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ANTICANCER ACTIVITY OF THYMOQUINONE AND ALLICIN ON CANCER PROSTATE (PC3) AND COLON CANCER (CACO2) CELL LINES, IN VITRO STUDY

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ABSTRACT

Thymoquinone and Allicine, natural products extracted from black seeds and garlic, respectively, are known as having anticancer activity. The activity of these two natural products was investigated on cancer prostate (PC3) cells and colon cancer (CaCO2) cells. The IC_{50} of these two compounds on PC3 cells was found to be 623.63 and 10.87 µg/ml, respectively and on CaCO2 cells was found to be 389.60 and 20.46 µg/ml, respectively. When using these concentrations in cell cycle analysis, it was found that the percent of G2-M phase and apoptosis was increased, while the percent of S phase was reduced for cells treated with these two compounds compared to control, untreated cells. Real time RT-PCR revealed that the activity of P53, caspase 3 and survivin was up-regulated, while the activity of Bcl2 was down-regulated compared to control cells. These results indicate that thymoquinone and Allicin have anticancer activity against cancer prostate (PC3) cells and colon cancer (CaCO2) cells.

KEYWORDS: Thymoquinone, Allicin, PC3, Caco2.

INTRODUCTION

Many natural agents and plant extracts have anticancer activity in many bioassay systems and animal models.[1,2] Black seeds (Nigella sativa) has long been used for treatment of many diseases.[3] The major bioactive constituent of the volatile oil of black seeds is thymoquinone (TQ). TQ shows anticancer activity against many types of cancer cells in vitro, including lung (LNM35), liver (HepG2), colon (HT29), melanoma (MDA-MB-435), and breast (MDA-MB-231 and MCF-7) cancer cells.[4] TQ exhibits antiproliferative effect, induces apoptosis, disrupts mitochondrial membrane potential and triggers the activation of caspases 8, 9 and 3 in myeloblastic leukemia HL-60 cells.[5]

Allicin, an organosulfur compound.[6] is mostly found in garlic (Allium sativum L.), and has various biological properties. Allicin exhibited numerous biological potentials such as anti-oxidant,[7] anti-microbial, and anticarcinogenic activities.[8,9] Allicin is able to inhibit the proliferation and survival of numerous tumors[10,11] involving colon, lung, cervix, breast, and gastric cancer. Apoptosis may be a mechanism of action of this phytochemical.[12,13] Numerous investigations established that allicin induce expression of pro-apoptotic proteins like Bax protein.[7] Allicin could enhance the level of mitochondrial cytochrome C, and Bax release from mitochondria that may be controlled by Bax which plays an important role in apoptosis. In addition, it has been reported that allicin effectively blocks the activity of telomerase in a dose and time-depended manner. It also induced cell cycle arrest at the G2/M phase and declined Bcl-2 expression as well.[14] Allicin inhibits TNF-α-induced VCAM-1 protein expression in MCF-7, but not in MDA-MB-231. Also, allicin significantly reduces tumor cell migration and invasion only in MCF-7 cells.[15] Allicin enhances 5-fluorouracil (5-FU) inducing cytotoxicity in HCC cells.[16]

The present study aims at investigating the anticancer activity of thymoquinone and allicin on cancer prostate (PC3) and colon cancer (CaCO2) cell lines.

MATERIALS AND METHODS

Phytochemicals

Thymoquinone was purchased from Santa Cruz Biotechnology (catalog # sc-215986). Allicine was prepared from garlic extract[17] as previously reported.[18] In brief, the peeled garlic bulbs were weighed (100 g), grounded thoroughly to obtain fine garlic juice and homogenized in 100 mL of 0.9% cold and sterile saline solution in a blender at high speed for 15 minutes then

filtered with muslin cloth. Aqueous extract of garlic was stored at −20° until use.[17] Proteins were separated using methanol (50:50, v/v) and supernatant was filtered through a 0.22 µm membrane. A 0.5-mL solution of filtrate was injected onto HPLC system equipped with a C18, Nucleosil 100 ODS (5 µm) semi-preparative column with a dimensions of 150 mm × 10 mm. The column was eluted with methanol– water (50:50, v/v). The allicin in the effluents was monitored at 220 nm. The fractions containing allicin was collected into 50 mL falcon tubes and stored at -80°C until use. To concentrate allicin, collected fractions were pooled and equal volume of non-polar solvent "diethyl ether" was added then the mixture was poured into a separating funnel; the funnel was shaken vigorously and left to stand for 15 minutes, until two distinct phases appeared. The organic phase was collected. Appropriate amount of double-distilled water was added and then the organic phase was evaporated under reduced pressure using a rotary evaporator at 33°C. The allicin solution was stored at -80°C for further analysis. The concentration of allicin was determined by analytical HPLC.[18]

Cell Lines

PC3 cells and CaCO2 cells were purchased from cell culture department, The Egyptian Holding Company for Biological products and vaccines (VACSERA), Giza, Egypt.

Viability assay

Cytotoxicity for test compounds was determined on PC3 and CaCO2 cells using MTT protocol . The effect of test compounds on cellular viability was evaluated using an assay based on the cleavage of the yellow dye MTT to purple formazan crystals by deshydrogenase activity in mitochondria, a conversion that occurs only in living cells.[19,20] Two-fold dilutions of tested samples were made in RPMI medium with 2% serum (maintenance medium). 0.1 ml of each dilution was tested in 3 different wells of 96 well tissue culture plate that was previously inoculated with 1 X 10^5 cells / ml (100 µl / well) and was incubated at 37°C for 24 hours to develop a complete monolayer sheet, leaving 3 wells as control, receiving only maintenance medium. Plate was incubated at 37°C and examined. Cells were checked for any physical signs of toxicity, e.g. partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. 20µl MTT solution (5mg/ml in PBS) (BIO BASIC CANADA INC) were added to each well, placed on a shaking table, 150rpm for 5 minutes, to thoroughly mix the MTT into the media. Plate were incubated (37°C, 5% CO2) for 1-5 hours to allow the MTT to be metabolized. The media was dumped off (dry plate on paper towels to remove residue if necessary). Formazan (MTT metabolic product) was resuspended in 200µl DMSO, then placed on a shaking table, 150rpm for 5 minutes, to thoroughly mix the formazan into the solvent. Optical density of the cellular homogenate was measured at 570 nm and background at 620 nm.

Cell cycle analysis

The effect of test compounds on cell proliferation was evaluated by measuring the distribution of the cells in the different phases of the cell cycle by flow cytometry, using ab139418 –Propidium Iodide Flow Cytometry Kit for Cell Cycle Analysis/BD. This determination was based on the measurement of the DNA content of nuclei labeled with propidium iodide.[21] Cell suspensions from either control cultures or treated cultures were prepared by trypsinization and washed with PBS. Cells (~100,000 – 600,000 cells) were pelleted at 500Xg for 5 min, then the supernatant was discarded. Cells washed again with PBS, spinned again at 500Xg for 5 min, then fixed with 66% ethanol on ice. The cells were stored at +4˚C for at least 3 hours, then equilibrated to room temperature. Cells were resuspended, then pelleted at 500Xg for 5 min. The supernatant were carefully aspirated without disrupting the pellet. Cells were washed with PBS, then pellted again as before and supernatant was removed. The cells were gently resuspended in 200 µL Propidium iodide + RNase staining solution, incubated at 37˚C in the dark for 20-30 min, then placed on ice (still in the dark). After resuspending the cells and passing through appropriate filter to remove cell aggregates, samples were run on flow cytometer. Propidium iodide fluorescence were collected in FL2 using 488 nm laser illumination.

Evaluation of P53, Bcl2, caspase 3 and survivin using Real Time RT-PCT

Total RNA was extracted from PC3 and Caco2 cell lines using RNeasy® Mini Kit (Qiagen®) by the method of purification of total RNA from animal cells using spin technology protocol in accordance with manufacturer's instructions.

Reverse transcription for total RNA and real-time PCR amplification for the cDNA using specific primers for P53, Bcl2, caspase 3, survivin and β actin (as internal standard) was performed using iScript™ One Step RT-PCR Kit with SYBR® Green (Bio-Rad Labotatories, Hercules, CA, USA). Primers were designed for P53, Bcl2, caspase 3, survivin and β actin using Rotor-Gene 6000 Series Software 1.7 (Corbett Research, a Division of Corbett Life Science). The primer sequences are shown in table 1 below. The reactions were performed in total volume of 50 µL, each reaction contained 2X SYBR® Green RT-PCR Reaction Mix (2X reaction buffer containing 0.4 mM of each dNTP (dATP, dCTP, dGTP, dTTP), magnesium chloride, iTaq DNA polymerase, 20 nM fluorescein, SYBR® Green I dye and stabilizers) 25 µL, Forward primer (10 µM) 1.5 µL, Reverse primer (10 µM) 1.5 µL, RNA template in nuclease-free H_2O (100 ng total RNA) 21 µL, and iScript Reverse Transcriptase for One-Step RT-PCR 1 µL.

The complete reaction mix was incubated in the real time thermal detection system (Rotorgene RT- PCR system) as follows. cDNA synthesis: 10 min at 50˚C; iScript Reverse transcriptase inactivation: 5 min at 95°C; PCR

cycling and detection (30 to 45 cycles): 10 sec at 95°C, 30 sec at 55°C to 60°C (data collection step).

Table 1: List of primers used in real time RT-PCR.

Primer	5'-3' sequence
p53 Forward	CCCCTCCTGGCCCCTGTCATCTTC
p53 Reverse	GCAGCGCCTCACAACCTCCGTCAT
bcl-2 Forward	CCTGTG GAT GAC TGA GTA CC
bcl-2 Reverse	GAGACA GCC AGG AGA AAT CA
Caspaes-3 Forward	TTC ATT ATT CAG GCC TGC CGA GG
Caspase-3 Reverse	TTC TGA CAG GCC ATG TCA TCC TCA
survivin Forward	TCG AAG AGA CCG CAA AGA AAG TGC
survivin Reverse	GAA TTG TGG CCG TTC TCC TTT CCT
β-actin Forward	GTGACATCCACACCCAGAGG
β-actin Reverse	ACAGGATGTCAAAACTGCCC

RESULTS

Viability assay

According to tables 2 and 3, IC50 values were found to be 623.63 and 10.87 µg/ml for both thymoquinone and Allicin respectively in PC3 cell, while in Caco2 cells, it was found to be 389.60 and 20.46 µg/ml respectively. Figs 1 and 2 are light microscope images of PC3 cells and Caco2 cells at different concentrations of the test compounds.

Table 2: IC_{50} values for thymoquinone and allicine on PC3 cells.

Cells and treatment	Concentration used (µg/ml)	O.D			Mean O.D	ST.E	Viability %	Toxicity %	IC50 (µg/ml)
PC3 control		0.254	0.263	0.257	0.258	0.002646	100	0	
PC3 cells treated with thymoquinone	1000	0.046	0.058	0.061	0.055	0.004583	21.31783	78.682170	623.63
	500	0.123	0.128	0.108	0.119667	0.006009	46.38243	53.617571	
	250	0.213	0.241	0.237	0.230333	0.008743	89.27649	10.723514	
	125	0.251	0.267	0.254	0.257333	0.00491	99.7416	0.2583979	
	62.5	0.253	0.269	0.264	0.262	0.004726	101.5504	0	
	31.25	0.256	0.257	0.268	0.260333	0.003844	100.9044	0	
PC3 cells treated with Allicin	96	0.023	0.024	0.021	0.022667	0.000882	8.78553	91.214470	10.87
	48	0.036	0.047	0.042	0.041667	0.00318	16.14987	83.850129	
	24	0.058	0.062	0.069	0.063	0.003215	24.4186	75.581395	
	12	0.097	0.111	0.109	0.105667	0.004372	40.95607	59.043927	
	6	0.213	0.225	0.236	0.224667	0.006642	87.0801	12.919896	
	3	0.256	0.258	0.258	0.257333	0.000667	99.7416	0.2583979	
	1.5	0.268	0.261	0.25	0.259667	0.005239	100.646	0	
	0.75	0.253	0.264	0.256	0.257667	0.003283	99.8708	0.1291989	

A

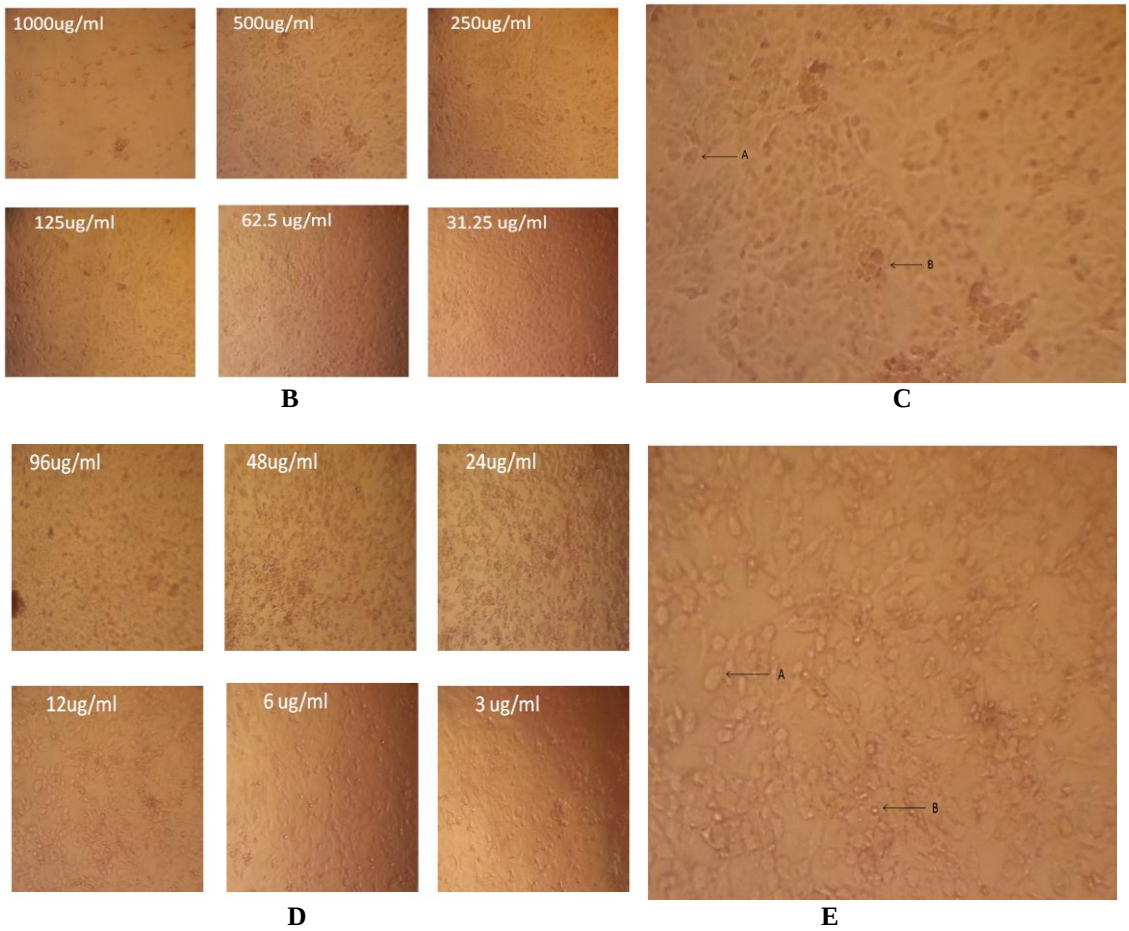


Figure 1: viability assay for PC3 cells treated with thymoquinone and allicin A: Control PC3 cells B: Effect of Thymoquinone on PC3 cells at different concentrations C: Effect of Thymoquinone on PC3 cells at 500 µg/ml thymoquinone. CPE is shown as follows; A: Rounding B: granulation. D: Effect of Allicin on PC3 cells at different concentrations E: Effect of Allicin on PC3 cells at 12 µg/ml Allicin. CPE is shown as follows; A: Rounding B: Shrinking.

Table 3: IC_{50} values for thymoquinone and allicine on Caco2 cells.

Cells and treatment	Concentration used (µg/ml)	O.D			Mean O.D	ST.E	Viability %	Toxicity %	IC50 (µg/ml)
Control Caco2		0.203	0.214	0.207	0.208	0.003215	100	0	
Caco2 cells treated with thymoquinone	1000	0.015	0.011	0.013	0.013	0.001155	6.25	93.75	389.60
	500	0.035	0.056	0.061	0.050667	0.007965	24.35897	75.641025	
	250	0.169	0.189	0.182	0.18	0.005859	86.53846	13.461538	
	125	0.203	0.216	0.211	0.21	0.003786	100.9615	0	
	62.5	0.213	0.208	0.206	0.209	0.002082	100.4808	0	
	31.25	0.21	0.204	0.208	0.207333	0.001764	99.67949	0.3205128	
Caco2 cells treated with Allicin	96	0.009	0.007	0.008	0.008	0.000577	3.846154	96.153846	20.46
	48	0.024	0.062	0.035	0.040333	0.011289	19.39103	80.608974	
	24	0.086	0.061	0.079	0.075333	0.007446	36.21795	63.782051	
	12	0.184	0.166	0.173	0.174333	0.005239	83.8141	16.185894	
	6	0.2	0.209	0.204	0.204333	0.002603	98.23718	1.7628205	
	3	0.21	0.208	0.209	0.209	0.000577	100.4808	0	
	1.5	0.216	0.201	0.21	0.209	0.004359	100.4808	0	
	0.75	0.209	0.203	0.207	0.206333	0.001764	99.19872	0.8012820	

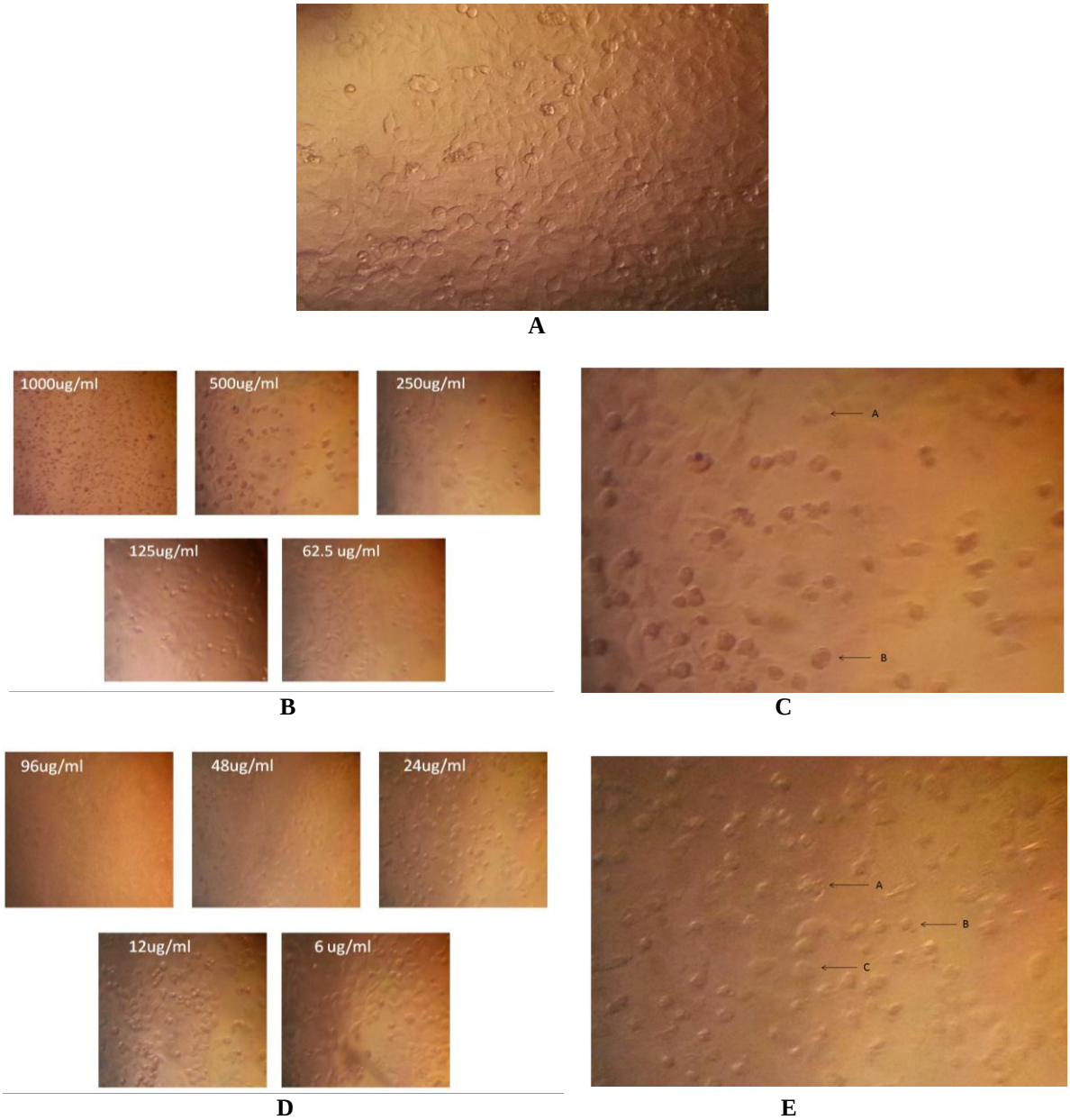


Figure 2: viability assay for Caco2 cells treated with thymoquinone and allicin A: Control Caco2 cells B: Effect of Thymoquinone on Caco2 cells at different concentrations C: Effect of Thymoquinone on Caco2 cells at 500 µg/ml thymoquinone CPE is shown as follows; A: Rounding B: granulation. D: Effect of Allicin on Caco2 cells at different concentrations E: Effect of Allicin on Caco2 cells at 24 µg/ml Allicin. CPE is shown as follows; A: Granulation B: Shrinkig C: Rounding.

Cell cycle analysis

It was observed that the percentage of S phase was reduced compared to control in both PC3 cells and Caco2 cells treated with thymoquinone and Allicin, while the percentage of both G2-M and apoptosis was increased in both cells treated with both compounds, which indicate the positive effect of test compounds on these cancer cells in vitro.

Percent apoptosis was increased in both cells for both compounds compared to control. Regarding PC3 cells, it was found that the percentage of apoptosis when using Thymoqinone at 3.8 mM (IC_{50}) was 8.36%, and when using Allicin at 66.9 µM (IC_{50}), was 12.35%, as compared to control, that was found to be 2.49%. Early and late apoptosis for PC3 cells treated with Thymoquinone at 3.8 mM was found to be 2.14 and 4.4, respectively, and necrosis was found to be 1.82%. When PC3 cells was treated with Allicin at 66.9 µM, early and late apoptosis was found to be 4.27 and 6.17%, respectively, and necrosis was found to be 1.91%. Tables 4 and 5 are representation of cell cycle phases in percentage and total, early and late apoptosis and necrosis for PC3 cells. Figure 3 is a graphic representation of table 4, and figure 4 is a represention of table 5. Figure 5 is propidium iodide histogram for control PC3 cells with DNA content color coded and ungated forward and side-scatter plot for control PC3

cells. Figure 6 is propidium iodide histogram for PC3 cells treated with thymoquinone with DNA content color coded and ungated forward and side-scatter plot for PC3 cells treated with Thymoquinone. Figure 7 is propidium iodide histogram for PC3 cells treated with allicin with DNA content color coded and ungated forward and side-scatter plot for PC3 cells treated with allicin.

Regarding Caco2 cells, the percentage of apoptosis was found to be 16.28% when treated with thymoquinone at a concentration 2.37 mM (IC_{50}), while it was found to be 22.12% when treated with allicin at a concentration 126 µM (IC_{50}), compared to control Caco2 cell, in which percent apoptosis was found to be 1.81%. Early and late apoptosis was also increased compared to control. Early and late apoptosis for Caco2 cells treated with thymoquinone at 2.37 mM concentration was found to be 5.17 and 8.05 % respectively, while for Caco2 cells treated with allicin at concentration 126 µM, the early and late apoptosis was found to be 4.89 and 11.49 % respectively. Early and late apoptosis for control Caco2 cells was found to be 0.68 and 0.43%, respectively, indicating the strong effect of thymoquinone and allicin on apoptosis. Cell necrosis was found to be 3.06 and 5.74% for Caco2 cells treated with thyoquinone and allicin respectively at the indicated concentrations, as compared to 0.7% for control Caco2 cells. Tables 6 and 7 are representation of cell cycle phases in percentage and total, early and late apoptosis and necrosis for PC3 cells, respectively. Figure 8 is a graphic representation of table 6, and figure 9 is a representation of table 7. Figure 10 is propidium iodide histogram for control Caco2 cells with DNA content color coded and ungated forward and side-scatter plot for control Caco2. Figure 11 is propidium iodide histogram for Caco2 cells treated with thymoquinone with DNA content color coded and ungated forward and side-scatter plot for Caco2 cells treated with Thymoquinone. Figure 12 is propidium iodide histogram for Caco2 cells treated with allicin with DNA content color coded and ungated forward and side-scatter plot for Caco2 cells treated with allicin.

Table 4: Percentages of cell cycle phases for PC3 cells treated with thymoquinone and allicin at concentrations 3.8 mM and 66.9 µM respectively.

Cells and treatment	%G0-G1	%S	%G2/M	%Apoptosis
Control PC3 cells	42.36	48.07	9.57	2.49
PC3 cells treated with 3.8 mM thymoquinone	53.49	25.39	21.12	8.36
PC3 cells treated with 66.9 µM Allicin	47.88	19.66	32.46	12.35

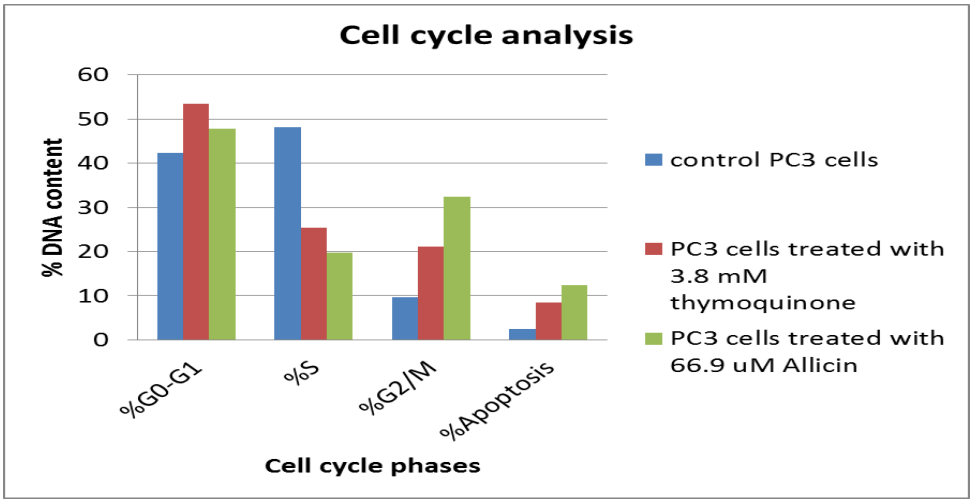


Figure 3: Graphic representation of cell cycle analysis for Thymoquinone-treated PC3 cells at concentration 3.8 mM and allicin-treated PC3 cells at concentration 66.9 µM compared to control.

Table 5: total, early and late apoptosis and necrosis for PC3 cells treated with thymoquinone and allicin at concentrations 3.8 mM and 66.9 µM respectively.

Cells and treatment	Apoptosis			Necrosis
	Total	Early	Late	
Control PC3 cells	2.49	1.21	0.46	0.82
PC3 cells treated with 3.8 mM thymoquinone	8.36	2.14	4.4	1.82
PC3 cells treated with 66.9 µM Allicin	12.35	4.27	6.17	1.91

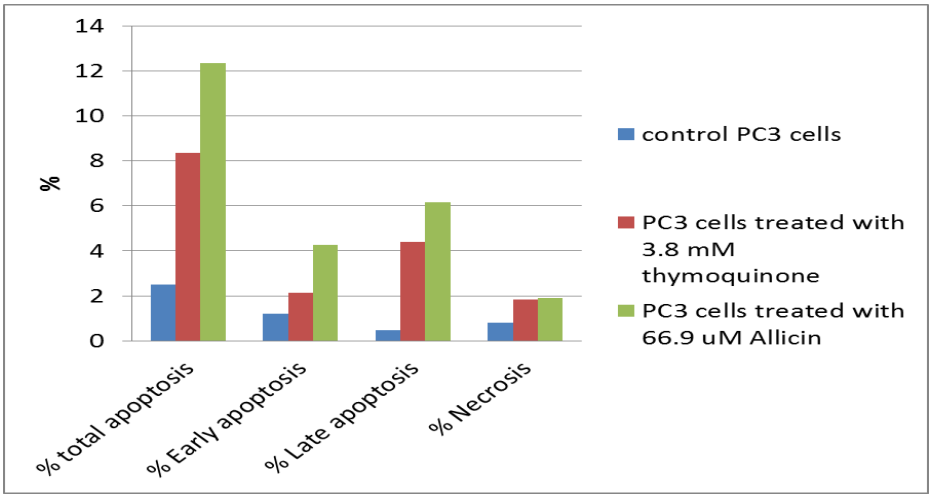
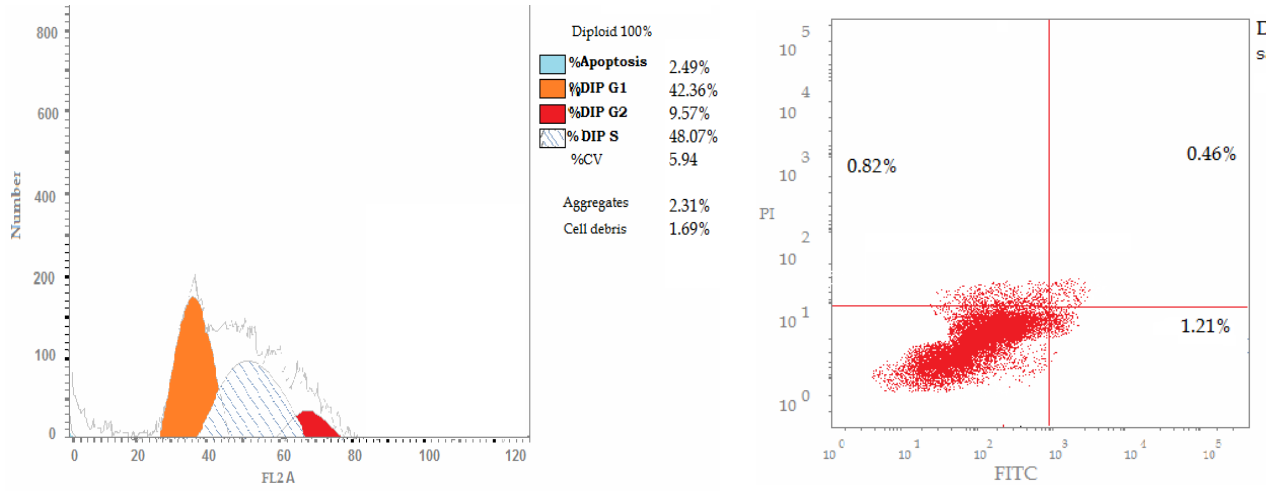
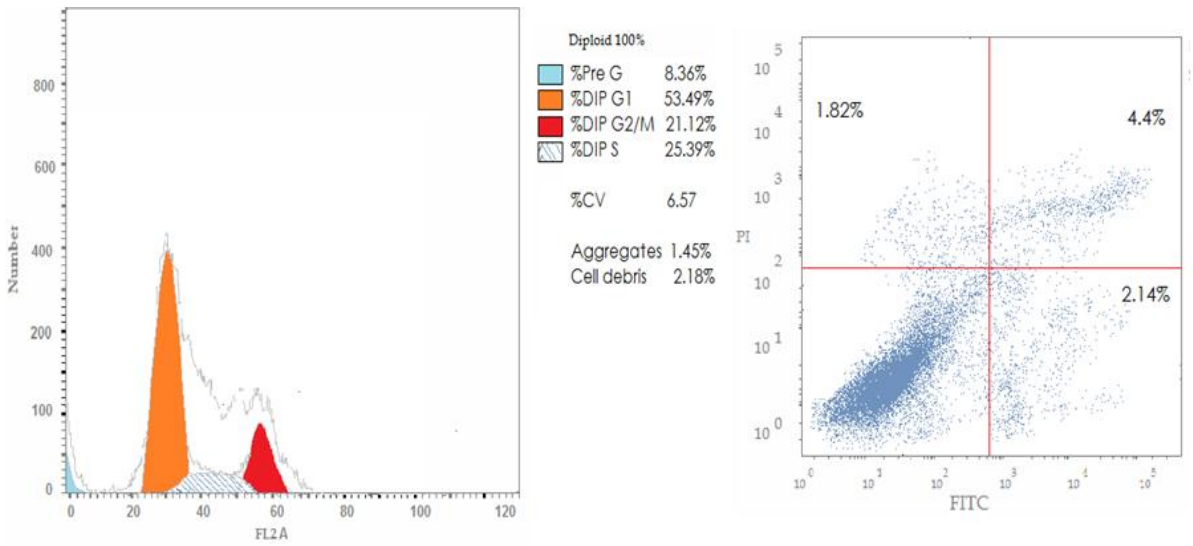


Figure 4: Graphic representation of total, early and late apoptosis and necrosis for PC3 cells treated with thymoquinone and allicin at concentrations 3.8 mM and 66.9 μM respectively.



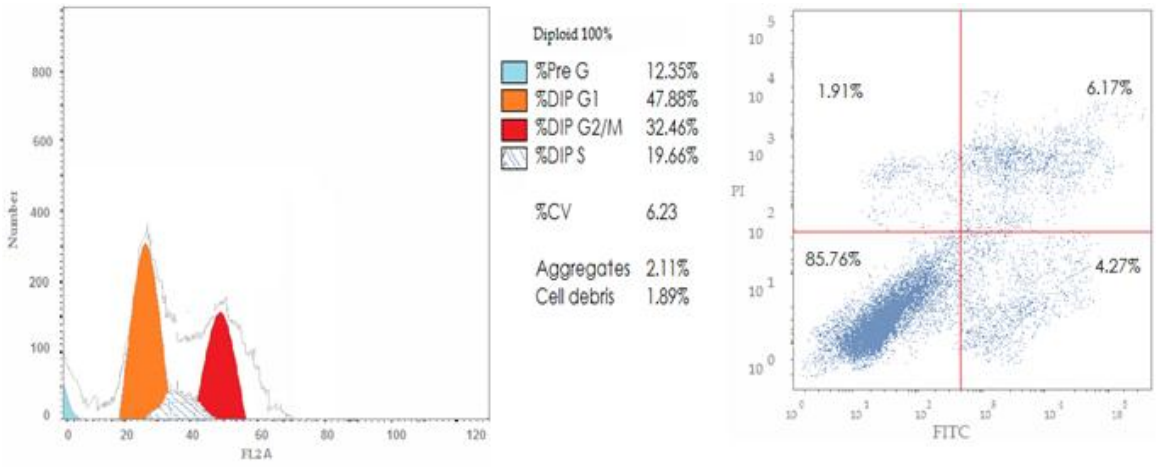
A **B**

Figure 5 A: propidium iodide histogram for control PC3 cells with DNA content color coded. B: ungated forward and side-scatter plot for control PC3 cells.



A **B**

Figure 6 A: propidium iodide histogram for PC3 cells treated with thymoquinone with DNA content color coded. B: is ungated forward and side-scatter plot for PC3 cells treated with Thymoquinone.



A **B**

Figure 7: A propidium iodide histogram for PC3 cells treated with allicin with DNA content color coded. B ungated forward and side-scatter plot for PC3 cells treated with allicin.

Table 6: Percentages of cell cycle phases for Caco2 cells treated with thymoquinone and allicin at concentrations 2.37 mM and 126 μM respectively compared to control.

Cells and treatment	%G0-G1	%S	%G2-M	%Apoptosis
Control Caco2	60.55	28.16	11.29	1.81
Caco2 cells treated with 2.37 mM thymoquinone	34.64	17.59	47.77	16.28
Caco2 cells treated with 126 μM Allicin	27.59	13.76	58.65	22.12

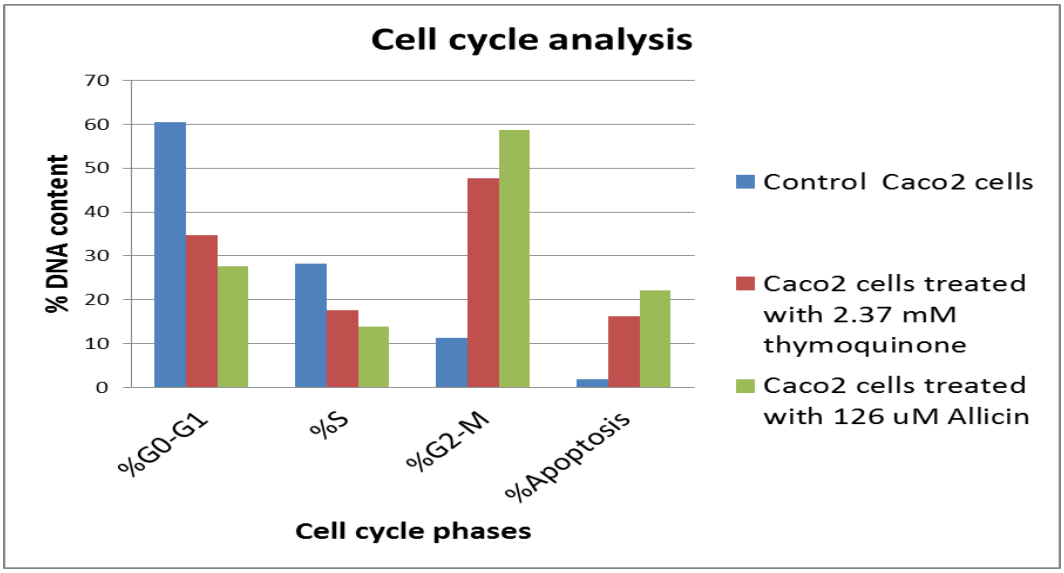


Figure 8: Graphic representation of cell cycle analysis for Thymoquinone-treated Caco2 cells at concentration 2.37 mM and allicin-treated Caco2 cells at concentration 126 μM compared to control.

Table 7: Total, early and late apoptosis and necrosis for Caco2 cells treated with thymoquinone (s1/PC3) and allicin (S2/PC3) at concentrations 2.37 mM and 126 μM respectively.

Cells and treatment	Apoptosis			Necrosis
	Total	Early	Late	
Control Caco2	1.81	0.68	0.43	0.7
Caco2 cells treated with 2.37 mM thymoquinone	16.28	5.17	8.05	3.06
Caco2 cells treated with 126 μM Allicin	22.12	4.89	11.49	5.74

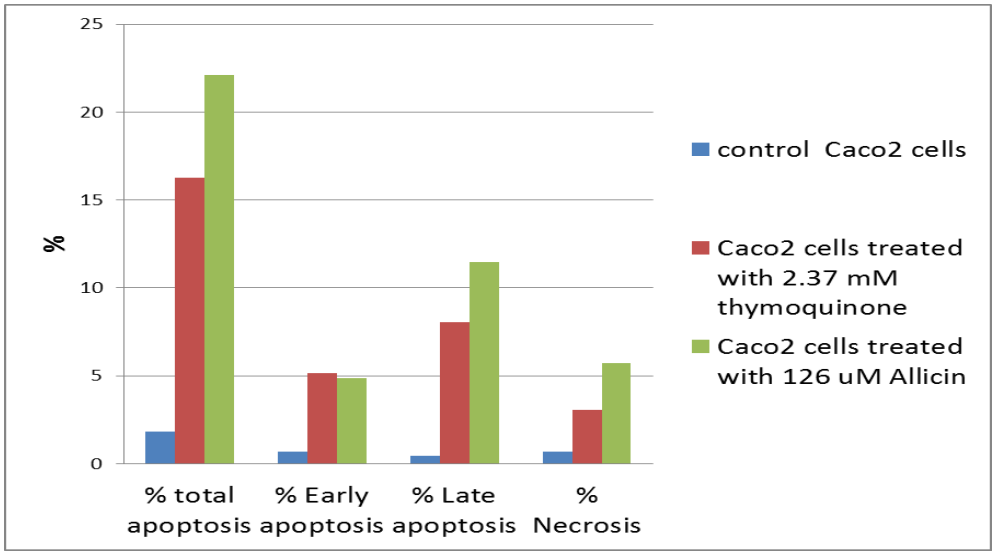
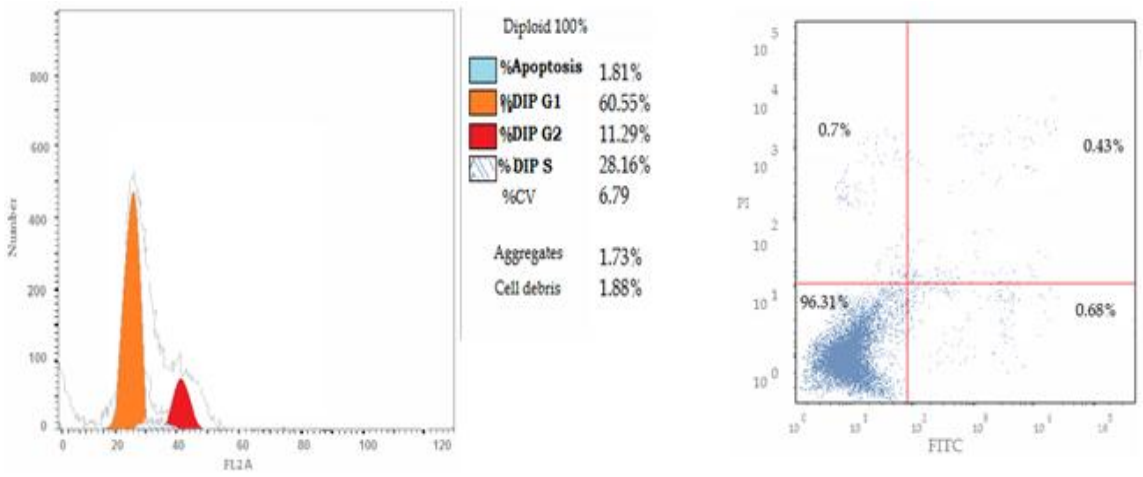
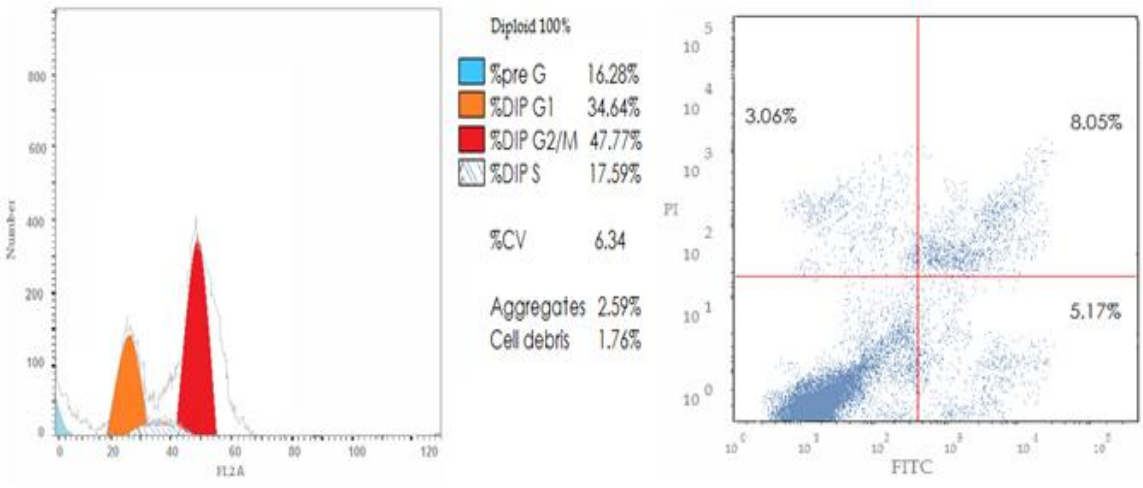


Figure 9: Graphic representation of total, early and late apoptosis and necrosis for Caco2 cells treated with thymoquinone and allicin at concentrations 2.37 mM and 126 μM respectively, compared to control.



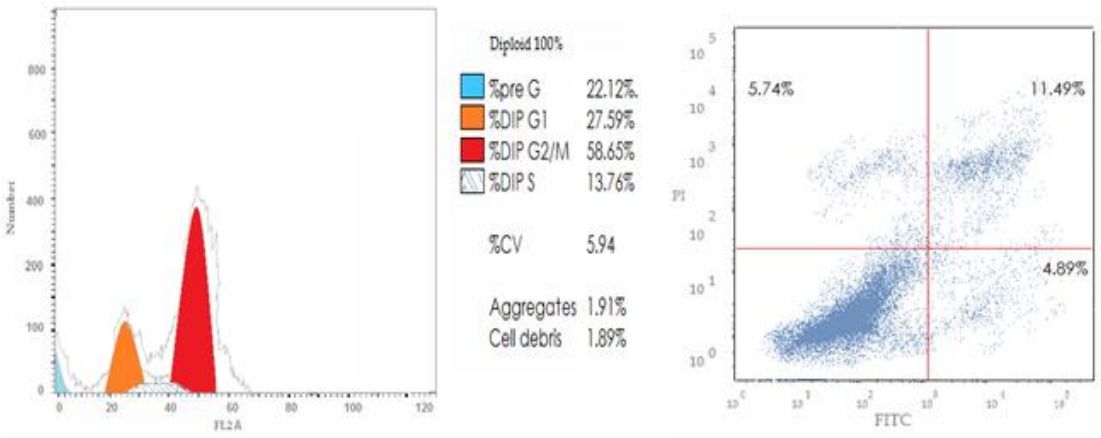
A **B**

Figure 10 A: propidium iodide histogram for control Caco2 cells with DNA content color coded. B: ungated forward and side-scatter plot for control Caco2 cells.



A **B**

Figure 11: A propidium iodide histogram for Caco2 cells treated with thymoquinone with DNA content color coded. B ungated forward and side-scatter plot for Caco2 cells treated with Thymoquinone.



A **B**

Figure 12 A: propidium iodide histogram for Caco2 cells treated with allicin with DNA content color coded. B: ungated forward and side-scatter plot for Caco2 cells treated with allicin.

Real time RT-PCR

Real time RT-PCR for P53, Bcl2 caspase 3 and survivin in PC3 and Caco2 cell each treated with thymoquinone and allicin at concentrations of IC_{50} compared to control indicated that the expression of P53, caspase 3 and survivin was increased as a fold change, while bcl2 was decreased. Tables 8 and 9 indicate the fold change for the expression of stated genes in PC3 cells and Caco2 cells treated with thymoquinone and allicin respectively, while figures 13 and 14 are graphic representations of tables 8 and 9, respectively.

Table 8: Fold change for P53, Bcl2, Caspase 3, and survivin compared to control in PC3 cells treated with thymoquinone and allicin at concentration 3.8 mM and 66.9 µM respectively.

Samples and treatment	P53	bcl2	Caspase 3	survivin
control PC3 cells	1	1	1	1
PC3 cells treated with 3.8 mM thimoquinone	7.723721	0.650476	8.018023	5.516454
Pc3 cells treated with 66.9 µM allicin	4.604295	0.42312	7.257026	4.193352

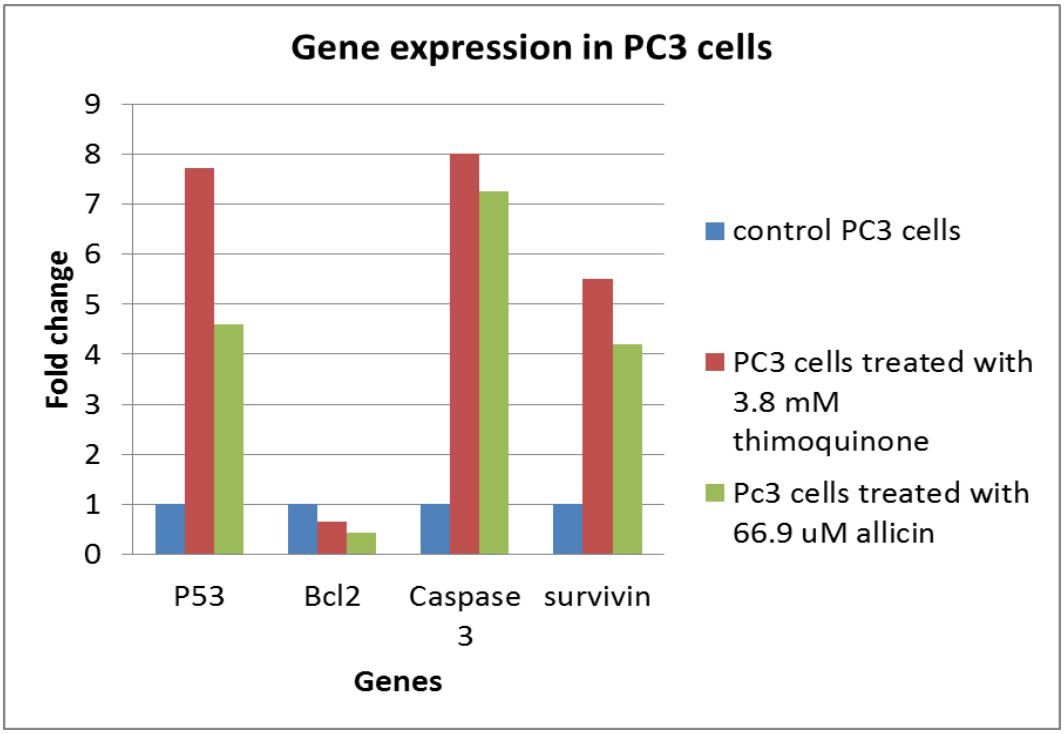


Figure 13: Graphical representation of Fold change for P53, Bcl2, Caspase 3, and survivin compared to control in PC3 cells treated with thymoquinone and allicin at concentration 3.8 mM and 66.9 µM respectively.

Table 9: Fold change for P53, Bcl2, Caspase 3, and survivin compared to control in PC3 cells treated with thymoquinone and allicin at concentration 3.8 mM and 66.9 µM respectively.

Samples	P53	bcl2	Casp3	survivin
control Caco2 cells	1	1	1	1
Caco2 cells treated with 2.37 mM thimoquinone	8.271823	0.280424	25.24157	10.28819
Caco2 cells treated with 126 µM allicin	3.128544	0.666897	6.209963	4.219569

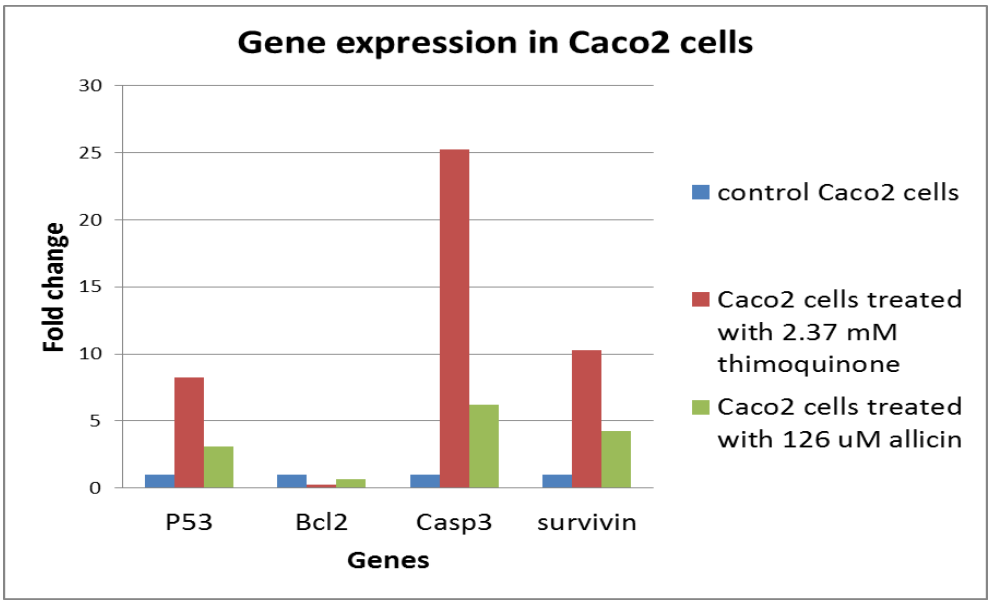


Figure 14: Graphical representation of Fold change for P53, Bcl2, Caspase 3, and survivin compared to control in Caco2 cells treated with thymoquinone and allicin at concentration 2.37 mM and 126 µM respectively.

DISCUSSION

Thymoquinone is a promising natural compound with significant in vitro and in vivo antineoplastic activities against different tumor cell lines. It is a potent inducer of cell cycle arrest and apoptosis.[22] Regarding allicin, it is now clear that it has anticancer effect by inducing apoptosis. Allicin also causes a redox-shift in human cell cultures,[23] that leads to the execution of cell death, both in a caspase-dependent[24] and caspase-independent manner.[10] Beside caspase activity, the apoptosis inducing factor (AIF), which contributes to the apoptotic DNA-laddering, is involved in allicin-induced cell death.[25]

In the present study, we investigated the effect of thymoquinone and allicin on cancer prostate (PC3) cells and colon cancer (CaCO2) cells. The effect was investigated by cell cycle analysis and real time RT-PCR for P53, Bcl2, caspase 3 and survivin. The IC_{50} was first determined to be used as a base for subsequent investigations.

In the present study, percent apoptosis for both types of cells treated with thymoquinone and allicin was increased compared to control cells, and P53, caspase 3 and survivin was upregulated while Bcl2 was downregulated. These observations are consistent with the findings of El Mahdy M et al,[5] which states that apoptosis of HL-60 cells is induced by thymoquinone . El Mahdy and his collogues also states that thymoquinone activates caspase 8 which in turn responsible for 2 subsequent events one of them is the activation of caspase 3 and that Bcl2 is downregulated that is in harmony with our results. There is another study that greatly support our study[26] in which thymoquinone was used to treat human cervical carcinoma cells (HeLa). In this study, HeLa cells treated with TQ for 72 hours showed a significant decrease in cell population at G0/G1 phase and significant increase of cell population at sub-G1 phase at 6.0, 10 and 30mg/ml ($p<0.05$), suggesting that TQ inhibited cell proliferation by induction of apoptosis in the cells. Expression of p53 detected by using the Human p53 ELISA showed that HeLa cells incubated with 10mg/ml of TQ for 72 hours resulted in significant up-regulation of the expression of the protein ($p<0.05$) compared to the control untreated sample. The doses used (which was at or close to the IC50 of thymoquinone twards HeLa cells at various incubation times as determined by using trypan blue dye exclusion method and MTT assay) was greatly higher than the doses that was used in our study which might be due to the difference in cells used. In another study,[27] thymoquinone was used as augmenting factor for two chemotherapeutic agents and was found to be responsible for down-regulation of nuclear factor-□B, Bcl-2 family on human pancreatic cancer cell lines BxPC-3 and HPAC which is also consistent with our results. Thymoquinone was also used for treatment of several types of cancer cells in vitro, namely lung (LNM35), liver (HepG2), colon (HT29), melanoma (MDA-MB-435), and breast (MDA-MB-231 and MCF-7) cancer cells. In all these cancer cells, Bcl2 level was

downregulated.[4] In another study, Thymoquinone exhibited an antiproliferative effect in a variety of colon cancer cells. Apoptosis induction was the hallmark of Thymoquinon's effect in DLD-1 cells as evidenced by flow cytometric analysis, M30 cytodeath assay, and activation of caspase-3.[28] Norsharina et al[29] used thymoquinone rich fraction extracted from Nigella sativa and commercially available thymoquinone on colon cancer (HT29), lymphoblastic leukemia (CEMSS) and promyelocytic leukemia (HL60) cells lines. Cell cycle analysis showed the increment of apoptosis in a time-dependent manner for all cell types. Our results is also in agreement with the studies of Ismail et al,[30] who investigated the effect of thymoquinone alone and in combination with resveratrol on hepatocellular carcinoma (HepG2) cells, and Salim et al,[31] who found decrease in viability of lymphocyte leukemia cells when treated with Thymoquinone. In the study of Ismail et al,[30] Thymoquinone increased caspase-3 enzyme by 77%, while in the study of Salim et al,[31], treatment of CEMss cells with TQ encouraged apoptosis with cell death-transducing signals by a down-regulation of Bcl-2 and up-regulation of Bax. In our study, the percent of apoptosis on PC3 cells and Caco2 cells treated with thymoquinone was found to be 8.36% and 16.28%, respectively, and necrosis was found to be 1.82% and 3.06%, respectively. This is harmonious with a study of Rooney and Ryan,[32] in which the effect of thymoquinone on four human cell lines was studied. In this study, thymoquinone induced less than 3% apoptosis in A549, HT-29 and MIA PaCa-2 cells, at doses 25-100μM for 24 hours. In the HEp-2 cell line, 25 μM thymoquinone elicited 21.2±3.3% apoptosis and 4.4±0.3% necrosis; and 50 μM elicited 33±5.6% apoptosis and 16.5±7.6 necrosis. One hundred μM thymoquinone induced more than 90% necrosis in HT-29, MIA PaCa-2 and HEp-2 cells, and 34.5±1.5% necrosis in A549 cells. The doses that was used in the study of Rooney and Ryan[32] was lower than the doses that was used in our study, which might be due to different cell lines that was used.

Allicin was previously reported as having hepatic protective effect and antitumor activity.[16] In our study, the IC50 was found to be 10.87 and 20.46 μg/ml for PC3 and CaCo2 cells respectively, while in a previous study,[16] it was 10.389 μg/ml and 10.004 μg/ml for SK-Hep-1 and BEL-7402 cells, respectively, which is consistent with our results. In this previous study, allicin induced apoptosis for SK-Hep-1 and BEL-7402 cells when used alone and in combination with 5-FU by increasing expression of caspase 3 and decreasing expression of Bcl2. In our study, Percent of G0-G1 and S phases was reduced compared to control, while percent of G2-M phase and apoptosis was increased for PC3 cells and Caco2 cells treated with allicin. This is consistent with the study of Tao et al,[11] in which the percentage of G0-G1 phase was reduced and the percentage of G2-M phase was increased when gastric cancer SGC7901 cells treated with three different concentration of allicin which were close to the concentration that was used in our study (3, 6 and 12 mg/L). In this study, the percentage of S phase was increased which is not consistent with our study. Allicin was found to activate caspase-3, among other caspases and induce apoptosis in SiHa cells (human cervical cancer cell line) and L-929 cells (murine fibrosarcoma cell line) at concentration of allicin 50 and 100 μM for both types of cells,[24] which is harmonious with our results. In another study, allicin at concentration of 90 μM induced decrease in Bcl-2 expression in U87MG human glioblastioma cells,[8] and this is consistent with our results.

CONCLUSION

According to our study, thymoquinone and allicin are promising phytochemicals that can be used in the treatment of several types of ancer.

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