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ORIGINAL ARTICLE

Apoptosis Induction and Reactive Oxygen Species Generation by *Artemisia absinthium* L. Leaf Extract in MCF-7 Breast Carcinoma Cells

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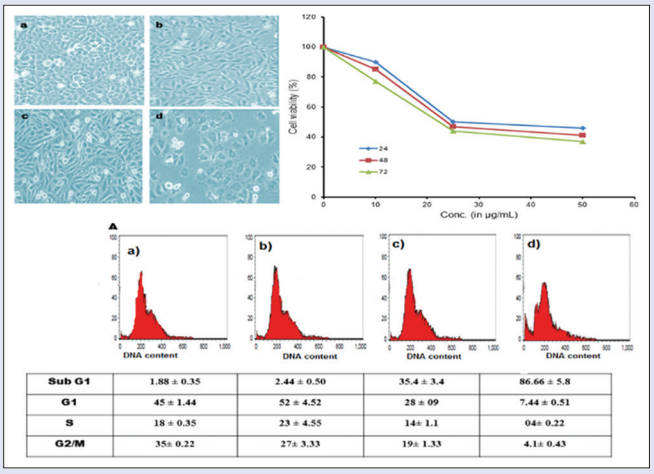
ABSTRACT

Background: Breast cancer is the commonly occurring cancer among women in both high-resource and low-resource settings, and the primary cause of death among women globally owing to suboptimal anticancer chemotherapy. This reflects the imperative need for better management of breast cancer among women. Therefore, the current study was conducted to evaluate the anticancer properties of *Artemisia absinthium* L. leaf extract on Michigan Cancer Foundation-7 (MCF-7) breast cancer cells. **Materials and Methods:** Leaf sample of *A. absinthium* L. was subjected to the Soxhlet extraction method with ethanol. The extract was concentrated to prepare the crude plant extract, which was tested for anticancer properties. The anticancer activity of extract was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and against (MCF-7) cells. Flow cytometry using propidium iodide staining was used for the determination of cell cycle distribution and DCFHDA staining for the analysis of reactive oxygen species (ROS) production. **Results:** MTT assay revealed that the leaf extract of *A. absinthium* L. reduced the cell viability of MCF-7 cancer cells. The IC_{50} of the crude extract was found to be 25 μg/mL. The results indicated that plant extract triggered the production of ROS and significantly reduced the mitochondrial membrane potential (ΔΨm). It also leads to the arrest of MCF-7 cells in sub-G1 stage of cell cycle and eventually induced apoptosis in a concentration-dependent manner as indicated by 4′-6-diamidino-2-phenylindole staining. Moreover, plant extract also reduced the colony-forming potential of MCF-7 cells in a dose-dependent manner. **Conclusion:** The present study demonstrated that ethanol extract of *A. absinthium* L. exhibited strong antiproliferative properties against breast cancer cells. Therefore, the extract can be used for the treatment for breast cancer directly or the chemical constituents may be used after the isolation.

Key words: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, anticancer agents, antiproliferative activity, apoptosis, *Artemisia absinthium* L., breast cancer

SUMMARY

- This is the first report of its kind in which the mechanism and anticancer activity of *Artemisia absinthium* L. leaf extracts against breast carcinoma cells were studied. Leaf extract of *A. absinthium* L. plant induced the alterations in intercellular reactive oxygen species, ΔΨm and cell cycle progression which ultimately culminates with apoptosis.



Sub G1	1.88 ± 0.35	2.44 ± 0.50	35.4 ± 3.4	86.66 ± 5.8
G1	45 ± 1.44	52 ± 4.52	28 ± 09	7.44 ± 0.51
S	18 ± 0.35	23 ± 4.55	14± 1.1	04± 0.22
G2/M	35± 0.22	27± 3.33	19± 1.33	4.1± 0.43

Abbreviations used: DMSO: Dimethyl sulfoxide; DAPI: 4′-6-diamidino-2-phenylindole; FBS: Fetal bovine serum; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ROS: Reactive oxygen species; WHO: World Health Organization; PBS: Phosphate-buffered saline.

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INTRODUCTION

Breast cancer is the frequently detected carcinoma among women globally, and the second-highest cause of cancer-related deaths in females worldwide.[1] In 2018, more than 2 million novel cases of breast carcinoma were diagnosed throughout the world.[1] Despite intense improvements in technology and chemotherapy, breast carcinoma continues to be a primary health problem and needs to be considered on top priority as a biomedical research problem. In women with the early stage of breast cancer, the mortality rate has been improved, but for those with the metastatic stage of tumor, the median survival rate is

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low (2 years). It is expected that the incidence and mortality rate of breast cancer patients may show a dramatic increase over the next 5–10 years.[1] In developing countries, the incidence rate of breast cancer patients is expected to increase by 55% and the mortality rate by 58% in the next 20 years compared to the current status.[2]

According to the WHO, around 65% of people in the world satisfactorily used herbal medicines and traditional medicines for various diseases.[3] Approximately 60% of the anticancer agents have either the plant or other natural source origins; still, there are vast numbers of medicinal plants that have antiproliferative properties, but they have not yet been fully explored for their anticancer potential.[4] Thus, only alternate to the harmful side effects associated with synthetic compounds is to use herbal medicine for the treatment of breast cancer.

Genus *Artemisia* belongs to the *Asteraceae* family comprising more than 800 species[5] and includes many important medicinal plants widely known for its biochemical diversity. *Artemisia* genus growing worldwide is the most popular source of traditional medicine. It is commonly used for the treatment of hepatitis, malaria, inflammatory disorders, and microbial infections.[6] Over the years, a large number of studies screened *Artemisia* species against various disorders, which led to the identification of many bioactive compounds as well as essentials oils. The present study was aimed to investigate the anticancer properties of *A. absinthium* L. leaf extract against MCF-7 breast carcinoma cells.

MATERIALS AND METHODS

Reagents

Dimethyl sulfoxide and many other common chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The antibodies used were supplied by the Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Plant material and preparation of extract

A. absinthium L. plant was procured from Xiansheng Drug Store (Nanjing, China) and assigned a Batch number: 1403086X. Fresh plant was washed under the running tap water, air-dried under the shade for 48 h in well-ventilated rooms, chopped, and then powdered in grinder. The powder of plant material obtained was stored under the cold conditions in airtight containers till used in further experiments. The dry *A. absinthium* L. plant powder weighing 500 g was extracted exhaustively by percolation with 95% ethanol using the soxhlet extractor. The extract was concentrated under vacuum at 50°C using rotary evaporation and then left over solvent was removed by water bath, air-dried for 24 h, and the lyophilized until the solvent was completely removed, yielding an ethanol extract weighing 170 g.

Cell culture

MCF-7 breast carcinoma cells were procured from the American Type Culture Collection (Manassas, VA, the United States). The cell cultures were carried in DMEM/high glucose mixed with 10% fetal bovine serum and antibiotics (1% penicillin-streptomycin). The culture of cells was performed at 37°C in an incubator under 95% air and 5% carbon dioxide.

Evaluation of cytotoxicity of plant extract against MCF-7 cells

Evaluation of cytotoxicity of plant leaf extract against MCF-7 cells was made using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MCF-7 cells at a density of 2×10^5 cells/well into 96 well plates were exposed to 10, 25, and 50 μg/mL *A. absinthium* L. extract or only with dimethyl sulfoxide (DMSO) (control). Afterward, cell viability was estimated for each treatment culture by MTT assay.[7] Following 24 h incubation, the medium in plates was replaced by fresh medium containing 10, 25, and 50 μg/mL *A. absinthium* L. extract and incubated for 72 h. Following which medium was removed and 30 μL MTT solution (0.5%) was put into the wells. Incubation of the cells with MTT solution was performed for 4 h at 37°C. Then, DMSO (150 μL) was put into the wells for dissolving purple-colored formazan precipitate formed. The ELISA reader (ELx800™; BioTek Instruments, Inc., Winooski, VT, USA) was employed for the measurement of absorbance for the determination of cell viability.

Analysis of cell cycle progression in MCF-7 cells

The MCF-7 cells were seeded in 96 well plates at a density of 2×10^5 cells/well. Following 24 h incubation, the cells were exposed to 10, 25, and 50 μg/mL *A. absinthium* L. extract for 24 h. The cells treated with DMSO vehicle were also included to be taken as a control group. DNA content estimation in the cells was quantified, and PBS was used to wash the cells followed by fixing in ethanol at −20°C. Subsequently, cells were resuspended in PBS containing 40 μg/mL propydium iodide, RNase A (0.1 mg/ml), and Triton X-100 (0.1%) under darkness for 40 min at 37°C. The analysis was performed by flow cytometry using the reported procedure.[8]

Evaluation of reactive oxygen species and mitochondrial membrane potential (ΔΨm)

MCF-7 cells were plated at a density of 2×10^5 cells/well in 96 well plates and cultured for 24 h. Then, cells were treated with 25 μg/mL *A. absinthium* L. extract for 6–72 h at 37°C. After that cells were collected, subjected to washing with PBS, and subsequently re-suspended in DCFHDA (10 μM) for reactive oxygen species (ROS) measurement. The cells were treated with DiOC6 (1 μmol/l) for the evaluation of ΔΨm under darkness at 37°C for 45 min. Flow cytometry was employed instantly for the measurement as previously reported.[9]

4′-6-diamidino-2-phenylindole staining

MCF-7 cells at 2×10^5 cells/well concentration were put in 6-well plates and exposed to 10, 25, and 50 μg/mL for 48 h. The apoptotic ratios of the cells exposed to the leaf extract were calculated by 4’-6-diamidino-2-phenylindole (DAPI) staining as previously reported.[8] The cells were then mounted onto slides, instantly subjected to fixing in paraformaldehyde (3%) and permeabilization in Triton X-100 (0.2%) in PBS for 10 min at 25°C. Subsequently, cells were stained with DAPI (5 μg/mL). Observation of the apoptotic nuclei was made using a fluorescence microscope (Motic, Germany) after DAPI staining.

Statistical analysis

The presented data are mean ± standard deviation of triplicate experiments performed independently. The analysis of the results was performed using the one-way ANOVA followed by the Student’s *t*-test. The SPSS software version 16.0 was used for the analysis of the data. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Cytotoxic effects of *Artemisia absinthium* L. extract on MCF-7 cells

Cytotoxicity of *A. absinthium* L. extract against MCF-7 cells was deduced using the MTT assay [Figure 1]. The cell viability was examined after MCF-7 cells were challenged with 10, 25, or 50 μg/mL doses of plant leaf extract for 24, 48, or 72 h. The plant extract exhibited concentration as well as time-based inhibitory effect on the viability of MCF-7 cells. On increasing the dose of *A. absinthium* L. extract from 10 to 50 μg/mL, the

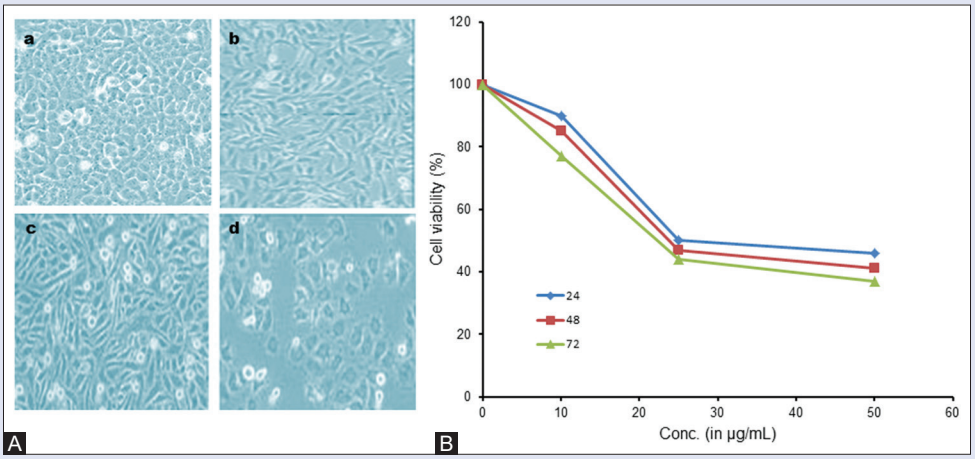

Figure 1: Ethanolic extract of *Artemisia absinthium* L. induced alterations in (A) cell morphology and (B) cell viability. MCF-7 cells were treated with 10, 25, and 50 µg/mL of plant extract for 24, 48, or 72 h. Each value is mean of three replicates ± standard deviation

cell viability showed a significant ($P < 0.05$) reduction [Figure 1a]. The reduction of cell viability was also observed in increasing the duration of treatment of MCF-7 cells to *A. absinthium* L. extract [Figure 1b].

Artemisia absinthium L extract causes apoptosis activation in MCF-7 cells

Ethanolic extract of *A. absinthium* L. induced apoptosis in MCF-7 cells [Figure 2]. DAPI staining indicated that leaf extract-treated cells showed condensation and fragmentation of chromatin in a concentration based manner. At 25 µg/mL dose of the plant extract, most of the cells showed apoptosis induction.

Effect of *Artemisia absinthium* L. extract on cell cycle distribution

A. absinthium L. extract led to the changes in cell cycle progression of MCF-7 carcinoma cells. The percentage of MCF-7 cells was significantly ($P < 0.02$) increased in G1 phase of cell cycle at 50 µg/mL of the plant extract [Figure 3]. Moreover, the proportion of MCF-7 cells in sub-G1 phase showed a marginal increase at 10 µg/mL. The cell population in sub-G1 phase showed a reasonable increase at 25 µg/mL, and a dramatic enhancement was observed at 50 µg/mL extract concentration.

Effect of *Artemisia absinthium* L. extract on reactive oxygen species and ΔΨm

Leaf extract of the plant increased the accretion of ROS level and reduced the mitochondrial membrane potential in MCF-7 cells [Figure 4]. The MCF-7 cells were treated with 25 µg/mL of plant extract for 12, 24, and 48 h to evaluate ROS production and ΔΨm levels. There was an increase in ROS and reduction of ΔΨm level significantly ($P < 0.05$) in the leaf extract treated MCF-7 cells. The leaf extract treatment augmented ROS levels from 12 to 72 h compared to the untreated control [Figure 4a]. Furthermore, the plant extracts considerably reduced ΔΨm level to around 37% at 48 h of exposure in MCF-7 cells compared to the control [Figure 5].

Artemisia absinthium L. extract reduces the formation of colonies in MCF-7 cells

The total number of viable MCF-7 cells that formed colonies was determined after exposure to 10, 25, and 50 µg/mL concentrations

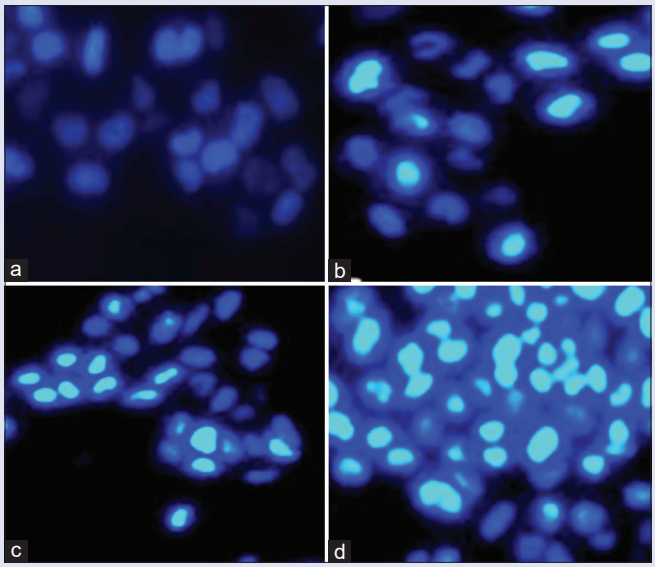

Figure 2: Effect of *Artemisia absinthium* L. on apoptosis induction. MCF-7 cells were treated with 0, 10, 25, and 50 µg/mL ethanolic extract of *Artemisia absinthium* L and apoptosis induction was determined by flow cytometry

of the plant extract [Figure 6]. The data revealed that the exposure of MCF-7 cells to plant leaf extract suppressed the percentage of colony formation significantly.

DISCUSSION

Breast carcinoma is the second common occurring cancer globally and continues to be the highest cause of deaths by cancer in females.[10] The mortality rates due to cancer are much lower than the incidence (~6–9/100,000). The treatment for breast cancer used presently consists of surgical intervention, radiotherapy, chemotherapy, and sometimes hormone therapy. However, elevated rate of relapse and progression to metastatic stage followed by suboptimal response to the chemotherapy or other treatments has been observed. This suggests the imperative requirement for better and selective treatments that are less toxic, more effective, and prevent the emergence of resistance.[11,12] Medicinal plants have been found to be an excellent source of therapeutic agents

with a broad spectrum of biological activities from ancient times. Anticancer agents from the natural sources or their derivatives such as paclitaxel are used as standard chemotherapeutics among others for cancer treatment.[4] In the present study, *A. absinthium* L. leaf extract was screened against breast carcinoma cells. The present study showed that the plant extract significantly suppressed MCF-7 cell proliferative potential with an IC_{50} of 25 µg/mL. Various physiological functions associated with proliferation, progression of cell cycle, metastasis, etc., are regulated by ROS. Moreover, ROS has vital significance in immune system regulation, functioning of several cell signaling pathways, and redox equilibrium of the cell. Excessive ROS generation causes damage to various cell proteins, DNA, lipids, and biomembranes, leading to the initiation of apoptosis.[13] For instance, disruption of ΔΨm by capsaicin leads to oxidative stress, resulting in pancreatic carcinoma cell apoptosis.[14] The current study demonstrated that *A. absinthium* L. leaf extract significantly altered ΔΨm in MCF-7 carcinoma cells. These observations provided a strong clue toward the role of ROS mediated ΔΨm alteration in apoptotic cells. Moreover, several genotoxic drugs exert their cytotoxic effects through DNA damage causing cell death.[15] Hence, a number of chemopreventive agents aim at apoptosis-inducing pathways.[14] For instance, several anticancer candidates such as cisplatin,[16] 5-fluorourcil,[17] and toxal[18] have been found to induce apoptosis specifically through apoptotic pathways. In addition, resistance to drug is partly explained by the ability of cancer cells to evade the apoptotic pathway.[19,20] Further, anticancer agents with apoptosis-activating potential may possess the ability to prevent the development of drug resistance. The data from the present study indicated that the treatment of the cells with *A. absinthium* L. leaf extract caused apoptosis induction *in vitro*. Moreover, *A. absinthium* L. leaf extract also decreased colony-forming potential of MCF-7 cells. *A. absinthium* L. leaf extract triggered the apoptosis in MCF-7 cells through the increase in the generation of intracellular ROS. The data from the present study are in agreement with the reported literature wherein anti-cancer agents target cancer cells partly by creating the high levels of intracellular ROS.[21,22] Other possible reasons for apoptosis induction by *A. absinthium* L. leaf extract may be due to cell cycle arrest in sub-G1 phase. Therefore, *A. absinthium* L. leaf extract might prove to be a potential candidate for the treatment of liver cancer.

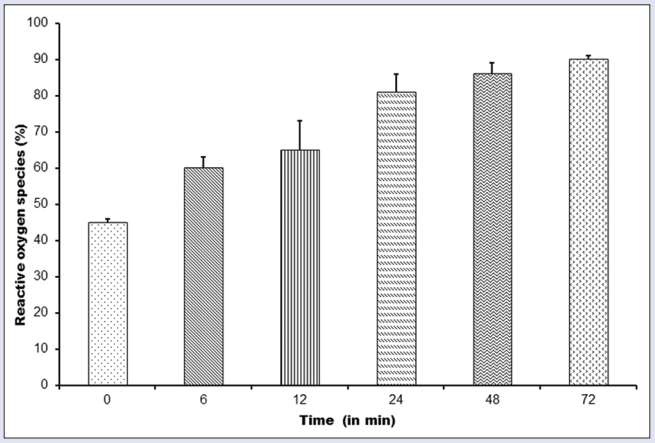

Figure 3: Determination of reactive oxygen species generation by 0.25 µg/mL of ethanolic extract of *Artemisia absinthium* L. Data shown in the figure are the average of three replicates ± standard deviation

CONCLUSION

In summary, the study showed that *A. absinthium* L. leaf extract exhibits considerable anticancer activity against breast carcinoma cells. The anti-proliferative potential of *A. absinthium* L. leaf extract may be due to its ability to induce apoptosis, promote ROS generation, alter ΔΨm, and arrest cell cycle. Therefore, *A. absinthium* L. leaf extract may be used for the development of treatment for breast cancer.

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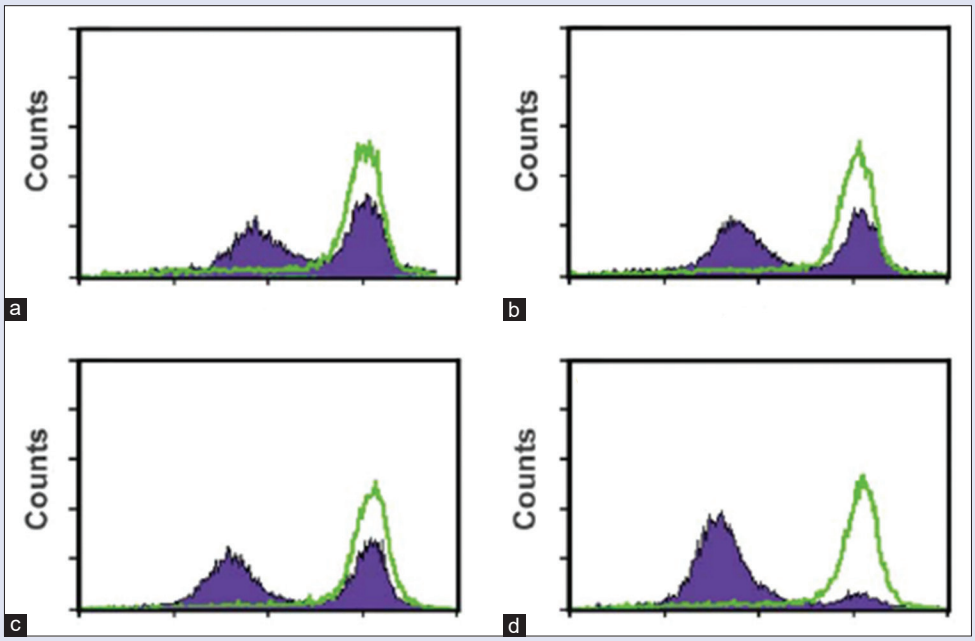

Figure 4: Evaluation of mitochondrial membrane potential by flow cytometry. MCF-7 cells at (a) 0, (b) 12, (c) 24, (d) 48 h of treatment with 25 µg/mL of ethanolic extract of *Artemisia absinthium* L were assessed for reactive oxygen species production

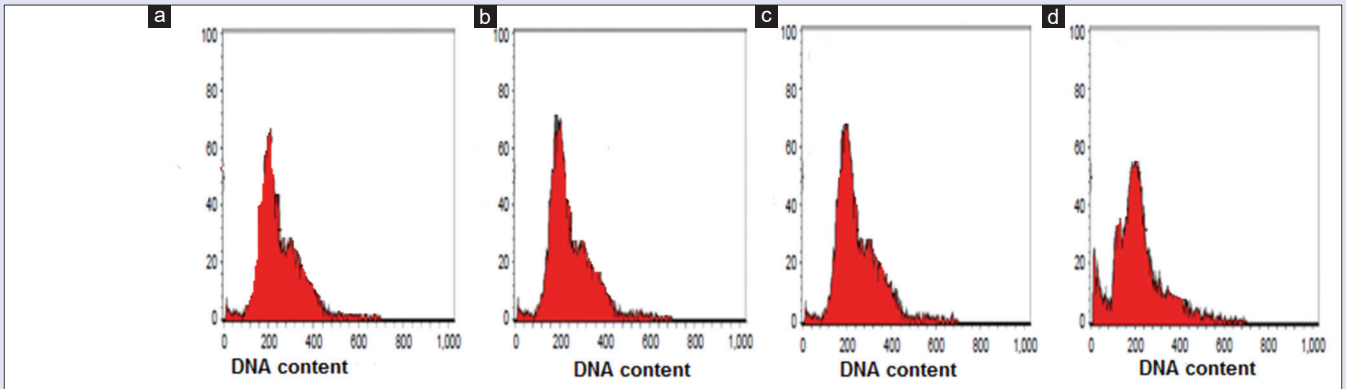

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Figure 5: Cell cycle distribution of MCF-7 cells. The cells after treatment with 0, 10, 25, and 50 µg/mL of ethanolic extract of *Artemisia absinthium* L were assessed for the distribution of DNA content

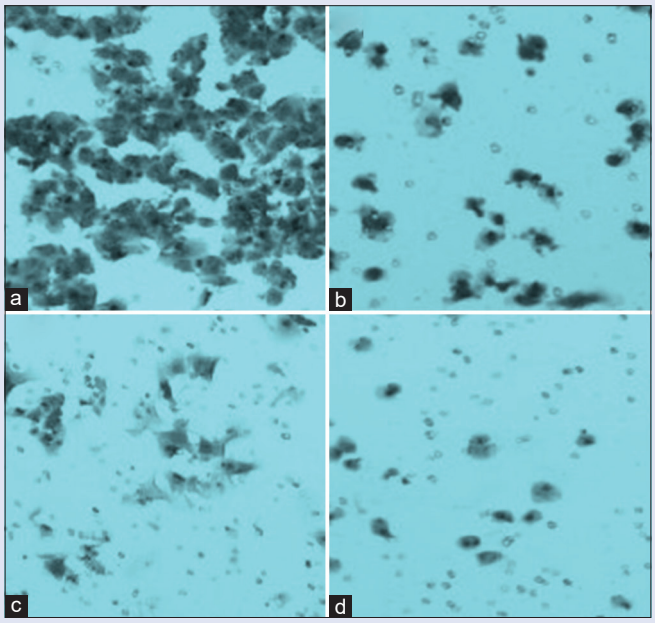

Figure 6: Clonogenic potential of MCF-7 cells treated with ethanolic extract of *Artemisia absinthium* L at 0, 10, 25, and 50 µg/mL

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Nil.

Conflicts of interest

There are no conflicts of interest.

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