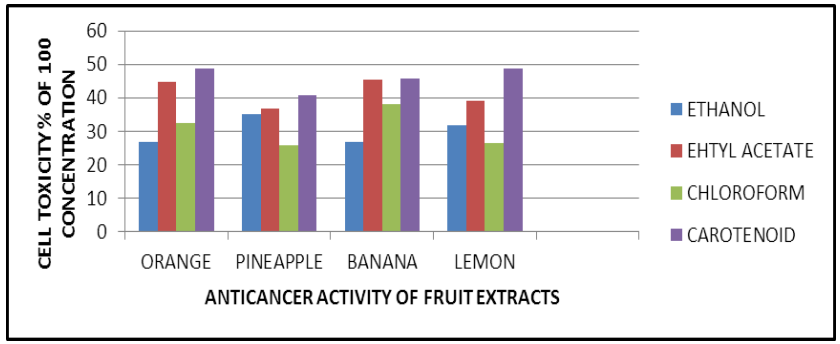

Figure 6: Anticancer activity of Fruit extracts at 100µl concentration

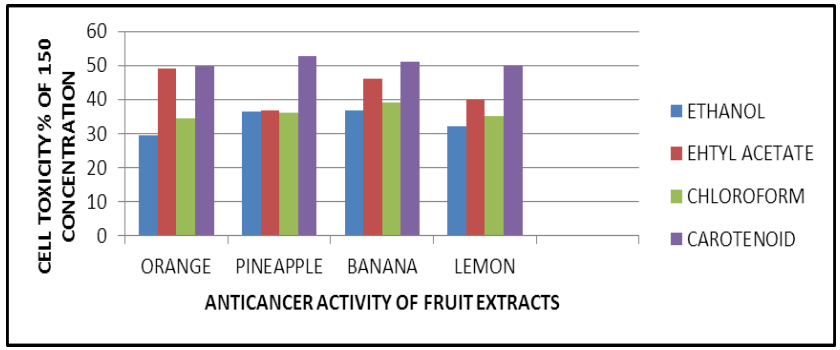

Figure 7: Anticancer activity of Fruit extracts at 150 µl concentration

CONCLUSION

The results of MTT assay on the human breast cancer cell lines, MCF 7 showed dose dependent increase in cytotoxicity of the extracts on the cancer cells. As the concentration of the extracts increasd, the cytotoxicity to the cells also increased suggesting the anticancer activity of the extracts. However, the cytotoxicity percentage was maximum in the isolated carotenoid pigment extracts than the crude extracts of all three solvents. Thus the present study reveals that the Fruits **Orange and Banana** to be the best in Anticancer activity and is highly recommended for consumption for prevention of dreadful diseases like cancer and for a healthy living in the long run.

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