

ORIGINAL RESEARCH ARTICLE

Supplementation with D-carvone Induces Cytotoxicity and Mitochondrial Mediated Apoptosis in Human Colon Cancer Cell Lines HT-29 and SW480**Rajamanickam Vinothkumar and Namasivayam Nalini****Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalai Nagar – 608 002, Tamil Nadu, India*

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ABSTRACT

Colon cancer is the third leading cause of morbidity and mortality throughout the world. The present study was aimed to explore the anticancer potential of D-carvone on human colon cancer cell lines HT-29 and SW480. The cells were treated with different concentrations of D-carvone (5-50 $\mu\text{g/ml}$) for the evaluation of IC_{50} doses on the colon cancer cells HT-29 and SW480 at different time point 24 h, 48 h and 72 h. The effect of 24 h IC_{50} dose of D-carvone on apoptotic morphology, mitochondrial membrane potential and oxidative stress markers were evaluated by specific staining techniques in a time dependent manner (24 h and 48 h). Our results revealed that treatment with D-carvone induced morphological changes and apoptotic characteristics like chromatin condensation and nuclear fragmentation in colon cancer cell lines such as HT-29 and SW480. Furthermore, our results revealed that treatment with D-carvone induced mitochondrial membrane potential ($\Delta\psi\text{m}$) collapse and significant ($p < 0.05$) changes in the oxidative stress markers (TBARS, SOD, CAT and GPx) as compared to the sham control. The findings of the present investigation demonstrated that D-carvone significantly suppressed growth and induced apoptosis in the colon cancer cell lines HT-29 and SW480 in a dose and time dependent manner. Thus, the study concludes that D-carvone is an effective anti-proliferative as well as anti-cancer agent.

Key words: Colon cancer, HT-29, SW480, D-carvone and Apoptosis.**1. INTRODUCTION**

Colon cancer continues to be a significant cause of cancer morbidity and mortality throughout the world. It is the third most common form of malignant disease in both men and women and the second most frequent cause of cancer related death in the United States and is the fastest emerging gastrointestinal cancer in the Asian Pacific regions ^[1]. In India, there are approximately 3.5 million cases of cancer, of which approximately 35,000 are found to be colon cancer. Colon cancer rate in India is currently increasing due to migration of rural population to the cities and changes in life style ^[2]. More specifically adopting western dietary habits, especially increasing the fat intake and decreasing carbohydrate intake, has been paralleled by an increase in colon cancer incidence in India. On the other hand, a number of studies have suggested that high consumption of fruits, vegetables, medicinal herbs and their constituents may

decreases the risk of colon cancer. Given the alarming increase in worldwide colon cancer population, there is a need for novel therapies which are effective with minimal adverse events.

Plant derived dietary agents consist of a wide variety of biologically active compounds and many of them have been used as traditional medicine for thousands of years ^[3]. The interest on bioactive compounds from herbal plants has increased in recent years due to their health benefits, particularly protection against a variety of ailments such as cardiovascular diseases and cancer ^[4]. D-carvone (**Figure 1**) is a monoterpene, present in the essential oils of many medicinal and aromatic plants (Caraway, dill and spearmint) that are endowed with many biological activities including antioxidant, antimicrobial, fungicidal and insecticidal properties ^[5]. Till date no investigation is available demonstrating the anticancer potential of D-carvone employing an *in*

vitro model of colon cancer. Therefore, the aim of the present study was to evaluate the anticancer effect of D-carvone on human colon cancer cell lines HT-29 and SW480.

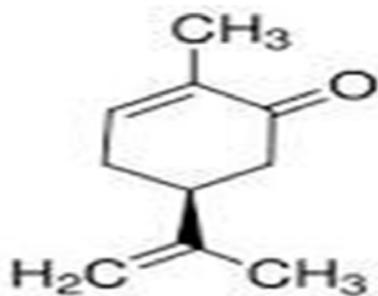


Figure 1: Structure of D-carvone

2. MATERIALS AND METHODS

Chemicals

D-carvone, ethidium bromide (EtBr), rhodamine 123, Hoechst 33258, fetal bovine serum (FBS), Dulbecco's Modified Eagles Medium (DMEM) and Roswell Park Memorial Institute 1640 medium (RPMI-1640), were purchased from Sigma Chemicals Co., St. Louis, MO, USA. Antibiotics, 0.25% trypsin EDTA, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl-2-tetrazolium bromide (MTT) were purchased from Hi-media Lab Ltd, Mumbai, India. All other chemicals and solvents utilized were of analytical grade quality and purchased from standard commercial suppliers.

Cell culture and maintenance

The established human colon cancer cell lines, HT-29 and SW480 were obtained from the National Center for Cell Science Pune, India. Normal colonic epithelial cells used in this study were obtained from previous experiment in this laboratory (data not yet published). Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) and Roswell Park Memorial Institute 1640 medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained as monolayers in 25 cm² plastic tissue culture flasks at 37°C in a humidified atmosphere of 5% CO₂ and 95% air incubator under standard conditions. The cells were harvested using 0.25% trypsin EDTA then washed in the culture medium to inactivate the trypsin before reseeded or analysis. The cells were fed every 2-3 days and sub-cultured once they reached 70-80% confluence. Cells were plated at an appropriate density; exponentially growing cells were used in all the experiments.

Preparation of drug

D-carvone was suspended in 1% dimethyl sulfoxide (DMSO) just before treatment and the final concentration of DMSO in the culture medium was 0.01% W/V. 0.01% DMSO was used as the sham control.

Study design I (IC₅₀ determination)

Cultured HT-29 and SW480 colon cancer cells were treated with different concentrations (5-50 µg) of D-carvone for 24 h, 48 h and 72 h respectively.

Study design II (Time dependent treatment)

Cultured HT-29 and SW480 cancer cells were treated with 24 h IC₅₀ dose of D-carvone in two time intervals 24 h and 48 h respectively. Cultured HT-29 and SW480 cancer cells were divided into three groups (I-III); in each group six individual samples were processed (*n* = 6).

- Group I : Sham control
- Group II : 24 h D- carvone treated
- Group III : 48 h D- carvone treated

Cytotoxicity assay

D-carvone was first dissolved quantitatively in 1% DMSO to prepare a stock solution (mg/ml). This solution was diluted to get various concentrations of the compound in the range of 5-50 µg, which were added to a series of wells containing 5x10³ cells per well for a period of 24 h, 48 h and 72 h. DMSO was used as the solvent control. A miniaturized viability assay using 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl-2-tetrazolium bromide (MTT) was carried out according to the method described by Mosmann^[6]. The cells were then assayed by the addition of 20 µl of 5 mg/ml MTT in phosphate-buffered saline (PBS). The plates were wrapped with aluminum foil and incubated for 3 h in dark at 37°C. The purple formazan product was dissolved by the addition of 100 µl of DMSO to each well. The absorbance was monitored at 570 nm (measurement) and 630 nm (reference) using a 96 well plate reader (Bio-Rad, Hercules, CA, USA). Data were collected for six replicates and used to calculate the respective means. The percentage inhibition was calculated, from this data, using the formula:

$$\frac{[(\text{Mean absorbance of control cells}) - (\text{Mean absorbance of treated cells})]}{\text{Mean absorbance of control cells}} \times 100$$

Apoptotic morphology analysis

Unstained live morphological assay

The HT-29 and SW480 cells were grown in glass cover slip (22x22 mm) placed in six well plates

at a density of 5×10^5 cells/well and allowed to settle for 24 h before treatment with the 24 h IC_{50} values of D-carvone. The medium was subsequently removed from each well of the treated and untreated HT-29 and SW480 cells, and a cover slip inverted and placed over the slide. The gross morphological changes in the treated and untreated sham control cells were observed using a differential interference phase contrast light microscope (Axio Scope A1, Carl Zeiss, Germany) and photographed.

Dual staining

Acridine orange and ethidium bromide staining were performed as described by Spector *et al.*^[7]. 25 μ l of cell suspension of each group (both attached, released to floating by trypsinization), containing 5×10^5 cells, were treated with acridine orange (AO) and ethidium bromide (EtBr) solution (1 part of 100 μ g/ml AO and 1 part of 100 μ g/ml EtBr in PBS) and examined using a fluorescent microscope with an UV filter (450-490 nm) and photographed.

Hoechst 33258 staining

Based on the method of Kasibhatla *et al.*^[8] the cell pathology was detected by staining of trypsinized cells (5×10^5 /ml) with 1 μ l of Hoechst 33258 (1 mg/ml, aqueous) for 10 min at 37 °C. A drop of cell suspension was placed on a glass slide and a cover slip was laid over to reduce light diffraction and cell pathology were observed using a fluorescent microscope (Axio Scope A1, Carl Zeiss, Germany) fitted with a 377-355 nm filter and the cells reflecting pathological changes were observed and photographed.

Mitochondrial membrane potential ($\Delta\psi_m$) using Rhodamine 123 fluorescent probe

Mitochondrial membrane potential of the treated and untreated cells were measured by the method of Johnson *et al.*^[9] using the fluorescent probe Rhodamine123. The cells (5×10^5) were grown in glass cover slip (22 \times 22 mm), placed in six well plates and treated with the D-carvone at the respective IC_{50} values. The cells were stained with Rhodamine123 dye after 24 h and 48 h exposure. The mitochondrial depolarization patterns of the cells were observed using a fluorescent microscope fitted with 485-545 nm filters and photographed.

Measurement of intracellular ROS

The development of intracellular ROS was measured by 2,7-diacetyl dichlorofluorescein (DCFH-DA), which first gets hydrolysed by the cellular esterases to DCFH, where it is oxidized

by ROS to yield fluorescent dichlorofluorescein (DCF). HT-29 and SW480 cells were treated with D-carvone and incubation was continued for 24 h and 48 h. After the treatment, cells were harvested and washed with PBS, incubated with 1 ml of 10 mM DCFH-DA in PBS for 30 min in dark. The fluorescence intensity was measured at 480 nm excitation and 530 nm emission (Shimadzu RF-5301, Spectrofluorimeter) and values were expressed as a percent of fluorescence, was calculated as follows:

$$F = [(F_{t30} - F_{t0})/F_{t0}] \times 100$$

Where; F_{t0} is the fluorescence at $t = 0$ min and F_{t30} the fluorescence at $t = 30$ min.

Measurement of lipid peroxidation byproducts and antioxidants

HT-29 and SW480 cells were seeded in a T₇₅ flask at a density of 1×10^6 cells/flask treated with 24 h IC_{50} values of D-carvone for 24 h and 48 h. The cells were harvested by trypsinization and washed with PBS. The cells were suspended in 130 mM KCl, 50 mM PBS and 10 μ M dithiothreitol and centrifuged at 20,000g for 15 min at 4 °C. The supernatant was collected and used for biochemical estimations. The concentrations of lipid peroxidation byproducts such as thiobarbituric acid reactive substances (TBARS) was measured by the method Niehaus and Samuelson,^[10] and the activities of enzymic antioxidants such as superoxide dismutase (SOD) was assayed by Kakkar *et al.*^[11], catalase (CAT) by the method of Sinha,^[12] and glutathione peroxidase (GPx) by the method of Rotruck *et al.*^[13].

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and a significant difference among treatment groups were evaluated by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant at $p < 0.05$. All statistical analyses were made using SPSS 17.0 software package (SPSS, Tokyo, Japan).

3. RESULTS

Effect of D-carvone on cell cytotoxicity

The MTT assay was used to make a preliminary assessment of the growth inhibitory potential of D-carvone on HT-29 and SW480 colon cancer cells, to find the respective IC_{50} values with varying concentrations of D-carvone (5-50 μ g) at different time points 24 h, 48 h and 72 h. The cytotoxic effect of D-carvone was determined

based on the concentrations of the compound required to reduce the survival of cells by 50% (IC_{50}). Treatment with D-carvone exhibited significant ($p < 0.05$) growth inhibition in a dose and time dependent manner and the IC_{50} values of D-carvone for each time point on HT-29 and SW480 colon cancer cells are represented in (Figure 2). The results clearly revealed that when the cells were treated with D-carvone for prolonged periods at the long time point (72 h), the IC_{50} values bear minimum [HT-29 (21.60 μ g) and SW480 (15.78 μ g)]. On the other hand when the cells treated with D-carvone for a short time (24 h and 48 h) the IC_{50} values were higher [24 h HT-29 (34.72 μ g) and SW480 (33.23 μ g): 48 h HT-29 (28.00 μ g) and SW480 (27.42 μ g)].

Effect of D-carvone on cell morphology reflecting the apoptotic features

The morphological changes in HT-29 and SW480 colon cancer cells treated with D-carvone were determined without staining by bright field microscope and the results are represented in (Figure 3). Cells (HT-29 and SW480) treated with fixed IC_{50} dose of D-carvone exhibited characteristic of apoptotic morphology, i.e., cell shrinkage, plasma membrane blebbing, loss of cell membrane integrity and impaction of nuclei. In addition, there were disassemble gaps between neighboring cells and remaining adherent cells had become more rounded which are characteristics of apoptosis that confirm cell death, in a time dependent manner (24 h and 48 h). No morphological change was observed in sham controls (0.01% DMSO treated cells).

To further characterize the cytotoxic potential and mode of cell death induced by D-carvone treatment at 24 h and 48 h, fluorescence cytochemical studies were carried out and the results are illustrated in (Figure 4). Cytochemical staining of cells using acridine orange (AO) and ethidium bromide (EtBr) revealed chromatin condensation, a hallmark of apoptosis, in D-carvone treated cells (24 h and 48 h). EtBr was selectively taken up by the condensed nuclei of apoptotic cells, whereas the nuclei of untreated sham control cells had only taken up AO (green). Early apoptotic cells with loss of membrane integrity showed several yellowish green coloured nuclei. The nuclei which had undergone chromatin condensation showed bright red and orange fluorescence depicting late apoptotic and necrotic. Sham control cells did not undergo these morphological changes and the

nuclei as well as cytoplasm revealed uniformly green fluorescence. From the data it is clear that the IC_{50} values of D-carvone induces marked changes in the cell morphology thus decreasing the number of viable cells tremendously.

We also visualised the nuclear morphology of sham control and D-carvone treated HT-29 and SW480 colon cancer cells using Hoechst 33258 staining. The sham control cells revealed normal nuclei, but many of the cells treated with D-carvone at the dose of fixed IC_{50} values (24 h) showed nuclear swelling, condensed chromatin, fragmented nuclei and apoptotic bodies, indicating the apoptosis inducing potential of D-carvone in a time dependent manner (24 h and 48 h), which are represented in (Figure 5).

Effect of D-carvone on mitochondrial membrane potential ($\Delta\Psi_m$)

The effect of D-carvone on mitochondrial membrane potential ($\Delta\Psi_m$) of HT-29 and SW480 colon cancer cells were explored by using the mitochondrial specific dye, Rhodamine 123 which are illustrated in (Figure 6). The sham control cells showed intense green fluorescence indicating no changes in the mitochondrial transmembrane potential. The cells treated with D-carvone showed progressive loss of green fluorescence at 24 h and 48 h due to mitochondrial transmembrane depolarization and showed highly diminished fluorescence at 48 h as compared to 24 h in both the HT-29 and SW480 cells, revealing complete collapse of the mitochondrial transmembrane potential.

Effect of D-carvone on intracellular ROS

(Figure 7) shows the levels of ROS generation in sham control and D-carvone treated cells, at 24 h and 48 h respectively. Treatment with D-carvone to HT-29 and SW480 cells showed significant ($p < 0.05$) increase in the intracellular ROS production in a time dependent manner (24 h and 48 h) as compared to the sham control cells, indicating ROS mediated apoptosis in cancer cells.

Effect of D-carvone on antioxidant and lipid peroxidation byproducts

(Table 1) denotes the changes in the levels/activities of thiobarbituric acid reactive substances (TBARS) and enzymic antioxidants such as SOD, CAT and GPx in the sham control and D-carvone treated cells. Treatment with D-carvone showed significantly ($p < 0.05$) elevated levels of lipid peroxidation byproducts such as TBARS. On the other hand, we observed a slight decrease in the activities of the enzymic antioxidants such as SOD, CAT and GPx, at 24 h

and 48 h time points as compared to the sham control.

4. DISCUSSION

Management of colorectal cancer (CRC) leaves few options for therapeutic treatment after initial surgery and many of them (therapeutic agents) induce severe side-effects. Natural products are known to be endowed with excellent properties as well as tolerability and reliability for the development of new drugs. Therefore, use of dietary agents against human diseases has evolved remarkably, since ancient times. Several preclinical trials were performed to investigate the effect of caraway, an essential oil on cancer models, including colon cancer [14, 15]. However, knowledge about the mechanism by which this compound can induce cell death and its antineoplastic potential is still unsatisfactory. Thus, our present study was designed to determine if D-carvone [major constituent of the essential oil from caraway] would help in colon cancer treatment.

We demonstrated the effects of D-carvone on cultured human colon cancer cell lines HT-29 and SW480 by exposing the cells to media containing D-carvone in the range of 5-50 μg concentrations at three different time points respectively 24 h, 48 h and 72 h. Supplementation with D-carvone to the culture medium inhibits growth of colon cancer cells (HT-29 and SW480) in a dose and time dependent manner, revealing the cytotoxic potential of D-carvone, although this IC_{50} dose did not affect the viability of normal colonic epithelial cells. In this context, several *in vitro* studies suggest that pharmacologically active agents from natural sources efficiently inhibit the viability of mammalian cancer cells at various time points [16-18].

Cytotoxicity tests generally possess a broad spectrum of sensitivity and are able to detect many novel anticancer drugs, which potentially inhibit the biochemical activity of a variety of cancer cells of animal and human origin. Both the cell lines required higher concentrations of D-carvone to induce 50% of cancer cell death [HT-29 24 h: 34.72 μg and 48 h 28.00 μg and SW480 24 h: 33.23 μg and 48 h 27.42 μg] whereas 72 h treatment period required lower concentrations of D-carvone [HT-29, 21.60 μg and SW480, 15.78 μg]. Previous studies in this laboratory also showed that a natural phytochemical hesperetin and its synthetic analogue inhibited cell growth and induced

cellular dysfunction and apoptosis in the HT-29 colon cancer cells by increasing oxidative stress at different time points [19].

Apoptosis is a physiological process leading to cell death far distinct from necrosis [20]. Apoptosis is responsible for the deletion of excess cells from normal tissues and for specific pathologic events. Thus, apoptosis could be a therapeutic target for cancer cells, at the same time cell death or apoptosis induced by harmful stimuli should be prevented in normal cells. Biochemically and morphologically distinct from cellular necrosis, apoptosis involves chromatin condensation, cell shrinkage, DNA fragmentation, plasma membrane blebbing and the formation of membrane enclosed apoptotic bodies [21, 22]. Therefore, induction of apoptosis has become a target strategy for antitumor drugs discovery in recent years and agents inducing apoptosis specifically in the tumor cells may be an ideal antitumor drug. Many chemotherapeutic agents have the ability to induce cell death and form apoptotic bodies. Notably treatment

with D-carvone to HT-29 and SW480 cells adopting live morphological analysis (unstained), AO/EtBr and Hoechst 33285 staining showed that the nuclei exhibited chromatin condensation and the formation of apoptotic bodies at two different time points (24h and 48 h). Moreover, D-carvone induced apoptosis in the cancer cells at the specific IC_{50} value, but at that dose, D-carvone did not inhibit the viability of normal human colonic epithelial cells. D-carvone exhibited large number of apoptotic bodies when the cell lines HT-29 and SW480 were treated with D-carvone for prolonged time periods i.e., 48 h and 72 h as compared to the 24 h treatment which could be attributed to the structural property of the compound. Moreover, the collapse in the mitochondrial membrane potential, confirms the contribution of D-carvone to induce mitochondrial mediated apoptosis in the cancer cells (HT-29 and SW480).

There are reports that suggest mitochondria as main targets for anticancer agents because they have a central role in the induction and regulation of both necrotic and apoptotic cell deaths [23]. In our study loss of mitochondrial membrane potential ($\Delta\psi\text{m}$) was observed on D-carvone treatment to HT-29 and SW480 colon cancer cells (staining with Rhodamine 123). In the non-apoptotic cancer cells the dye (Rhodamine 123) accumulated and aggregated within the

mitochondria, resulting in bright green fluorescence while the apoptotic cells revealed weak staining underlining the anticancer effects of D-carvone.

Decreased accumulation of Rhodamine 123 indicates a collapse in the mitochondrial membrane potential, which correlates with a high amount of ROS generation, observed in the present study on treatment with D-carvone to HT-29 and SW480 colon cancer cells. ROS is involved in triggering apoptotic signalling by inducing depolarization of the mitochondrial membrane ($\Delta\psi_m$) which eventually leads to an increase in the levels of pro-apoptotic molecules intracellularly^[24]. Furthermore, enhanced lipid peroxidation byproducts (TBARS) and decreased activities of antioxidant enzymes (SOD, CAT and GPx) observed in the present study correlates with D-carvone induced ROS production. In addition, decrease in the antioxidant enzymes and increase in TBARS on D-carvone treatment to the cancer cells as compared to the untreated sham control cells further confirms D-carvone as an effective stimulator of ROS production. Similar results were obtained in earlier studies with colon cancer cells and Hela cells, when these cells were exposed to other plant derived phytochemicals^[19, 25]. Thus, reduction in the activities of antioxidant enzymes can result in a number of deleterious

effects due to the accumulation of superoxide radicals and H_2O_2 in the mitochondria of cells undergoing apoptosis. Thus over all D-carvone exerts a beneficial action in cancer cells in the presence of low antioxidant defence. These effects by D-carvone could be associated with inhibition of cell proliferation, induction of tumor cell death and alterations in the levels of oxidative stress markers.

Induction of apoptosis is an ideal cancer therapy strategy. In our study D-carvone exploits this process by selectively inducing cell death through the ROS dependent apoptotic pathway in HT-29 and SW480 colon cancer cells. Thus our findings suggest that D-carvone selectively possesses potent anticancer properties. Further, molecular studies and preclinical trails are warranted to know the exact mechanism of apoptosis by D-carvone.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

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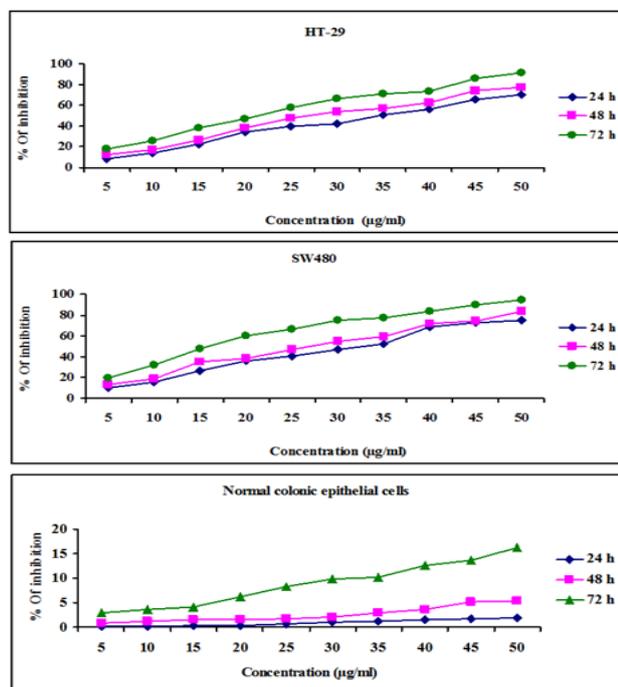


Figure 2: Effect of D-carvone on cell viability (MMT assay) in the HT-29, SW480 cells and normal colonic epithelial cells. The cells were treated with different concentrations of D-carvone (5-50 µg/ml), which affected cell viability in a time dependent manner 24 h, 48 h and 72 h respectively. Data are expressed as mean \pm SD of six independent experiments

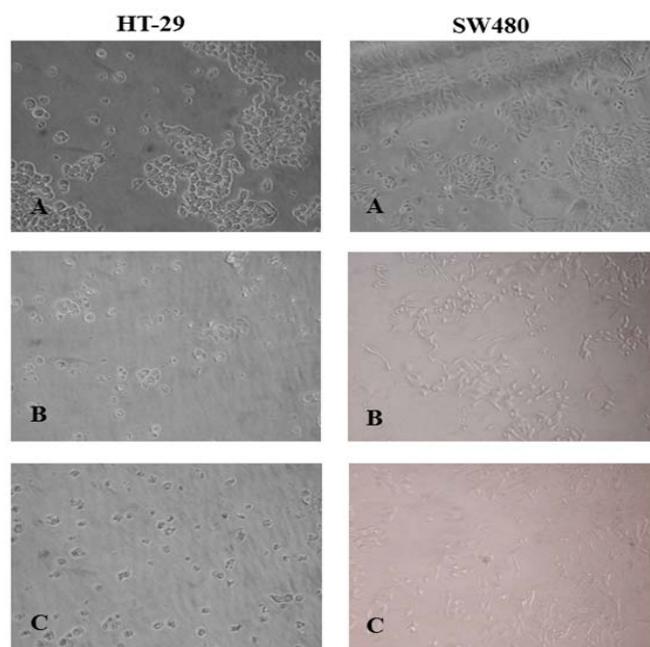


Figure 3: Effect of D-carvone on the cell morphology in the HT-29 and SW480 cells. (A) Sham control cells show well defined cellular morphology. (B and C) D-carvone treated (24 h and 48 h) cells show characteristics of apoptosis i.e., cell shrinkage, plasma membrane blebbing, loss of cell membrane integrity and impaction of nuclei.

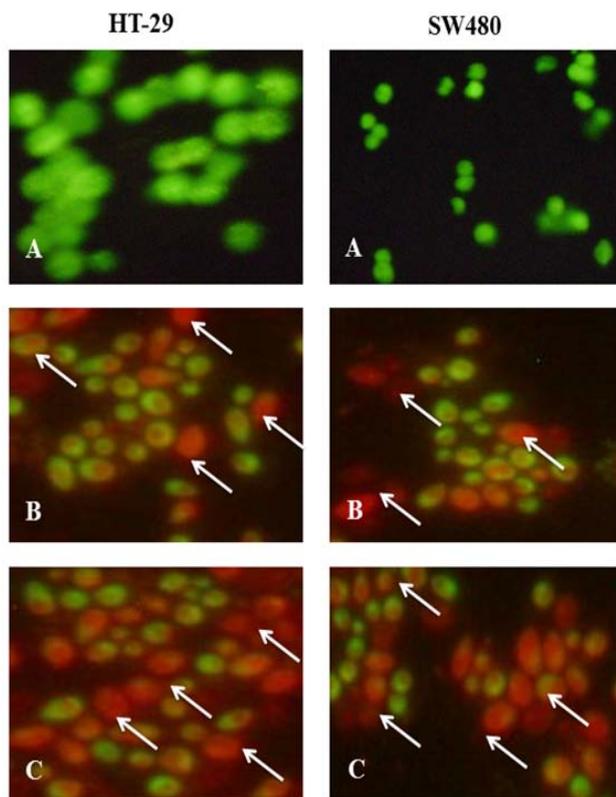


Figure 4: Effect of D-carvone on the cell morphology by AO/EtBr stain in the HT-29 and SW480 cells. (A) Sham control show uniformly green fluorescing, viable cells (B and C). D-carvone treated cells show chromatin condensation indicating late apoptosis and necrosis, which fluoresce uniformly in bright red and orange (arrowheads).

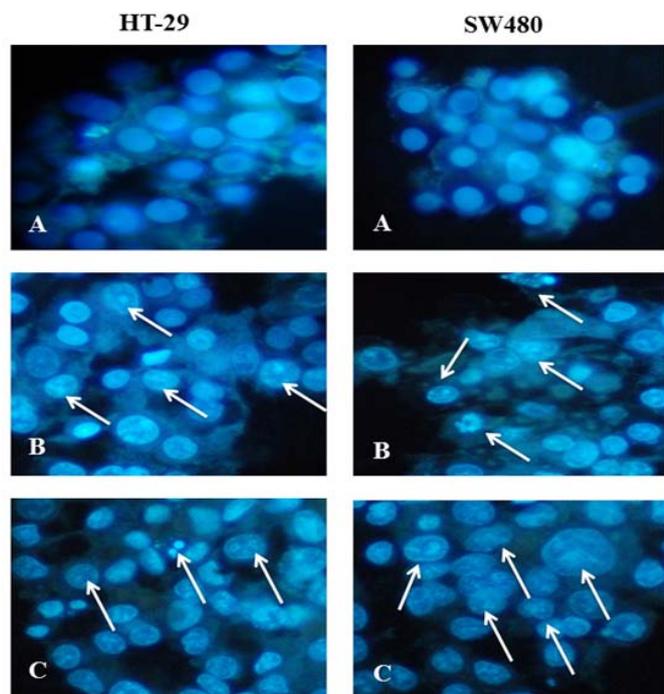


Figure 5: Effect of D-carvone on nuclear morphology (Hoechst 33258 staining) in the HT-29 and SW480 cells. (A) Sham control shows normal nuclei. (B and C) D-carvone treated cells show nuclear swelling, condensed chromatin, fragmented nuclei and apoptotic bodies (arrowheads).

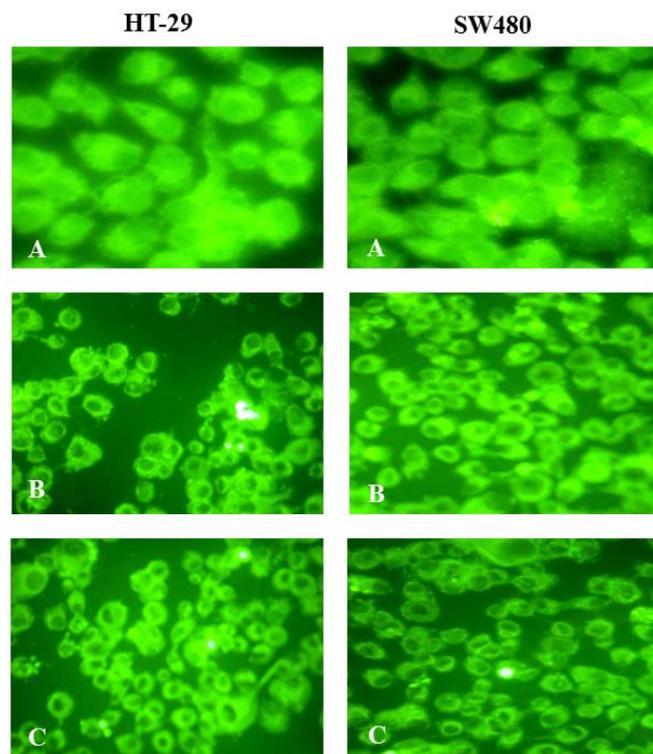


Figure 6: Effect of D-carvone on mitochondrial membrane potential ($\Delta\psi_m$) (Rhodamine 123 staining) in the HT-29 and SW480 cells. (A) Sham control shows intense green fluorescence indicating no changes in mitochondrial transmembrane potential. (B and C) D-carvone treated cells show weak green fluorescence at 24 h and 48 h due to mitochondrial transmembrane depolarization.

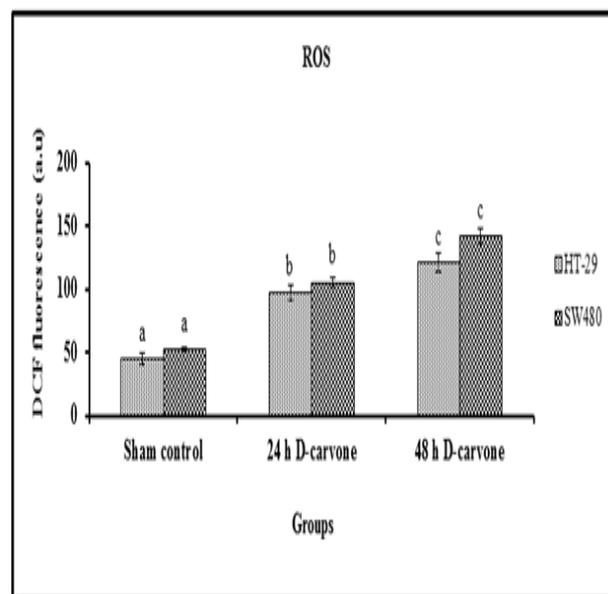


Figure 7: Effect of D-carvone on ROS generation in sham control and D-carvone treated HT-29 and SW480 cells. Data are presented as the means \pm SD of six independent experiments in each group. Values not sharing a common superscript letter are (^{a-c}) differ significantly at $p < 0.05$ (DMRT).

Table 1: Effect of D-carvone on TBARS and antioxidant enzymes (SOD, CAT and GPx)

Cell lines	HT-29			SW480		
Groups	I	II	III	I	II	III
TBARS ^A	9.14±0.68 ^a	14.57±0.94 ^b	15.88±1.33 ^c	7.68±0.58 ^a	11.35±0.86 ^b	12.97±0.92 ^b
SOD ^B	16.41±1.26 ^a	13.01±1.10 ^b	12.59±0.80 ^b	12.83±1.07 ^a	10.23±0.71 ^b	8.47±0.75 ^c
CAT ^C	10.57±0.79 ^a	8.82±0.47 ^b	7.64±0.42 ^c	8.65±0.57 ^a	7.12±0.50 ^b	6.84±0.34 ^b
GPx ^D	7.45±0.68 ^a	6.36±0.47 ^b	6.08±0.54 ^b	8.41±0.41 ^a	6.80±0.55 ^b	5.83±0.52 ^b

^Anmoles/ml of cell lysate; ^B50% NBT reduction/min/mg protein; ^Cμmoles of H₂O₂ utilized/min/mg protein; ^Dμmoles of GSH utilized/min/mg protein. Data are presented as the means ± SD of six independent experiments in each group. Values not sharing a common superscript letter are (a-c) differ significantly at *p*<0.05 (DMRT).

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