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ORIGINAL ARTICLE

Geraniol and geranyl acetate induce potent anticancer effects in colon cancer Colo-205 cells by inducing apoptosis, DNA damage and cell cycle arrest

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Summary

Purpose: *Colon cancer ranks second in mortality among all human malignancies, creating thus a need for exploration of novel molecules that would prove effective, cost-effective and with lower toxicity. In the recent past monoterpenes have gained tremendous attention for their anticancer activity. In the present study we evaluated the anticancer effects of two important monoterpenes, geraniol and geranyl acetate against colo-205 cancer cells.*

Methods: *The antiproliferative activity was determined by MTT assay. Apoptosis was assessed by DAPI staining and DNA damage was checked by comet assay. The cell cycle analysis was carried out by flow cytometry and protein expression was examined by western blotting.*

Results: *The results showed that both geraniol and geranyl acetate exhibited significant anticancer activity against colo-205 cancer cell line with IC_{50} values of 20 and 30 μM respectively. To find out the underlying mechanism, DAPI staining was carried out and it was observed that both the monoterpenes, geraniol and geranyl acetate, induced apoptosis in colo-205 cells. The apoptosis was also associated with upregulation of Bax and downregulation of Bcl-2 expressions, indicative of mitochondrial apoptosis. Moreover, these two monoterpenes could trigger DNA damage and G2/M cell cycle arrest in colo-205 cells.*

Conclusions: *Taken together, we propose that geraniol and geranyl acetate may prove to be important lead molecular candidates for the treatment of colon cancer. Their anticancer activity can be attributed to the ability to trigger apoptosis, DNA damage and cell cycle arrest.*

Key words: *apoptosis, colon cancer, geraniol, geranyl acetate, monoterpenes*

Introduction

Plants produce a diverse assortment of secondary metabolites which have evolved since millions of years to carry on vital physiological functions [1]. Among plant secondary metabolites monoterpenes constitute a large group of non-nutritive dietary metabolites that are present in the essential oils of several plant species which include but are not limited to citrus, mint, cherry and several other herbs [2]. Physiologically, they play vital roles in plants as they may help plants to lure pollinators, to carry out the act of pollination or act as chemo-repellents to keep away the predators. Chemically, monoterpenes consist of ten carbon isoperenoids and are biosynthesized by mevalonate pathway. Monoterpenes are biosynthesized in plants but have not been reported from fungi, mammals or any other species [3]. In the last few decades monoterpenes have gained tremendous attention for their health promoting properties. They have been found to have antioxi-

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dant, antimicrobial and anticancer properties [4]. Several of the monoterpenes have been shown to exhibit anticancer activity and have the capacity to inhibit the development or progression of cancer, and to inhibit the proliferation of the existing tumors [5]. The anticancer properties of monoterpenes are discovered every now and then. For instance, limonene, a monoterpene, has been shown to exhibit anticancer properties against different types of cancers [4]. Geraniol and geranyl acetate (Figure 1) are two important monoterpenes and are found in the essential oils of several plant species [6]. They have been shown to be of tremendous pharmacological potential. In the present study we evaluated the anticancer activity of geraniol and geranyl acetate against the colon cancer Colo-205 cell line. Among the gastrointestinal cancers, colon cancer is one of most prevalent types of cancer [7]. Over the years there has been drastic alterations in human lifestyles, accompanied with increased incidence of cancer around the globe. Studies have reported that colon cancer is the second most common cause of cancer related deaths among malignant tumors [8]. Currently, the treatment of colon cancer involves surgery followed by chemotherapy. However, the prognosis for colon cancer is rather poor and the mortality rate is high [9]. Therefore there is an urgent need to develop novel treatment strategies or explore novel targets for the treatment of this disease. The purpose of this study was to evaluate the anticancer activity of geraniol and geranyl acetate against the colon cancer Colo-205 cell line.

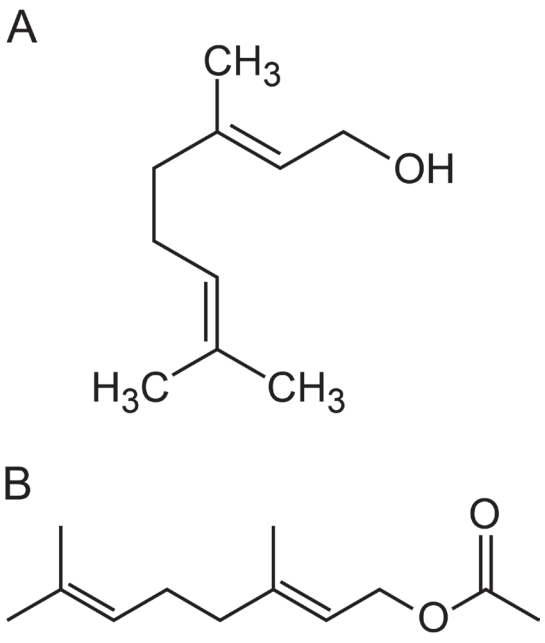

Figure 1. Chemical structures of **(A)** Geraniol and **(B)** Geranyl acetate.

Methods

Chemicals, Reagents and culture conditions

The following chemicals were used in the present study; DAPI, RNase A triton X-100 dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine, antibiotics were procured from Invitrogen Life Technologies (Carlsbad, CA, USA). Human colon cancer colo-205 line was purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% FBS, penicillin and streptomycin (100 U/mL each) and maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

MTT assay

The *cell* viability of colon Colo-205 cancer cells was assessed by MTT assay. In brief, Colo-205 cells were cultured in a 96-well plates at a density of 5×10^3 cells in each well. Then the cells were incubated overnight and the medium was removed and replaced with a new medium with geraniol and geranyl acetate separately at different concentrations (0-100 µM) for 24 hrs. Thereafter, MTT solution at a concentration of 0.5 mg/ml was added for the last 4 hrs of incubation and the absorbance was measured at 570 nm.

Apoptosis assay

Colon cancer colo-205 cells were separately seeded at a density of 2×10^5 cells/well in 6-well plates and administered 20 µM of geraniol and 30 µM of geranyl acetate separately and incubated for 24 hrs. DAPI staining was carried by incubating the cells in 6-well plates with DAPI. The cells were then washed with PBS, fixed in formaldehyde (10%) and then washed again with PBS. The DAPI stained cells were then examined by fluorescence microscope.

Comet assay

DNA damage triggered by geraniol and geranyl acetate was assessed by the comet assay. Briefly, geraniol and geranyl acetate-treated colo-205 colon cancer cells were harvested and suspended in cold PBS. The cells in the 0.5% low melting point agarose were placed on a slide precoated with a layer of 1% regular agarose. Afterwards, these two layers were allowed to solidify at 4°C, and then suspended in a cold lysis buffer for 50 min at 4°C. Then, the gel slides were allowed to dry and the dried slides were soaked in fresh electrophoresis solution for 25 min. Then, electrophoresis was carried out at 300 mA, 25 V for 25 min at 4°C. This was followed by staining with ethidium bromide (20 µg/ml) for 12 min, and neutralization of the slides with 0.4 M Tris-HCl (pH 7.5). Finally, the slides were washed and observed under a fluorescent microscope (BX51; Olympus, Tokyo, Japan). The parameters representing DNA damage were obtained and recorded for 100 cells per sample by CASP software (Linco Software Inc. Carlsbad, CA, USA).

Cell cycle analysis

For estimating the distribution of Colo-205 colon cancer cells in different phases of the cell cycle, the treated cells were harvested and washed twice with PBS. Thereafter the cells were fixed with ethanol (70%) for about an hour and washed again by PBS. The cells were finally resuspended in solution of propidium iodide (PI) (50 µl/ml) and RNase1 (250 µg/ml). This was followed by incubation for 30 min at room temperature and fluorescence-activated cell sorting cater-plus cytometer using 10, 000 cells/group.

Western blotting

Total protein from cancer and normal cells was isolated in RIPA lysis buffer. Equal protein extracts from each group were run on SDS PAGE and then transferred to a polyvinylidene fluoride membrane. This was followed by blocking with 5% non-fat milk and incubation at room temperature for 1 hr. Thereafter, the membranes were incubated with a specific primary antibody at 4 °C overnight. This was followed by washing in washing buffer and incubation for 1 hr with the suitable secondary antibody. The protein bands of interest were visualised by an ECL Advanced Western Blot Detection Kit. (GE Healthcare, Chicago, IL, USA).

Statistics

All the experiments were carried out in triplicate and the values were expressed as mean ± SD. Differences between the control and treatments were analyzed using Student's t test, and statistical significance was considered at *p<0.01, **p<0.001 and ***p<0.0001. The Graph Pad prism 7 software (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analyses.

Results

Geraniol and geranyl acetate exert antiproliferative effects on Colo-205 cells

To assess the antiproliferative effects of the monoterpenes geraniol and geranyl acetate the Colo-205 cells were treated with these monoterpenes and the cell viability was assessed by MTT assay. The results of MTT assay revealed that both geraniol (Figure 2) and geranyl acetate (Figure 3) exhibited significant anticancer activity on the colo-205 cells which was concentration-dependent. However, it was observed that geraniol exhibited comparatively better anticancer activity against colo-205 cancer cells as compared to the geranyl acetate. The IC_{50} of geraniol against colo-205 colon cancer cells was 20 µM as compared to the IC_{50} of geranyl acetate which was 30 µM.

Geraniol and geranyl acetate induce apoptosis in Colo-205 cells

Since monoterpenes have been shown to exert antiproliferative effects via induction of apoptosis [10], we investigated by DAPI staining whether geraniol (Figure 4A-B) and geranyl acetate (Figure 5A-B) monoterpenes also trigger apoptosis in colo-205 colon cancer cells. The colo-205 colon cancer cells were first treated with geraniol and geranyl acetate separately at IC_{50} concentrations, subjected to DAPI and observed under fluorescence microscope. It was observed that both geraniol and geranyl acetate induced apoptosis in colo-205 colon cancer as evidenced from the increased number of cells with white color nuclei. To examine whether geraniol and geranyl acetate induced mitochondrial-related apoptosis we checked the expression of Bax and Bcl-2 proteins (Figure 6). The results clearly showed that upon geraniol and geranyl acetate treatment separately increased the expression of Bax and decreased the Bcl-2 proteins, indicative of mitochondrial apoptosis.

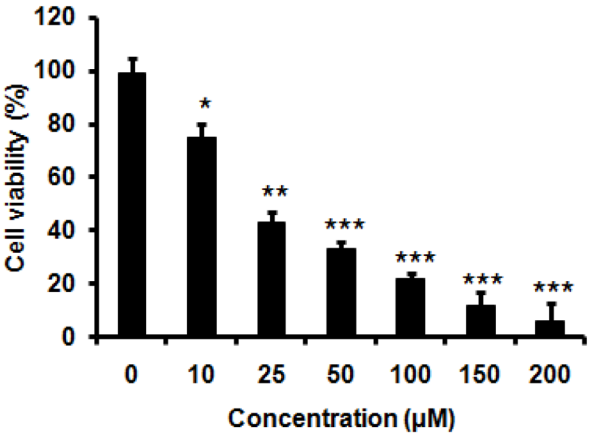

Figure 2. Assessment of cell viability of Colo-205 cells at indicated concentrations of geraniol. The experiments were carried out in triplicate and expressed as mean ± SD. The values were considered as significant at *p<0.01, **p<0.001 and ***p<0.0001.

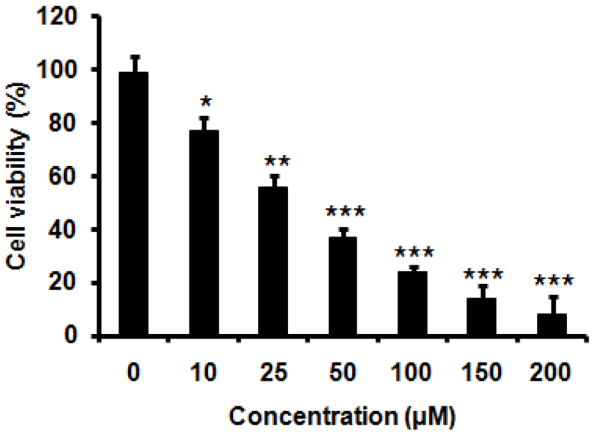

Figure 3. Assessment of cell viability of Colo-205 cells at indicated concentrations of geranyl acetate. The experiments were carried out in triplicate and expressed as mean ± SD. The values were considered as significant at *p<0.01, **p<0.001 and ***p<0.0001.

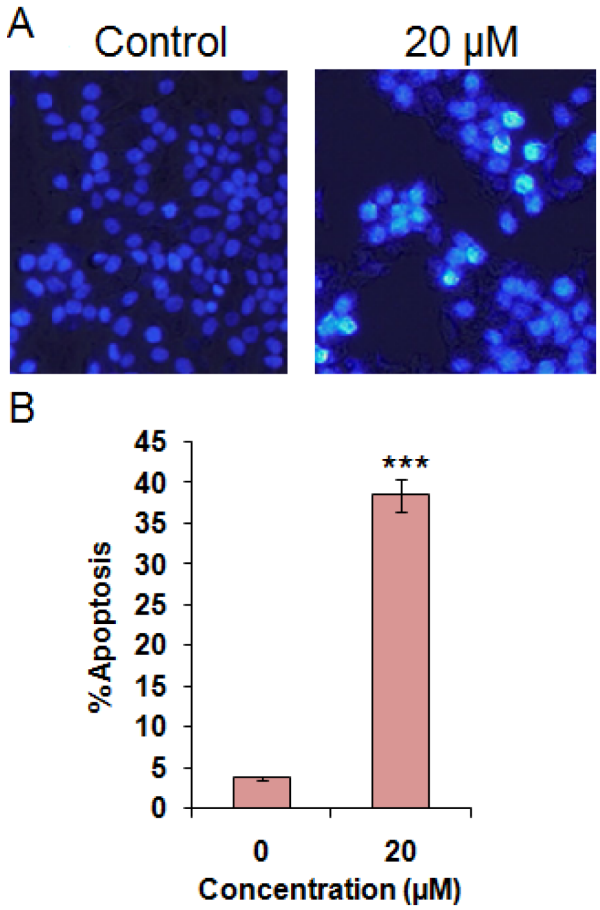

Figure 4. Induction of apoptosis in Colo-205 cells at IC_{50} concentrations of geraniol as indicated by **(A)** DAPI staining and **(B)** quantification of % apoptotic cells. The experiments were carried out in triplicate and expressed as mean ± SD. The values were considered as significant at ***p<0.0001.

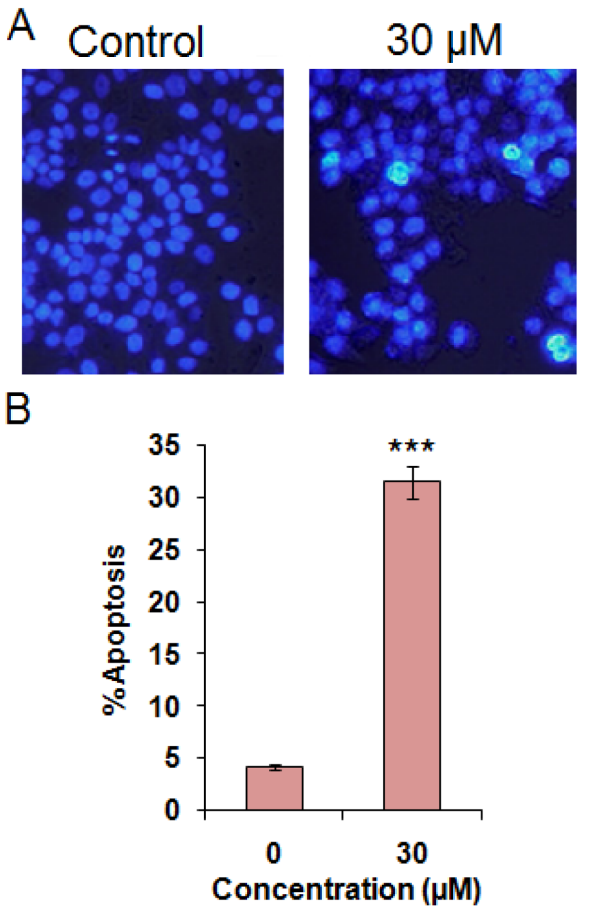

Figure 5. Induction of apoptosis in Colo-205 cells at IC_{50} concentrations of geranyl acetate as indicated by **(A)** DAPI staining and **(B)** quantification of % apoptotic cells. The experiments were carried out in triplicate and expressed as mean ± SD. The values were considered as significant at ***p<0.0001.

Geraniol and geranyl acetate induce DNA damage in Colo-205 cells

We also assessed whether geraniol and geranyl acetate caused DNA damage in colo-205 colon cancer cells by comet assay. The results of comet assay showed that both geraniol (Figure 7A-B) and geranyl acetate (Figure 8A-B) induced DNA damage in colon cancer colo-205 cells at IC_{50} concentrations. The DNA damage was evidenced from the formation of tail DNA.

Geraniol and geranyl acetate trigger G2/M cell cycle arrest in Colo-205 cells

The distribution of Colo-205 cells in the different cell cycle phases after treatment with geraniol and geranyl acetate separately was studied at their respective IC_{50} concentrations. The results showed that both geraniol (Figure 9) and geranyl acetate (Figure 10) led to the accumulation of colo-205 colon cancer cells in G2/M phase of the cell cycle, ultimately leading to G2/M cell cycle arrest.

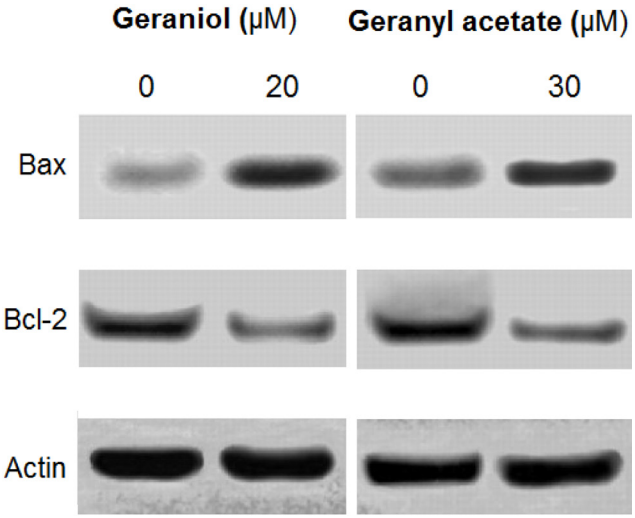

Figure 6. Expression of Bax and Bcl2 in Colo-205 cells at IC_{50} concentrations of geraniol and geranyl acetate as indicated by western blotting. The experiments were carried out in triplicate. The Figure shows that geraniol and geranyl acetate increase the expression of Bax and decrease the expression of Bcl-2.

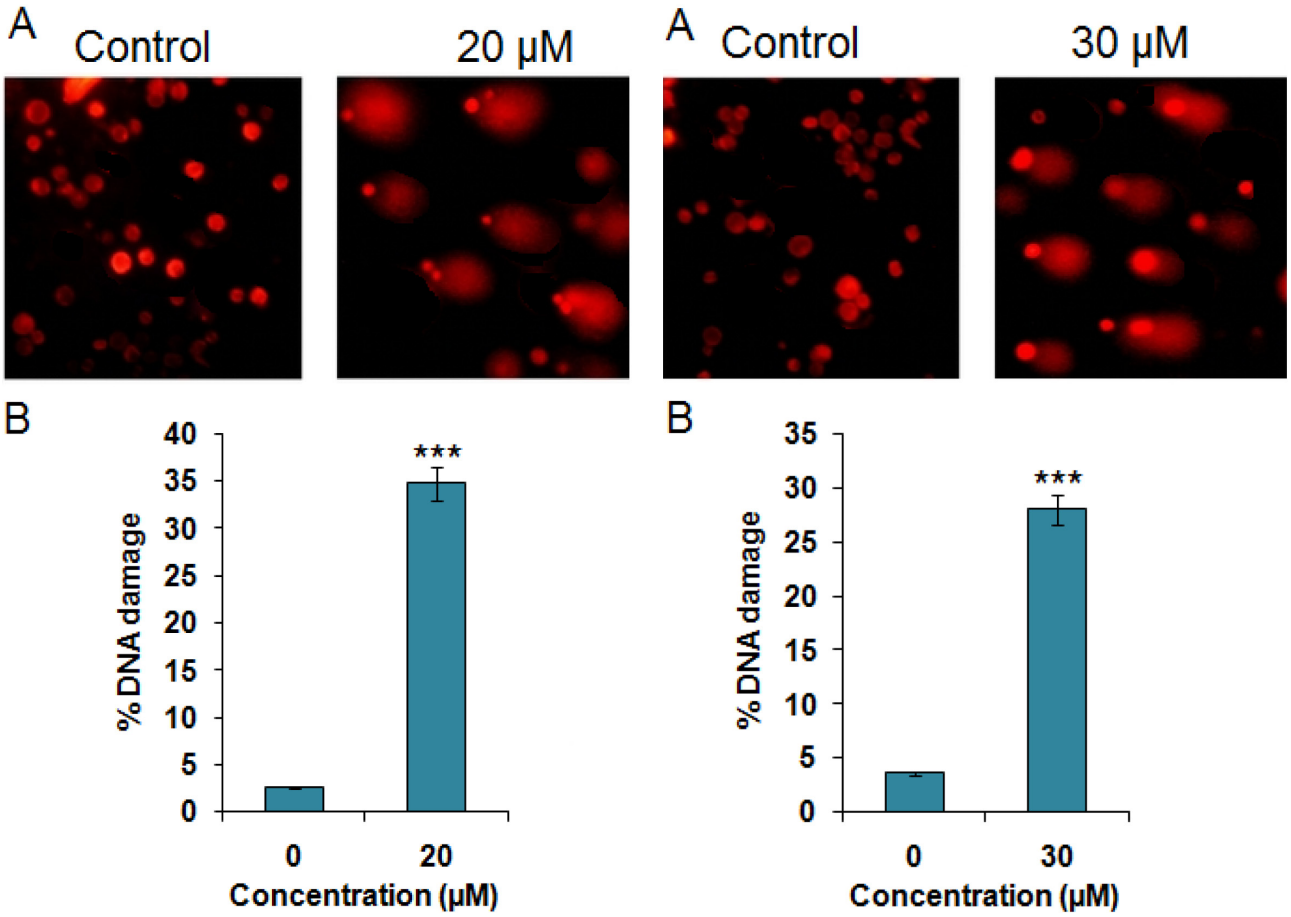

Figure 7. Induction of DNA damage in Colo-205 cells at IC_{50} concentrations of geraniol as indicated by **(A)** Comet assay and **(B)** quantification of % DNA damage. The experiments were carried out in triplicate and expressed as mean ± SD. The values were considered as significant at ***p<0.0001.

Figure 8. Induction of DNA damage in Colo-205 cells at IC_{50} concentrations of geranyl acetate as indicated by **(A)** Comet assay and **(B)** quantification of % DNA damage. The experiments were carried out in triplicate and expressed as mean ± SD. The values were considered as significant at ***p<0.0001.

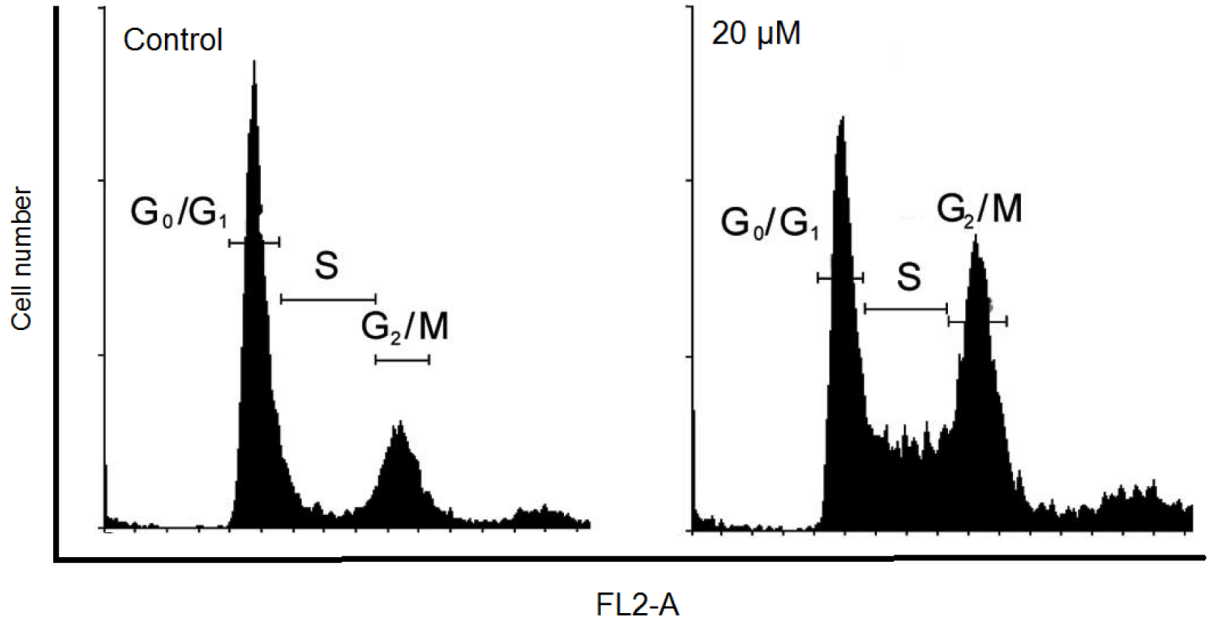

Figure 9. Induction of cell cycle arrest in Colo-205 cells at IC_{50} concentrations of geraniol as indicated by flow cytometry. The experiments were carried out in triplicate. The results of this Figure indicate that geraniol induces G2/M cell cycle arrest in Colo-205 cells. FL2-A indicates pulse area.

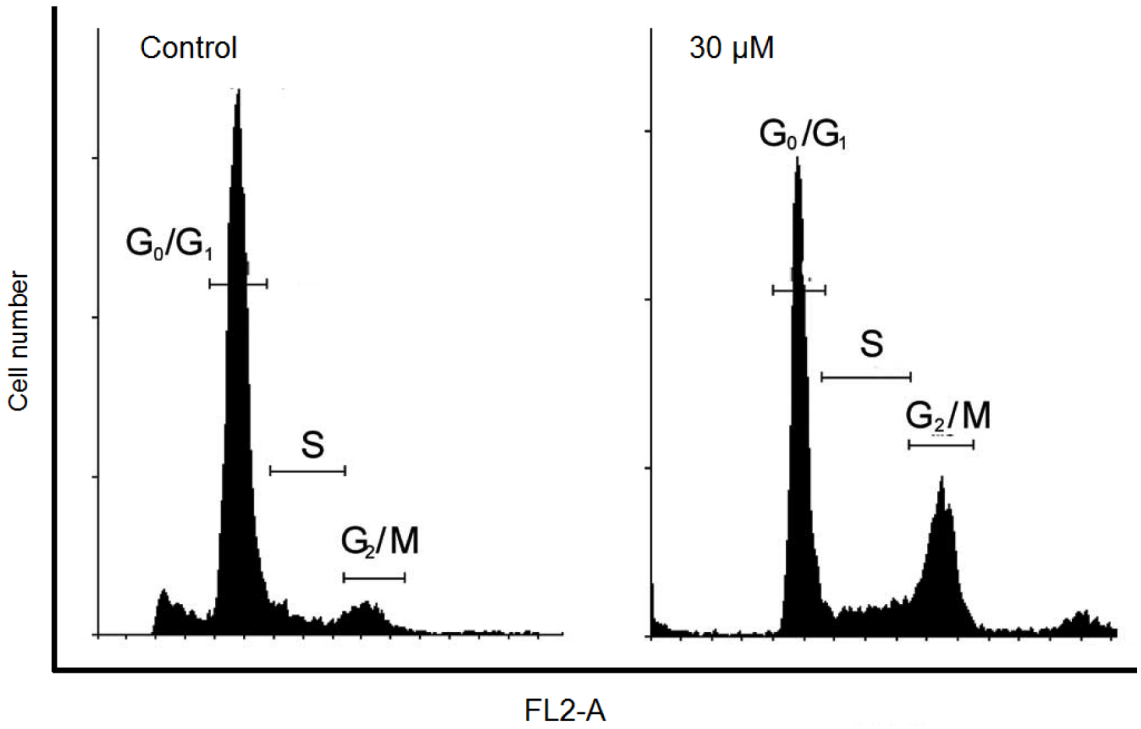

Figure 10. Induction of cell cycle arrest in Colo-205 cells at IC_{50} concentrations of geranyl acetate as indicated by flow cytometry. The experiments were carried out in triplicate. The results of this Figure indicate that geranyl acetate induces G2/M cell cycle arrest in Colo-205 cells. FL2-A indicates pulse area.

Discussion

Monoterpenes have drawn considerable attention in last few decades as anticancer agents. They have been reported to exhibit anticancer activity against breast, skin and liver cancers and new activities are discovered every now and then [2-4]. Colon cancer, being the second most common reason for cancer-related deaths [8], demands exploration of novel molecules that would prove effective, cost-effective and with lower toxicity. In the current study, investigated were the anticancer effects of two monoterpenes, geraniol and geranyl acetate, against colo-205 cancer cells. The results showed that both monoterpenes exhibit considerable anticancer activity. However, it was observed that geraniol was comparatively more effective with an IC_{50} of 20 μM as compared to geranyl acetate which showed an IC_{50} of 30 μM. To further investigate the underlying mechanism of geranyl acetate and geraniol we used DAPI staining and observed that both monoterpenes exerted anticancer effects via induction of apoptosis. Apoptosis involves a form of cell death that includes programmed series of actions without releasing any harmful chemicals. It an important mechanism by which several of the chemotherapeutic drugs exert their anti-proliferative activities [12]. Our results are in concordance with previous studies wherein monoterpenes, such as limonene, have been reported to trigger apoptosis in cancer cells [6,13-15]. In order to find out if the geraniol and geranyl acetate-induced apoptosis follows the mitochondrial pathway, we determined the expression of Bax and Bcl-2 proteins. Western blotting results showed that the expression of Bax was upregulated and that of Bcl-2 was downregulated upon treatment with these monoterpenes. We also investigated if these monoterpenes induced DNA damage in cancer cells by comet assay. Our results revealed that both geraniol and geranyl acetate induced DNA damage in colo-205 cancer cells at IC_{50} concentrations. Another important mechanism that has been reported to contribute to the anticancer effects of many well known drugs is cell cycle arrest [11]. Some anticancer drugs halt the progression of cells from one phase of the cell cycle to another by targeting specific proteins, leading to the accumulation of cancer cells at a particular phase. Arrest of the cell cycle prevents the cancer cells to develop into tumors and to spread to other parts of the body [11,16-18]. In the present study we observed that both monoterpenes, geraniol and geranyl acetate, caused G2/M cell cycle arrest of colo-205 cancer cells at IC_{50} concentrations. Taken together these results suggest that geraniol and geranyl acetate are promising lead molecules for the treatment of colon cancer.

Conclusion

From the results of this study we conclude that the monoterpenes geraniol and geranyl acetate, present in the essential oils of several plant species, exhibit significant anticancer activity against colon cancer cells. The anticancer activity can be attributed to the ability to trigger apoptosis, DNA damage and cell cycle arrest. Our results suggest that geraniol and geranyl acetate may prove promising lead molecules for the development of new cancer therapies and deserve further investigation.

Conflict of interests

The authors declare no conflict of interests.

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