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D-carvone inhibits growth, migration, cell cycle at G_0/G_1 phase and induces apoptosis in A431 cells by disrupting mitochondrial membrane potential

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ABSTRACT

The simultaneous control over multiple hallmarks is an effective and viable strategy in the treatment of life-threatening diseases like cancer. This work plan was designed to investigate the outcome of D-carvone on viability, movement, and reactive oxygen species production, level of mitochondrial membrane potential, apoptosis and cell cycle events against A431 skin cancer cell lines. Cells were treated with D-carvone on A431 cell line in a dosage reliant approach; the cytotoxicity of the drug was observed and confirmed as IC_{50} values of the D-carvone to investigate further parameters. We examined the efficiency of D-carvone on apoptosis induction by dual staining method, cell cycle distribution using flow cytometric analysis, activation of oxidative stress were evaluated by measuring ROS and MMP. And the migration efficiency of cancer cells was determined by transwell migration assay. The statistical analysis of all data was analyzed using one-way analysis of variance (ANOVA) followed by post hoc test. In most results were observed, strong cytotoxic activity of D-carvone (30 µg) had towards- A431 (human epidermoid carcinoma cells) in the MTT analysis. Further, it shows that the D-carvone 25, 30 and 35 µg/ml concentration capable of inducing apoptosis in A431 cells in a dose-responsive approach through the generation of reactive oxygen species, as well as leading to loss of mitochondrial membrane potential. Moreover, we observed cell cycle arrest in G_0/G_1 phase at an application of D-carvone 35 µg/ml and movement of cancer cells significantly reduced in D-carvone 30 µg treated groups in dose reliant manner. Overall this study concludes that D-carvone is a potential antiproliferative as well as anti-cancer agent and may be developed into a lead to treating human skin cancer.

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INTRODUCTION

Skin is the most important and largest organ in our body, its role is very important to protect our body from environmental pollution, preserving internal cellular settings, and ultimately organism continued existence (Zmijewski *et al.,* 2011). Nowadays skin cancer is a serious threat to human health. Due to environmental pollution and UV radiation, abnormal cell proliferation and molecular changes in organisms will occur (Ji *et al.,* 2015). The abnormal development of cells of skin leads to cancer that can spread to other parts of the body (metastasis) (Cakir *et al.,* 2012; Gloster *et al.,* 1996). The incidence of skin cancer is increasing worldwide, among all cancers, 40% of cases are skin cancer (Fartasch *et al.,* 2012). In India skin cancer, accounting for approximately 1-2% (Khullar *et al.,* 2014).

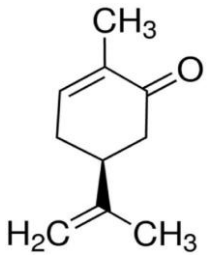

Figure 1: D-carvone (Chemical structure)

Chemotherapy is the majority hunted behind cure in the early stages among drugs that induce apoptosis of proliferating cancer cells. Without side effects as well as low cost are important for those drugs (Kuriakose *et al.,* 2016). Therefore, necessitate for the discovery of alternative natural means had been precisely emphasized. Previous studies from our laboratory evaluated natural compounds like, vanillic acid (Sindhu *et al.,* 2015), β-sitosterol (Shamila *et al.,* 2017; Sharmila *et al.,* 2016), Linalool (data not published) and Umbelliferone (Vijayalakshmi *et al.,* 2017) for apoptosis-inducing effect and cell cycle regulatory role in various in vivo and in vitro carcinogenesis models.

D-carvone (Figure 1) is a cyclic, dietary monoterpene compound abundantly found in the Caraway seeds as an essential oil. D-carvone has been shown to exert various positive biological effects, such as anticancer, anti-hypertensive, lipid-lowering and anti-proliferative effects (Sharma *et al.,* 2010). Dill, spearmint and Angelica are other important constituents of essential oil. Recently reported that the D-carvone has effectively induced cytotoxicity in human colon cancer cell lines (HT-29 and SW-480), in different concentrations as well as they proved in another one study, thus D-carvone prevented DMH- 1,2-dimethylhydrazine induced colon neoplastic transformation in animal model (Vinothkumar *et al.,* 2013; Vinothkumar *et al.,* 2013). Rajeshwari et al. proved that the D-carvone have free radical scavenging activity in in vitro study. Further, they evaluated the antioxidant influence of D-carvone in L-NAME induced hypertensive rats (Rajeshwari *et al.,* 2014; Rajeshwari *et al.,* 2015).

Human skin keratinocytes are natural targets of threatening agents. A431 (ATCC-CRC1555) is a cell line from an epidermoid carcinoma of the human vulva of an 85-year-old female patient. A431 cell line is a specific choice to analyze or discovering new anticancer drugs in vitro. This cell line is utilized by researchers to study the molecular aspects of skin carcinogenesis as well as to investigate the cytotoxic/ anti-cell proliferative ability of natural remedies or synthetic entities (Giard *et al.,* 1973).

In general, apoptosis is a regular cellular activity to clear the cells which are at the end of their life or unrepairable condition. Accumulation of unwanted genetic alterations in altered cells leads to out of control. Compounds which facilitate the removal of improperly stimulated cells are the best means of protection mechanism (Hickman 1992). Walzl et al. reported that quantification and detection of the cell death and modes give central information for common physiological questions. The cytotoxic action by dose- and time-dependency manner is used for drug development during in vitro studies in different cell types (Walzl *et al.,* 2014). Cell cycle arrest at various phases by natural and synthetic entities are taken into account as loyal modes of protective and therapeutic strategy (Zhou 2018).

Nonetheless, a few research articles have been published as regards the anticancer effect of D-carvone. However, the mechanism of action of this compound in skin cancer cell line is still not known. Thus, this report was aimed to assess the anticancer activities of D-carvone in A431- human epidermoid carcinoma cells using cell cycle analysis, stimulation of apoptosis, cell viability assay and cell migration assay as endpoints.

MATERIALS & METHODS

Reagents and chemicals

The following chemicals and reagents were used for this current study was procured from the mentioned companies: D-carvone, Dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Penicillin (antibiotic), 3-(4,5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium bromide (MTT), acridine orange (AO), ethidium bromide (EB) from Sigma Aldrich chemicals Pvt Ltd. Bangalore, Mitochondrial membrane potential assay kit, Propidium iodide (100µg/ml) and Ribonuclease I (stock 100 mg/ml) (Sigma, USA), Total reactive oxygen species (ROS) kit, (Invitrogen, USA), 1X Phosphate buffer saline (PBS), Crystal violet (Himedia, India), 96 well tissue culture plate wash beaker (Tarson, India), Transwell 24 well plate with 8.0µm pore insert (Corning, USA).

A431 cell culture and maintenance

A431 cell line was procured from NCCS, Pune, India. Cells were cultured in a fibronectin-coated tissue culture flask (T25, T75), supplemented with DMEM- Dulbecco's modified Eagle's Medium, 10% FBS- fetal bovine serum and penicillin+ streptomycin (100µg/ml each) mixture in a 5% CO_2 incubator at 37˚C. The cell line was maintained with regular passaging.

Preparation of D-carvone

D-carvone was suspended in 1% dimethyl sulfoxide (DMSO) just before treatment and ending

concentration of DMSO in the culture medium was 0.01% DMSO was used as vehicle-treated control. IC_{50} value of the drug was determined using a different concentration of D-carvone (5-45µg) in A431 cell line for 24 hours.

Cytotoxicity assay (MTT assay)

D-carvone stock solution (mg/ml) was first dissolved quantitatively in 1% DMSO. This solution was diluted to get various concentrations of the compound in the range of 5-80 µg, which were added to a series of wells containing 5×10^3 cells per well for 24 hours. DMSO was used as a 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 1983). The cells were then assayed by the addition of 20µl of 5mg/ml MTT in PBS. The plate was wrapped with aluminium foil and incubated for 3 h in the 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). The percentage inhibition was calculated, from this data, using the formula:

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

After 24 hours, treatment of D-carvone treated cells in various concentrations (5-45 µg/ml), the gross morphological changes in the unstained, treated and untreated vehicle control cells were observed using a differential interference phase contrast light microscope.

Measurement of mitochondrial membrane potential

The A431 cells (5,000-20,000 cells/well) were seeded in a 24 well plate in a DMEM and treated with 0.01% DMSO as control and D-carvone at the concentration of 25, 30 and 35 µg in a serum-free medium. The measurement of mitochondrial membrane potential for the treated and control cells was carried out according to the manufacturer's instruction. Briefly, the cells were incubated with 100 µl/well of JC-10 dye loading solution and plate was protected from light. The plate was incubated for 30-60 minutes in a 5% CO_2 incubator at 37˚C. After incubation, 100 µl/well assay buffer B was added to each sample/well. Finally, the plate was centrifuged at 800 rpm for 2 minutes and the fluorescence was observed at 490/525nm and 540/590nm.

Reactive oxygen specious (ROS) measurement

The D-carvone was tested for ROS using A431 cells. Briefly, the cultured A431 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^6 cells/ml into 24-well tissue culture plate in DMEM medium containing 10% FBS and 1% antibiotic solution for 24-48 hours at 37˚C. The wells were washed with sterile PBS and treated with various concentrations of a D-carvone (25, 30 and 35 µg/ml) in a serum-free DMEM medium and the cells were incubated at 37˚C in a humidified 5% CO_2 incubator for 24 h. After the incubation period, 100 µl of 1X ROS assay solution was added to the wells and mixed gently. The plate was incubated for 60 minutes in a 37˚C incubator with 5% CO_2. Finally, the plate was centrifuged at 800 rpm for minutes and the production of ROS was evaluated immediately within an hour and examined at least 100 cells by fluorescence microscope (Nikon, Eclipse TS 100, Japan) using a fluorescent filter at 520 nm.

Dual staining (acridine orange (AO)/ethidium bromide (EB) method)

Dual stain by AO/EB dye method was followed as described in research article Baskin *et al.,* 2006. A431 cells were trypsinized and 25 µl of cell suspension of each group containing 5×10^5 cells seeded in 6-well plate. After treatment with a various concentration of D-carvone 25, 30 and 35 µg/ml to A431 cells for 24h, the control and D-carvone treated cells were washed by cold PBS and then stained with a mixture of AO (100 µg ml^{-1}) and EB (100 µg ml^{-1}) at room temperature for 5 min. The stained A431 cells were observed by a fluorescence microscope (Nikon, Eclipse TS 100, Japan) at 40 x magnifications.

A431 Cell cycle analysis by FACS (Flow cytometer)

The various concentration of D-carvone was tested for cell cycle analysis, using A431 cells by propidium iodide staining method (Nicoletti *et al.,* 1991). Briefly, the cultured A431 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 2×10^6 cells/ml into a 6-well tissue culture plate in DMEM medium containing 10% FBS and 1% antibiotic solution for 24-48 hour at 37˚C. The wells were washed with sterile phosphate buffer saline and treated with 0.01% of DMSO (35 µl) and 25 µl, 30 µl, 35 µl of D-carvone in a serum-free DMEM medium and incubated at 37˚C in 5% CO_2 incubator for 24 hours. After incubation, the cells were harvested by trypsinization and washed in PBS by centrifuging at 1500 rpm for 5 min. Furthermore, the cells were fixed for 30 min at 4˚C using cold 70% ethanol by adding dropwise to the cell pellet. After incubation, the cells were washed with sterile PBS for twice by centrifuging at 1500 rpm for 5 min and discarded the supernatant. Finally, the cell pellet was treated with 50 µl of RNase (100µg/ml) and 500 µl of PI (100µg/ml) and kept at 4˚C until flow cytometry analysis.

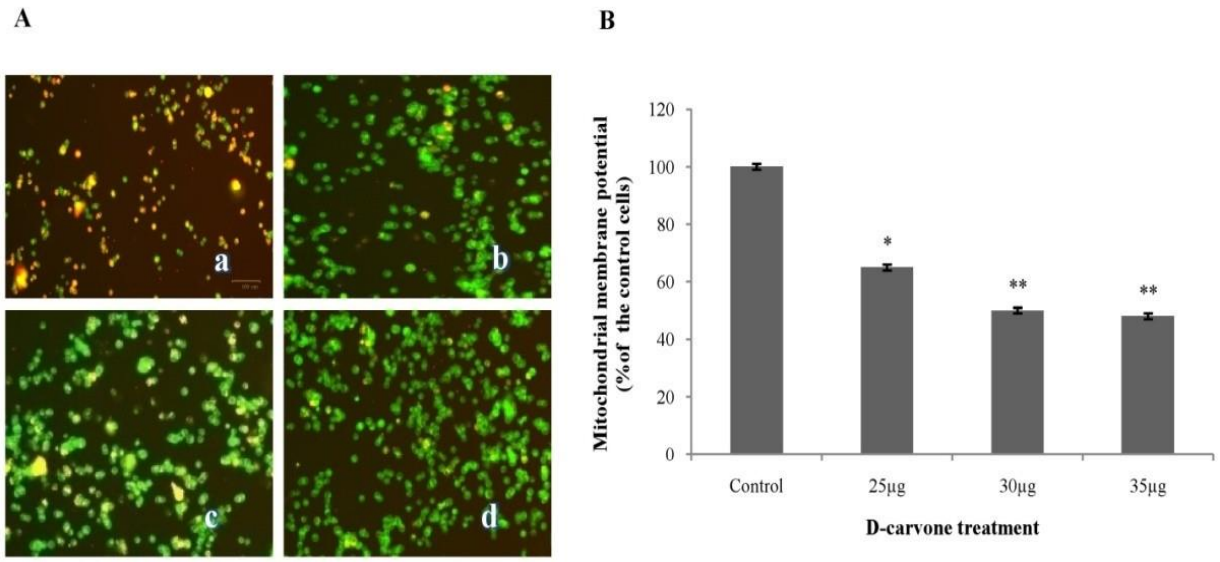

Figure 3: Effect of D-carvone on mitochondrial membrane potential in A431 cells

A. (a) Vehicle control-treated (0.01% DMSO) cells show JC-10 concentrated in the mitochondrial matrix where it forms red fluorescent aggregates. D-carvone (25, 30, 35 µg/ml) treated cells (b,c and d) shows the apoptotic and necrotic cells, there JC-10 exists in monomeric form and stains the cells in green. B. Quantification of MMP in the Fluorescence microscope. Data are expressed as mean ± Standard Deviation (SD) of experimentally 4 independent values. * indicates variations that were significant with statistical analysis. * p<0.05; ** p<0.01.

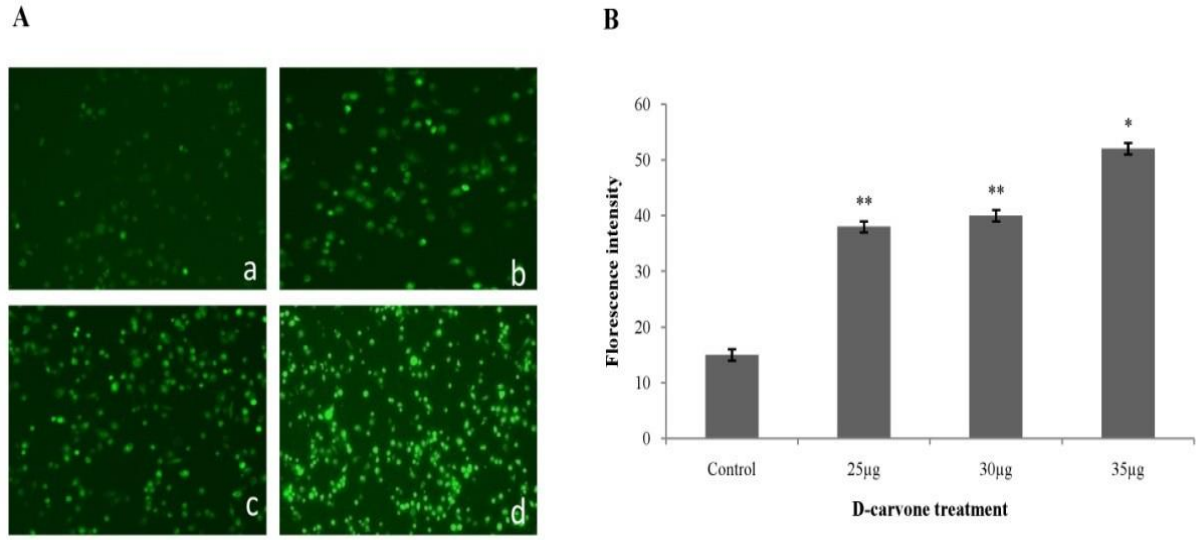

Figure 4: Effect of D-carvone on ROS production in A431 cells

A. Cells were treated with D-carvone at various concentrations, stained with ROS assay solution. The production of ROS was evaluated by fluorescence microscopy. (a) Vehicle-treated (0.01% DMSO), shows dark green fluorescence. D-carvone (25, 30, 35 µg/ml) treated cells (b,c and d) shows the bright and visible green fluorescence in A431 cells. Data are expressed as mean ± Standard Deviation (SD) of experimentally 4 independent values. * indicates variations that were significant with statistical analysis. * p<0.05; ** p<0.01

Transwell migration assay

This assay has been commonly used for studying the motility of diverse types of cells as well as metastatic cancer cells (Hughes *et al.,* 2018). The effect of D-carvone on A431 cells invasion by in vitro was assessed using Transwell chambers (8 µm pore size, Corning, USA) method as described in the previous report (Dong *et al.,* 2018). Briefly, the cultured A431 cells (Human skin carcinoma cells) cells were harvested by trypsinization, pooled in a 15 ml tube. Cells were plated in serum-free medium, and medium containing 10% FBS in the lower chamber served as the chemoattractant (Treated with 0.01% of DMSO (35 µl), 25 µg, 30 µg, and 35 µg of D-carvone). After 24 hr incubation in a humidified atmosphere containing 5% CO_2 incubator at 37˚C, non-invasive cells on the upper surface were removed with a cotton swab. The

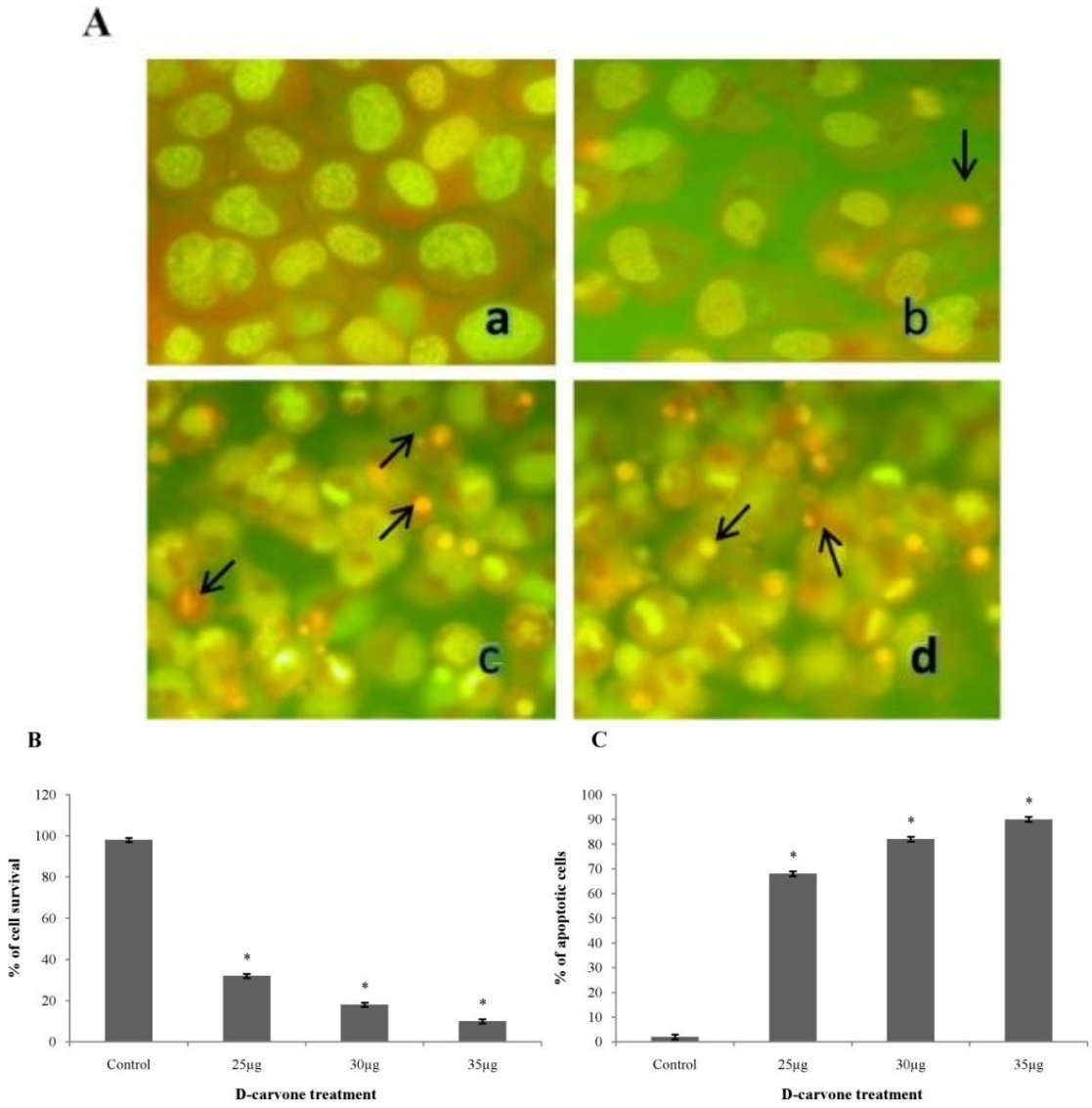

Figure 5: Effect of D-carvone on the cell morphology by AO/EB stain in the A431 cells
A. (a) Vehicle treated cells shows uniformly green fluorescing, viable cells. D-carvone (25, 30 and 35 µg/ml) shows cells with chromatin condensation, indicating late apoptosis and necrosis, which fluoresce uniformly in bright red and orange (arrowheads). B. Percentage of apoptotic cells was calculated by scoring apoptotic and visible cells. Data are expressed as mean ± Standard Deviation (SD) of experimentally 4 independent values. * indicates variations that were significant with statistical analysis. * $p<0.05$; ** $p<0.01$

invasive cells on the lower chamber were fixed with 75% ethanol and then stained with 0.5% crystal violet and images were captured. Results were presented as images of invading A431 cells.

Statistical data analysis

The results were statistically compared and analyzed by one-way analysis of variance (ANOVA) data are expressed as mean ± standard deviation (SD) statistical comparisons were performed by, followed by Duncan's multiple range test (DMRT) using SPSS version 17.0 for windows software analysis. The results were considered statistically significant values $p<0.05$.

OBSERVED RESULTS

Cytotoxicity outcome of D-carvone on A431 cells

The MTT analyze used to make a beginning measurement of the growth inhibitory probable of D-carvone on A431 cells to find the respective IC_{50} values with various concentrations of D-carvone (5-45µg/ml) at 24 hours. The cytotoxic effect of

D-carvone was determined based on the concentrations of the drug needed to decrease the survival of cells by 50% (IC_{50}). Treatment with D-carvone exhibited significant ($p<0.05$) growth inhibition in a dose-dependent manner and the IC_{50} value of D-carvone for 24 hours (Figure 2B). The results

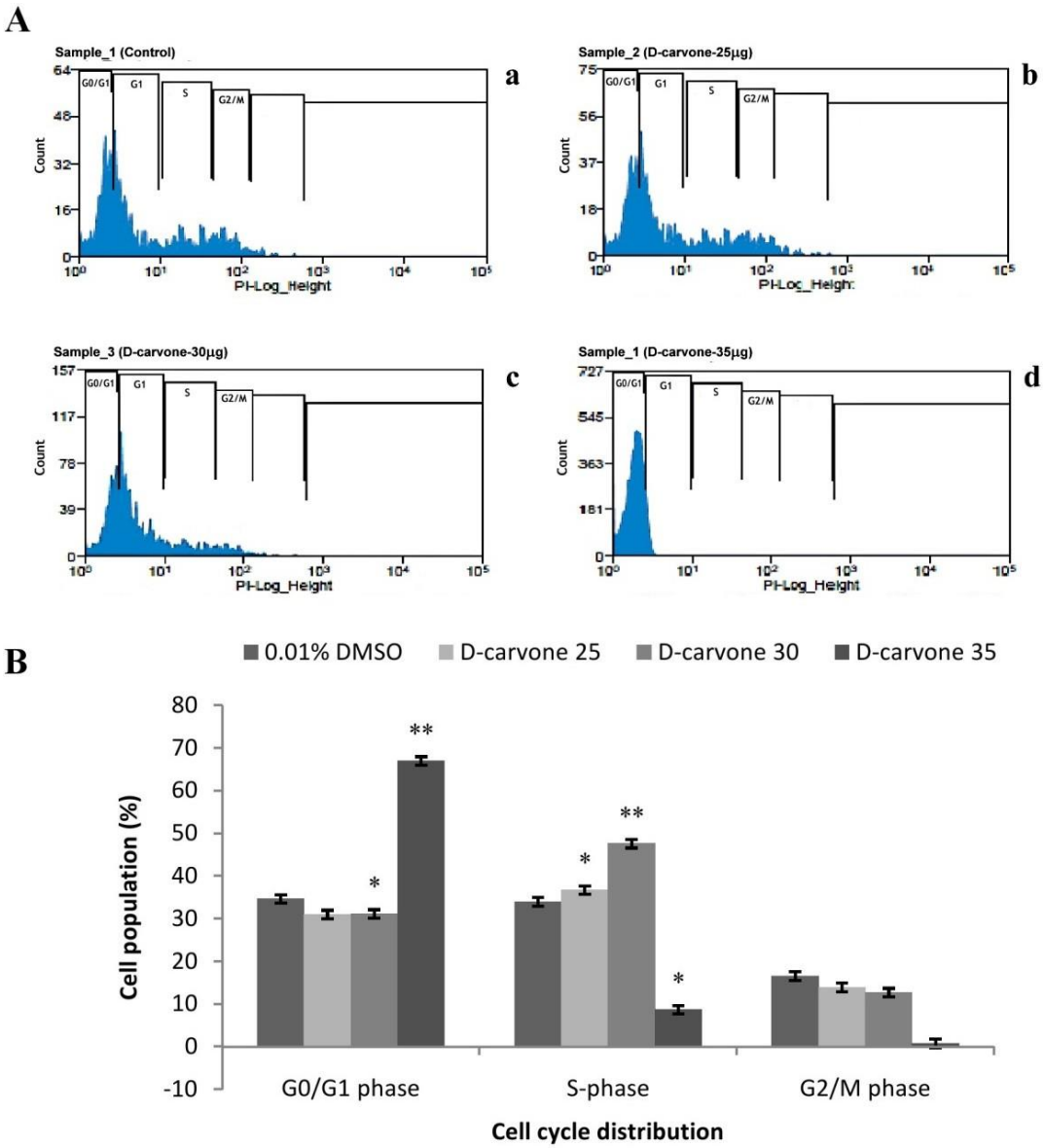

Figure 6: Effect of D-carvone on cell cycle distribution in A431 cells

Cell cycle distributions of vehicle-treated and D-carvone treated A431 cells with various concentrations (25, 30 and 35 µg/ml) for 24h. (a) Vehicle-treated cells; (b) D-carvone- 25 µg; (c) D-carvone- 30 µg; (d) D-carvone- 35 µg ;(B) Quantification of cell cycle distribution and histograms showing the number of the channel (horizontal axis) against DNA content (vertical axis). Data are expressed as mean ± Standard Deviation (SD) of experimentally 4 independent values. * indicates variations that were significant with statistical analysis. * $p<0.05$; ** $p<0.01$

revealed that when cells were treated with D-carvone for 24 hours, graph intersected in 30.00 µg.

Mitochondrial Membrane Potential (Δψm) on control and D-carvone treated A431 cells

Apoptosis induction in a cell can be determined with mitochondrial membrane potential (MMP). MMP was assessed with dye JC-10. In this experiment, we observed that A431 cells treated with D-carvone at the concentration of 25, 30, and 35 µg showed green fluorescence indicating the dose-dependent loss of mitochondrial potential. Therefore the induction of apoptosis, whereas vehicle-treated (0.01%DMSO 35µl) cells (Figure 3a) displayed a high intensity of orange fluorescence with JC-10 aggregates.

A431 generating reactive oxygen specious in control and D-carvone treated cells

The level of ROS generation in vehicle-treated (0.01% DMSO) and D-carvone treated cells, at 24 h (Figure 4). Treatment with D-carvone to A431 cells significantly increased the intracellular ROS generation in a dose-dependent manner (25, 30 and 35 µg) indicating ROS mediated apoptosis in cancer cells, whereas in the vehicle (Figure 4a) treated cells shows weak fluorescent background indicating low ROS production.

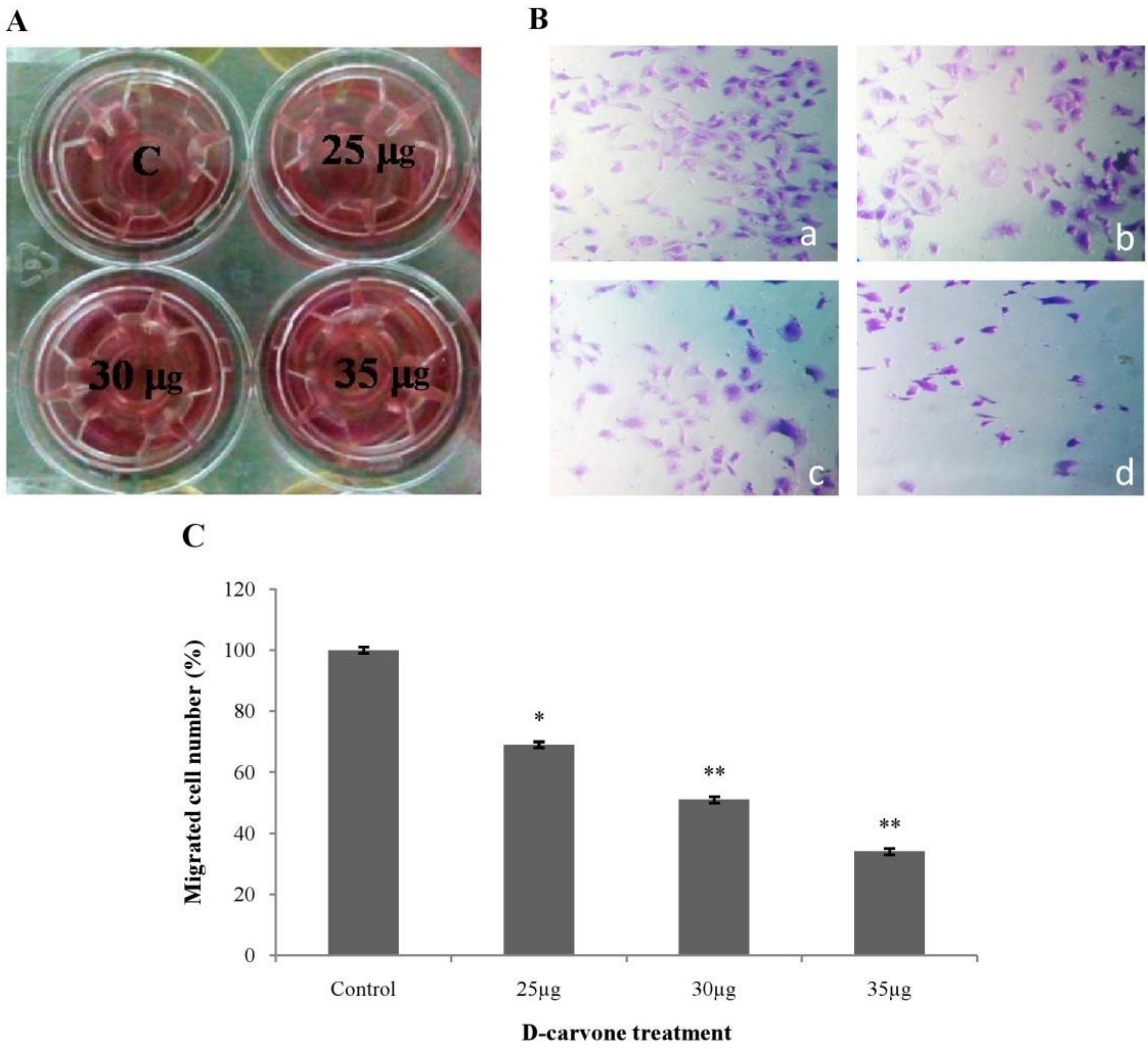

Figure 7: Effect of D-carvone on cell migration through transwell membrane against human epidermoid carcinoma (A431) cells

A. The invasive cells on the lower chamber were fixed. B. (a) Vehicle treated cells. (b) D-carvone- 25 µg; (c) D-carvone- 30 µg; (d) D-carvone- 35 µg shows inhibition in the cell migration; images were captured at 40 x and quantified (C). Data are expressed as mean ± Standard Deviation (SD) of experimentally 4 independent values. * indicates variations that were significant with statistical analysis. * $p<0.05$; ** $p<0.01$

Effect of D-carvone on apoptotic morphological changes using dual staining

Vehicle-treated cells exhibited uniform bright green nuclei with organized structure (Figure 5). Early apoptotic cells were noticed more in 25 µg/ml D-carvone treated group. 30 and 35 µg/ml D-carvone treated cells were representing a late apoptotic stage of cells due to EB intercalation into DNA. Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin. Necrotic cells have a uniform orange to red nuclei with condensed structure. The maximum increased quantity of apoptotic cells was observed at 25, 30 and 35 µg/ml concentration of D-carvone treated cells than compared to the vehicle-treated group (0.01% DMSO 35µl) (Figure 5a).

Effect of D-carvone on cell cycle arrest

We evaluated the cell cycle distribution after D-carvone treatment by flow cytometry. Vehicle-treated cells with 35µl of 0.01% DMSO (Figure 6), the percentage of G0/G1 phase were 34.67 %, S phase was 34.29 % and in the G2/M phase were observed 16.57%. After treatment with 25 µg of D-carvone, the G0/G1 phase was observed in 31.01 %, S-phase was increased to 36.74 % and G2/M phase showed 12.72 %. Similarly, treatment with 30 µg of D-carvone showed 31.15 % of G0/G1

phase, 47.73 % of S-phase and 10.93 % of G2/M phase. In addition, D-carvone treatment with high dose at 35 µg showed 90.28 % of G0/G1 phase, 8.74 % of S-phase and 0.8 % of G2/M phase. This finding indicates that the cell cycle distribution was arrested significantly in the G0/G1 phase

when A431 cells are treated with D-carvone particularly at the concentration of 35µg.

D-carvone enhanced inhibition of cell migration

Mortality rates are hiked always in metastasis stage of skin cancer. Increased migrations followed by invasion are the key features for spreading cancer cells into secondary organs, via the blood vessels mediated extravasations (Friedl *et al.,* 2003; Nguyen *et al.,* 2007). Therefore, it is imperative to investigate whether D-carvone can inhibit skin cancer cells (A431) migration. The transmembrane migration assay was used to evaluate the effects of D-carvone on A431 cell migration. Results of transmembrane migration assay (Figure 7 b, c and d), in the presence of different concentration of D-carvone (25, 30 and 35 µg/ml) treated A431 cells, the migration of cells were significantly inhibited in a dose-reliant manner compared with vehicle-treated cells (0.01% DMSO 35µl) in 24h. Altogether, these results are strong evidence to D-carvone has anti-metastatic effects in A431 cells.

DISCUSSION

Viability and migration of cancer cells are the critical hallmark capabilities of any cancer for independent growth and continuous existence (Buddhan *et al.,* 2017). MTT assay is important and commonly used by researchers, to test the anti-cell proliferative efficacy of the anticancer agents including natural products and their synthetic compounds (Sargent *et al.,* 2003). In previous reports, D-carvone has cytotoxicity on HT-29 and SW480 colon cancer cells were found that 50% of cell viability were arrested at the concentration of (34.72µg) in HT-29 and (33.23 µg) in SW480 (Vinothkumar *et al.,* 2013). In general D-carvone treatment significantly reduced the viability of A431 cells by all the doses exposed in the present study. For 24 h treatment, 30 µg concentrations of D-carvone treated cells showed 50 % inhibition that was concluded as IC_{50} in this study.

Apoptosis stimulated by natural or synthetic anticancer agents and many of those agents execute their antitumor activity through ROS-mediated apoptotic signalling on mitochondria. Reactive Oxygen Species (ROS) are byproducts of cellular respiration and have a central role in apoptotic cell death (Kong *et al.,* 2016). Increased levels of cellular ROS lead to the failure of mitochondrial membrane potential. It causes the release of pro-apoptotic signals consequently cells to undergo nuclear chromatin condensation and apoptosis (Fan *et al.,* 2016; Brodska *et al.,* 2011). Treatment of A431 cells with D-carvone resulted in the mitochondria dysfunction, accompanied with ROS level was significantly augmented by different concentration of D-carvone. High ROS accumulation resulted in D-carvone treated cells were also observed with loss of mitochondrial membrane potential which causes apoptosis. This could be related to prooxidant nature of D-carvone as well as corroborated with previous reports (Vinothkumar *et al.,* 2013). Ye et al. were reported that cryptotanshinone induces ROS-mitochondrial pathway in melanoma cells. Further, they confirmed that cryptotanshinone repressed the cancer cell movement and invasion of cancer cells too (Ye *et al.,* 2016).

It was proven that loss of cell cycle regulation is one of the hallmarks of cancer. Therefore, agents which target cell cycle machinery in a regulatory way are a promising one in the management/treatment of cancer (Buolamwini 2000; Collins *et al.,* 1997). Anticarcinogenic effect of the drug may regulate the cell cycle mechanism, resulting in the arrest of cells in diverse phases of the cell cycle and this manner reducing the cancerous cells growth and proliferation (Hajduch *et al.,* 1999; McDonald *et al.,* 2000). Here, we investigated the contribution and machinery of D-carvone on cell cycle regulation and apoptosis in skin cancer-derived cells (A431). The data of cell distribution at each phase revealed that D-carvone inhibited cell growth and proliferation of A431 cells induced by G_0/G_1 phase arrest in a dose-reliant manner. Salehi et al. reported that the Zataria Multiflora (ZEO) induces G_1 and G_2/M phase cell cycle arrest in monolayer, but in MDA-MB-231 cell spheroids, they observed ZEO induced S phase arrested in the cell cycle (Salehi *et al.,* doi: 10.1038s/41598-017-02633-z). Mantena et al. have revealed that berberine treatment in varying concentration and time duration significantly arrested A431 cells in the G_0/G_1 phase (Mantena et al. 2006). Antonsson et al. demonstrated that Staurosporine has blocked the progression of G_2/M phase in the Human leukemic cell line (U-937) followed by down-regulation of cyclin-B1 and CDK1 (Antonsson *et al.,* 2009). These above findings correlate with our results.

Apoptotic morphology was assessed by dual staining (acridine orange/ethidium bromide) assay, which is apoptotic cells are characterized by the red-orange fluorescence, while viable cells and early apoptotic cells and exhibit bright green and green fluorescence respectively (Buddhan *et al.,* 2017). D-carvone apoptotic potential was confirmed; by A431 cells exhibit red-orange fluorescence. Our observation that exposure of A431 cells to the D-carvone for 24h caused an increase in the population of apoptotic cells gradually. Also, we noticed an increase in late apoptotic cells accompanied by a decrease in early apoptotic cells after 24h treatment with D-carvone. Dikmen et al. revealed induction of apoptosis in HL -60 (human

promyelocytic leukaemia) cells treated with curcumin in in vitro study. Curcumin-untreated cells and treated cells were subjected to an acridine orange(AO) and ethidium bromide(EB) stained HL - 60 cells showed the role of curcumin as ROS mediated apoptosis-inducing agent (Dikmen *et al.,* 2006). It has been showed diasmin treated A431 cells undergo apoptosis as evidenced by orange to red nuclei with condensed chromatin, while diasmin untreated cells showed uniformly green in colour (Buddhan *et al.,* 2017). Therefore, both the results positively went with our present findings.

Kumar et al. reported that cell migration is an essential physiological event occurs for the duration of embryonic development, tissue organization, disease enlargement as well as cancer progression (Kumar *et al.,* 2018). Migration and invasion are associated with metastasis of malignant cells can degrade the extracellular matrix and promote cell mobility from the primary neoplastic clone into surrounding normal tissues. Alterations in the extracellular matrix dimensionality, elasticity and cross-linking help cancer cell invasion (You *et al.,* 2018; Ukaji *et al.,* 2017; Shin *et al.,* 2016). In the present study, D-carvone treated A431 cells transmigrated through the membrane, are very low in number when compared with control cells. From the present findings, D-carvone treatment was found to reduce the migration and invasion abilities of human epidermoid cancer cells (A431). Similar results illustrated that Hinokiflavone has potent ability to suppress melanoma cell movement and invasion in a dose reliant manner (Yang *et al.,* 2018).

CONCLUSION

Our study indicates that cell growth inhibition, induced by G_0/G_1 phase arrest; mediate apoptotic cell death via mitochondrial ROS production and controls cell migration of human epidermoid carcinoma A431 cells treated with D-carvone in various concentrations. We suggest that D-carvone could be developed as a skin cancer chemotherapeutic agent. Further, in-depth research is required to ascertain D-carvone antitumor activity of other tumours in vivo and the precise molecular mechanism.

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