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Research article

In-vitro evaluation of Epherda Foemenia for anti-oxidant and anti-cancer properties

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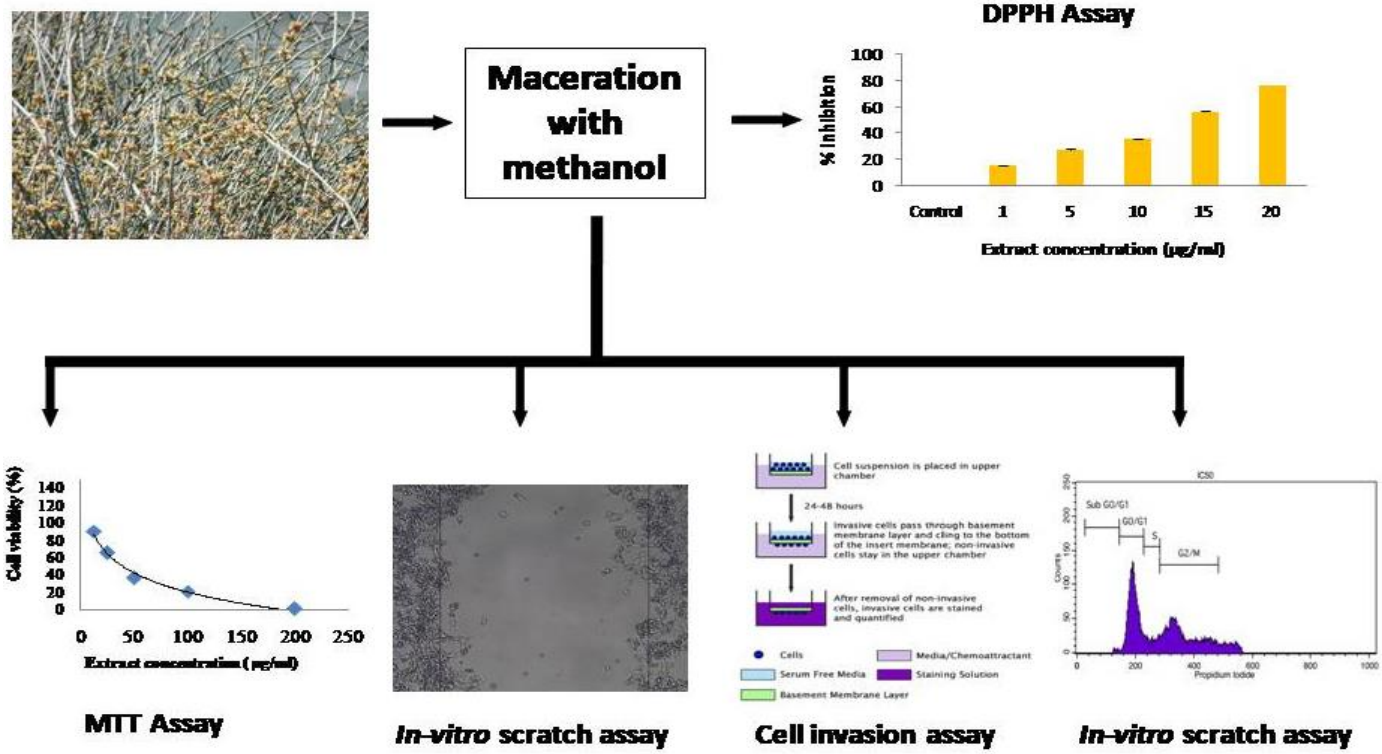
ABSTRACT

Ephedra foeminea has been increasingly recognized for its anti-cancer properties. The current study evaluated the anti-oxidant and anti-cancer activities of the methanol extract of *E. foeminea* (EFME). The extract was evaluated for cytotoxic effects against mouse breast cancer 4T1 cells, and camptothecin was used as a standard anti-cancer drug for comparison. The anti-oxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging effect while the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, cell invasion assay, scratch assay and cell cycle analysis were used for evaluation of its anti-cancer potential.



In the MTT assay on 4T1 cells, viability was preserved at ≥86% when treated at 12.5 µg/ml, with an IC_{50} concentration of 40.09 µg/ml. Further, the extract increased the cell invasion in the invasion assay, while a reduced cell migration was observed in the scratch assay. Cell cycle analysis using flow cytometry demonstrated a good anti-cancer effect of EFME with cell cycle arrest in the G2/M phase. Moreover, a remarkable anti-oxidant effect was observed in the DPPH assay. These findings indicated the EFME exhibits significant anti-cancer, and anti-oxidant properties but the effects were observed at a higher concentration of 40.09 µg/ml.

Keywords: Apoptosis, Cytotoxic, Camptothecin, DNA fragmentation, Flow cytometry.

INTRODUCTION

The death rate from cancer continues to rise worldwide despite advances in its treatment techniques. Traditional cancer treatments encompass radiotherapy, chemotherapy, and surgical interventions. Yet, their efficacy is often hindered by profound toxicity and drug resistance [1, 2].

Ephedra foeminea (Ephedraceae) is a medicinal plant indigenous to the Eastern Mediterranean region and widely found in dry and semi-dry habitats worldwide [3]. In traditional medicine, *ephedra* species are used to treat asthma, bronchitis, colds, fever, allergies, and rheumatism [4]. *E. foeminea* extracts are reported to contain several phytocompounds, such as flavonoids, alkaloids, organic acids, polysaccharides, tannins, volatile oils, and other active ingredients [5]. The use of ephedra genus for medicinal purposes dates back thousands of years and is used traditionally for anti-oxidant, antimicrobial, anti-inflammatory, anti-allergy, and antiproliferation activities [6,7]. Notably, several other species of *Ephedra* contain alkaloids like ephedrine and pseudoephedrine, which have sympathomimetic effects on the cardiovascular and respiratory systems [8]. Essential oils and other secondary metabolites play a significant role in these beneficial bioactivities [9, 10].

Recent research has highlighted the phytochemical and anti-cancer capabilities of *E. alata* extracts on various cancer cell lines [11]. A study by Al-Saraireh et al. [3] revealed that *E. foeminea* methanolic extract has significant anti-proliferative efficacy against several cancer cell lines in a concentration-dependent manner. In a similar study, Mpingirika et al. [12] found that the ethanol, ethyl acetate, and water extracts of *E. foeminea* aerial parts decreased the viability of osteosarcoma U2OS cells by affecting beta-catenin expression, a key factor in osteosarcoma cell proliferation and migration. The current study determined the anti-cancer and anti-oxidant potential of methanolic extract of *E. foeminea* against 4T1breast cancer cells and determined the mechanisms involved in its anti-cancer effect. The breast cancer cells were selected because of its widespread traditional use in the treatment of breast cancer in North Africa and Middle East regions.

MATERIALS AND METHODS

Plant collection and preparation of extract

The plant was purchased from the local market and identified by a departmental botanist. A voucher specimen (CAMS/CLS/2023/001) has been kept in the institute for future studies. *Ephedra foeminea* leaves were macerated in methanol to produce the methanolic extract [13]. The yield was measured in percentage, and preliminary phytochemical analysis was performed on the resulting extract.

Anti-oxidant activity of *E. foemenia* extract

The ***E.*** *Foemenia* methanolic extract (EFME) was evaluated for its free radical scavenging activity using DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay [14]. In brief, 100 µl of both EFME and ascorbic acid (standard) were introduced into each well and mixed with 0.1 mm DPPH reagent (4mg/100ml). The total volume of the reaction mixture was adjusted to 200 µl followed by incubation at 37°C for 30 min in the dark. The absorbance at 595 nm was determined using a UV-Vis ELISA reader (ELX-800, BioTek, USA).

Anti-cancer properties of *E. Foemenia* methanol extracts

In vitro cytotoxicity assay (MTT assay)

The 4T1 cells were seeded into a 96-well plate (20,000 cells/well) and grown overnight. EFME at different concentrations or camptothecin (CPT) (Merck, USA) were added, followed by incubation at 37°C under 5% $CO_2$for 24 h. Following this, spent media was removed from the wells, and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was introduced to each well, and incubated for 3 h. Dimethyl sulfoxide (100 µl) was added after removing the MTT reagent. The absorbance was determined at 570 nm and 630 nm used as reference wavelength, and theIC50 value was determined.

Invasion assay

This was carried out using the Cell Invasion Assay Kit (Catalog Number: CBA-110, Cell BiolabsInc). The invasion chamber plate was warmed to room temperature for 10 min and Dulbecco's Modified Eagle Medium was added to rehydrate the basement membrane layer of the cell culture inserts, followed by incubation at room temperature for 1 h. To a cell suspension (4T1) containing 0.2 x 106cells/ml in serum free media, extract and camptothecin- 8 µM (inhibitor of cell invasion) or leptin-1.2 NM (stimulator of cell invasion)(Merck, USA) were added. The rehydration medium was carefully removed, and media containing fetal bovine serum (10%) or chemo-attractant was added to the lower well of the invasion plate. A cell suspension was added to the inside of each insert and incubated for 24h at 37ºC in 5% CO2 atmosphere. The media was aspirated

carefully from the inside of the insert. The non-invasive cells were removed and the inserts were transferred to a well containing Cell Stain Solution (400 µl), and incubated for 10 min at room temperature, and washed followed by counting of the invasive cells. Each insert was transferred to an empty well, followed by addition of Extraction Solution (200 µl) per well followed by incubation for 10 min and the absorbance was measured at OD 560nm.

***In-vitro* scratch assay**

The 4T1 cells were grown in Dulbecco's Modified Eagle Medium with high glucose media containing fetal bovine serum (10%).Into a 6-well plate, the 4T1 cells were seeded at a density of 150,000cells for 24h. The monolayer was scratched with a new 10 µl pipette tip across the center of the well. A cross was created in each by scratching one line perpendicular to the other line. After scratching, the wells were gently washed with PBS to remove the detached cells and replenished with fresh medium along with extract or camptothecin. The cells were grown for 48h, and the cell images were taken at different time intervals (ex: 0, 24, and 48 h). The gap distance was quantitatively evaluated using Image J software. The calculation was done using the following equation.

Wound healing (%) = [(initial area-final area) /initial area)] x 100

Cell cycle analysis

The 4T1cells($2x10^5$cells/ml) were cultured ina6-wellplate followed by removal of spent medium and washing with 1ml 1XPBS.The cells were then IC_{50} concentration of EFME or CPT. The cells were then collected by trypsinization and harvested directly into 5ml storage vials. It was then centrifuged at 1800rpm for 5min at room temperature. The cells were washed with 1xPBS and centrifuged againat1800rpmfor5minat room temperature. The cells were then fixed with pre-chilled absolute ethanol for 1h at 4°C to fix and permeabilize the cells.

Centrifuged the cells again at 1800 rpm for 5min and removed the ethanol. To remove excess of ethanol, wash the cells with 1ml of 1xPBS and centrifuged at 1800rpm for 5min at room temperature. To ensure staining of DNA and no other nucleic acids, the cell pellet was treated with 400µL propidium iodide/RNase staining buffer followed by incubation for 15 to 20 min at room temperature in dark. The samples were analyzed by flow cytometry in PI/RNase solution without washing.

Statistical analysis

Experiments were repeated three times, and data was analyzed using one-way ANOVA followed by Tukey's post-test. GraphPad Prism 8.0 was used to analyze the data collected statistically.

RESULTS AND DISCUSSION

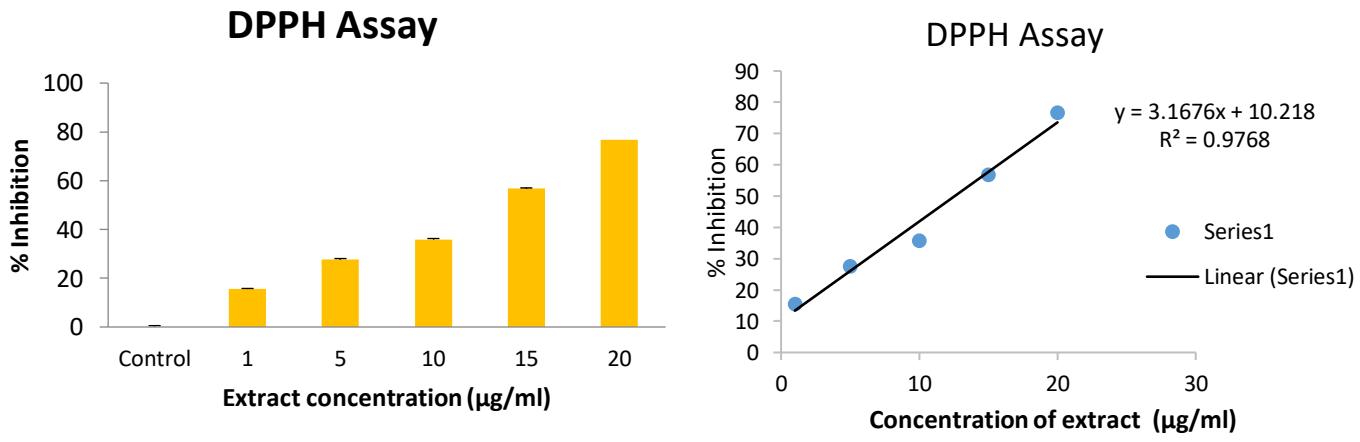
Preliminary phytochemical analysis

The percentage yield of the extract was 9.34% w/w. Preliminary phytochemical analysis revealed the presence of carbohydrates, proteins, glycosides, flavonoids, and alkaloids. Natural substances, such as anti-oxidants, with potential anti-cancer attributes can eradicate transformed or malignant cells without causing damage to normal cells. Cancer cells have shown great potential for apoptosis induction by natural plant compounds [15]. Anti-oxidants, which are bioactive compounds, are found extensively throughout the plant world. Their use has been documented in ancient medicinal practices to treat various ailments, including cancer [16, 17]. Consequently, the current study has focused on studying the anti-oxidant and anti-cancer effects of the methanol extract of *E. foeminea*.

Anti-oxidant activity of *E. foeminea* methanol extract (EFME)

The anti-oxidant effect of EFME was compared with ascorbic acid, a standard anti-oxidant agent. Both EFME and ascorbic acid showed dose-dependent anti-oxidant action. The correlation equation justifies the acceptability of the standard graph with r^2= 0.986. The percentage inhibition of DPPH radical enhanced from 15.57% to a maximum of 76.79% with an increased dose of *E. foeminea* methanolic extract (Figure 1). A maximum of 93.44% DPPH scavenging was possible with 100 µg/ml of ascorbic acid. According to data obtained, an IC_{50} value of ascorbic acid to scavenge DPPH was 8.2 µg/ml. With 100 µg/ml of *E. foeminea* methanolic extract, 76.79% DPPH scavenging, the IC50 value for the extract was calculated to be 12.56 µg/ml.

Figure 1: Anti-oxidant activity of EFME in DPPH assay

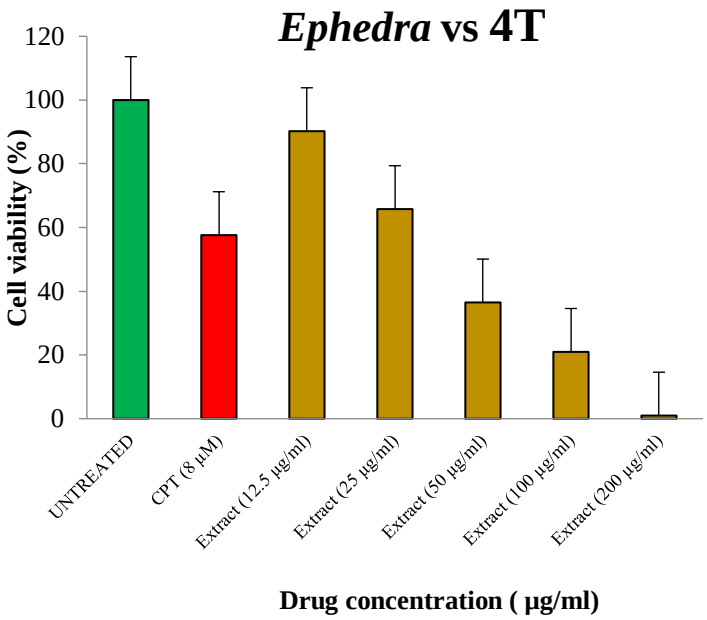


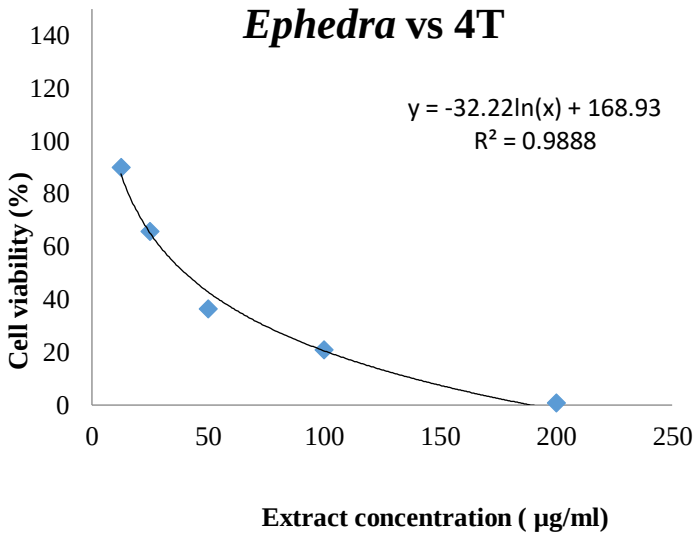
The *E. foeminea* extract exhibited an excellent anti-oxidant effect in the DPPH radical scavenging assay. A recent study reported the anti-oxidant effect of dichloromethane/methanol, methanol, ethanol, and water of *E. foeminea*, where dichloromethane/methanol and methanolic extracts showed excellent anti-oxidant effects in ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assay as compared to ethanol and water extracts. Further, several chemical constituents, including limonene, kaempferol, stearic acid, riboflavin, and naringenin were identified [18]. Another study also reported anti-oxidant and anti-diabetic effects of aqueous extract of *E. foemenia* and identified limonene (aliphatic hydrocarbon), flavonoids such as kaempferol, kaempferol-3-rhamnoside, and quercetin, vitamins such as thiamine, and riboflavin, naringenin, and ferulic acid as main constituents [19]. The anti-oxidant effect of *E. foemenia* fruits was also reported on the endothelial cells [20]. The present study determined the antioxidant effect of methanolic extract of whole plant of *E. foemenia* in the DPPH assay *in-vitro.*

Cytotoxicity evaluation

The cytotoxic characteristics of *E. foeminea* methanol crude extract were examined through an MTT assay on mouse breast cancer cell line, 4T1. A positive correlation was observed between cytotoxicity and varying concentrations of the methanol extract with r^2 values of 0.961 (Figure 2). The IC_{50} was found to be 40.09 µg/ml.

Figure 2: Cytotoxicity activity evaluation by MTT assay of various breast cancer cell lines after treatment for 24 h with standard (camptothecin8.5 µM) and methanol extract of *E. foeminea*(from 12.5 to 200 µM).





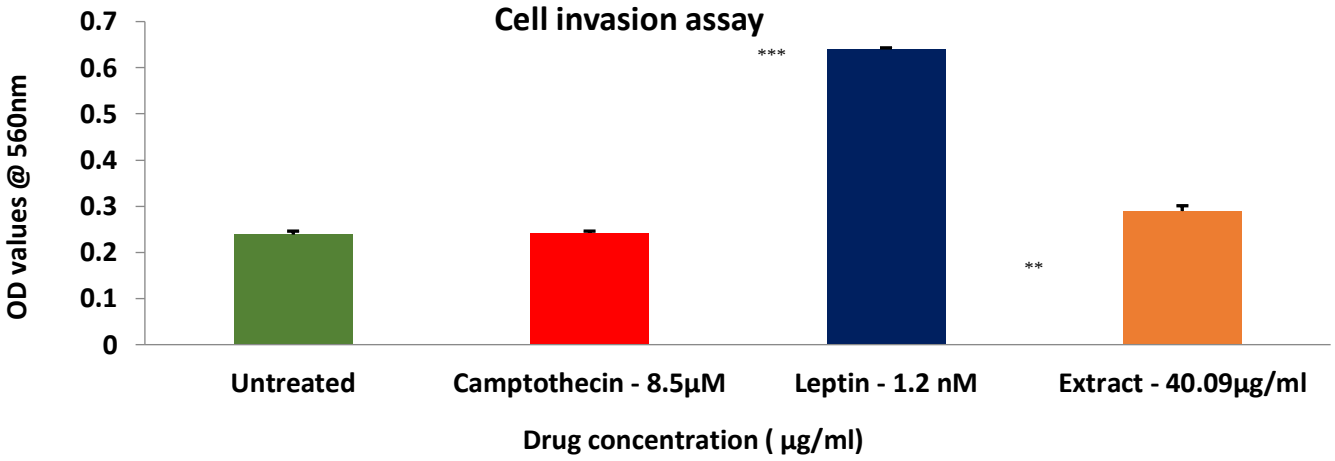
The cytotoxicity of the extract was determined using the conventional MTT assay to arrive at its IC_{50}, which was used for further analysis. The IC_{50} was found to be 40.09 µg/ml. CPT used as a reference drug here showed cytotoxic activity of 57.61% for 4T-1 mouse breast cancer cells. In comparison, the methanol extract of *E. foeminea* at 12.5 µM concentration showed 90.23% and decreased on increasing the concentration to 200 µM/ml with IC50 of 40.09. However, the concentration at which anti-cancer was observed is considered to be high. The American National Cancer Institute (NCI) has established criteria for cytotoxicity of the crude plant extracts and botanicals, and these criteria indicate that an IC_{50} value of less than 20 µg/ml or 10 µM after incubation for 48 hours or 72 hours is required to assign any crude plant extract or botanical as sufficiently cytotoxic [21]. An upper IC_{50} value of 30 µg/ml has been fixed for promising crude extracts for further purification. However, the results of the present study indicate that *Ephedra foeminea* may not be an effective anti-cancer agent as claimed by traditional users of the plant for the treatment of breast cancer. Furthermore, the current study results support earlier reports that this plant is not an effective anti-cancer drug, and our results are contradictory to those that claimed that ephedra extract is a good anti-cancer agent [12, 22-24]. Mpingirika et al [12] reported anticancer effect on the human osteosarcoma U20S cell and the IC_{50} was found to be at concentrations above 250 µg/ml. Though this concentration was very high, the authors concluded that *E. foemenia* is an effective anticancer agent against U20S cells. Jaradat et al [22] and Alsharif [23] described the traditional use of this herb in West Bank of Palestine, where it is widely used in the treatment of breast cancer though our study revealed that it is not a potent anti-cancer drug *in-vitro*. Maayan ET al [24] studied the effect of water extract, ethanol extract and fruit juice of *E. foemenia* fruits on MDA-MB-231 breast cancer cells, A549 lung carcinoma cells, HaCaT skin keratinocytes, and HCT116 epithelial colorectal carcinoma cells using just one concentration of each of the extracts or juice. They reported that ethanol extract and fruit juice possesses anticancer effects at 2

mg/ml and 0.6% respectively. Our study determined the effect at IC_{50} concentrations against breast cancer 4T1 cells. However, it is worth mentioning here that our study does not completely rule out the cytotoxic effect of ephedra against cancer cell lines. It only showed that the plant extract may not be an effective anti-cancer agent as per the criteria set by the American National Cancer Institute NCI). Camptothecin (CPT), a broad-spectrum anti-cancer agent, was used to check the accuracy of the protocol [25]. The IC_{50} of camptothecin (8.5 µM) determined as per in-house protocols, was used for evaluation.

Invasion assay

Measurement of cell migration after different treatments showed that leptin, a standard drug reduced the cell invasion significantly when compared to untreated control ($P<0.001$). A similar effect but less effect was observed after treatment with the extract ($P<0.01$), while camptothecin did not affect the cell invasion significantly as compared to untreated control (Figure3).

Figure 3: Effect of ephedra extract on cell invasion. All values are mean ± SEM, n=3, ***P<0.001, **P<0.01 compared to control.

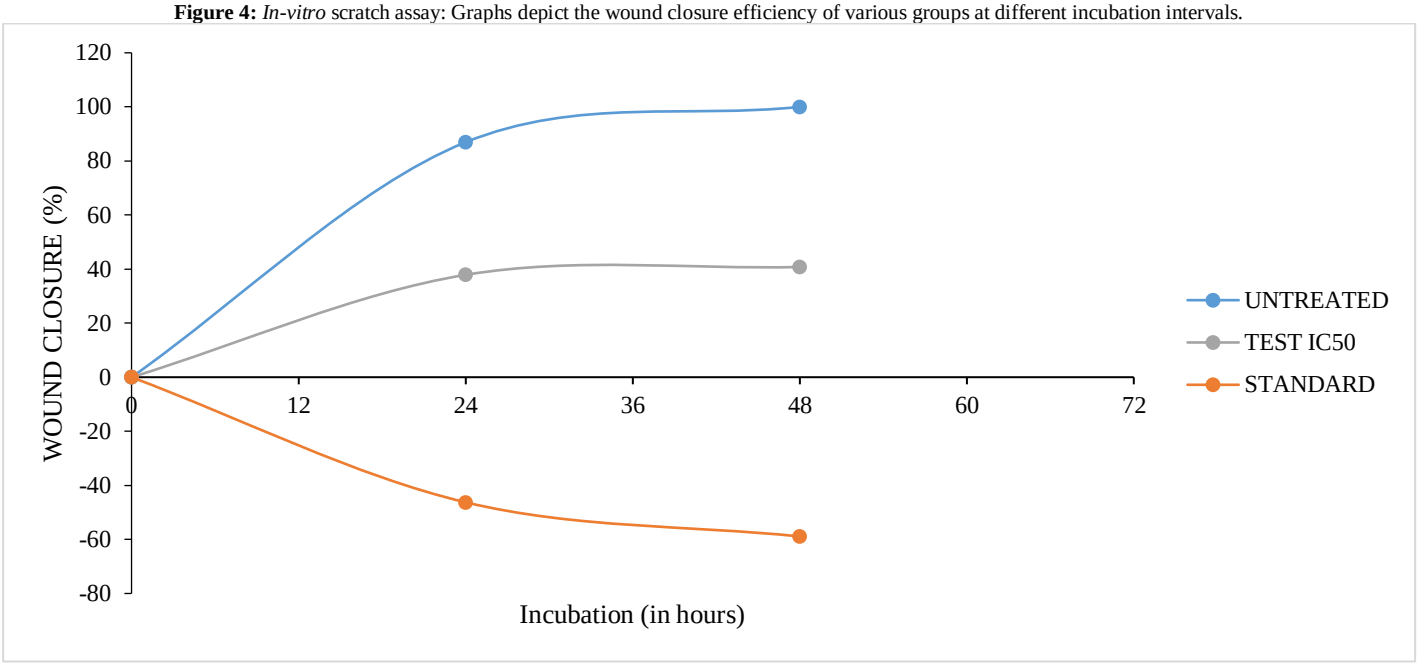


Invasion of cancer cells into normal tissues leads to morbidity and mortality in cancer patients [26]. The Cell Invasion Assay Kit uses basement membrane-coated inserts to assess the invasive properties of tumor cells. Ephedra extract was not effective in reducing cell invasion.

***In-vitro* scratch assay**

The results of the *in-vitro* scratch assay suggested that IC_{50} concentrations of the extract induced the cell migration of 4T1 cells after 24 and 48 h. The IC_{50} of the standard drug camptothecin stopped the cell migration after 24 and 48 h of incubation (Figure 4 and Figure 5). The *in-vitro* scratch assay is used to measure cell migration. The ephedra extract reduced cell migration in the scratch assay.

Figure 4: *In-vitro* scratch assay: Graphs depict the wound closure efficiency of various groups at different incubation intervals.



Cell cycle analysis

The effect of ephedra extract on the cell cycle progression of the 4T 1cell line showed that extract at IC_{50} concentration showed a high % of cells in the G2/M phase when compared to untreated cells, indicating cell cycle arrest at the G2/M phase (Figure 6 and Figure 7).

Figure 5: Image analysis of*in-vitro* scratch assay. Light microscopy images of 4T1 captured at 0, 24 and 48 h.

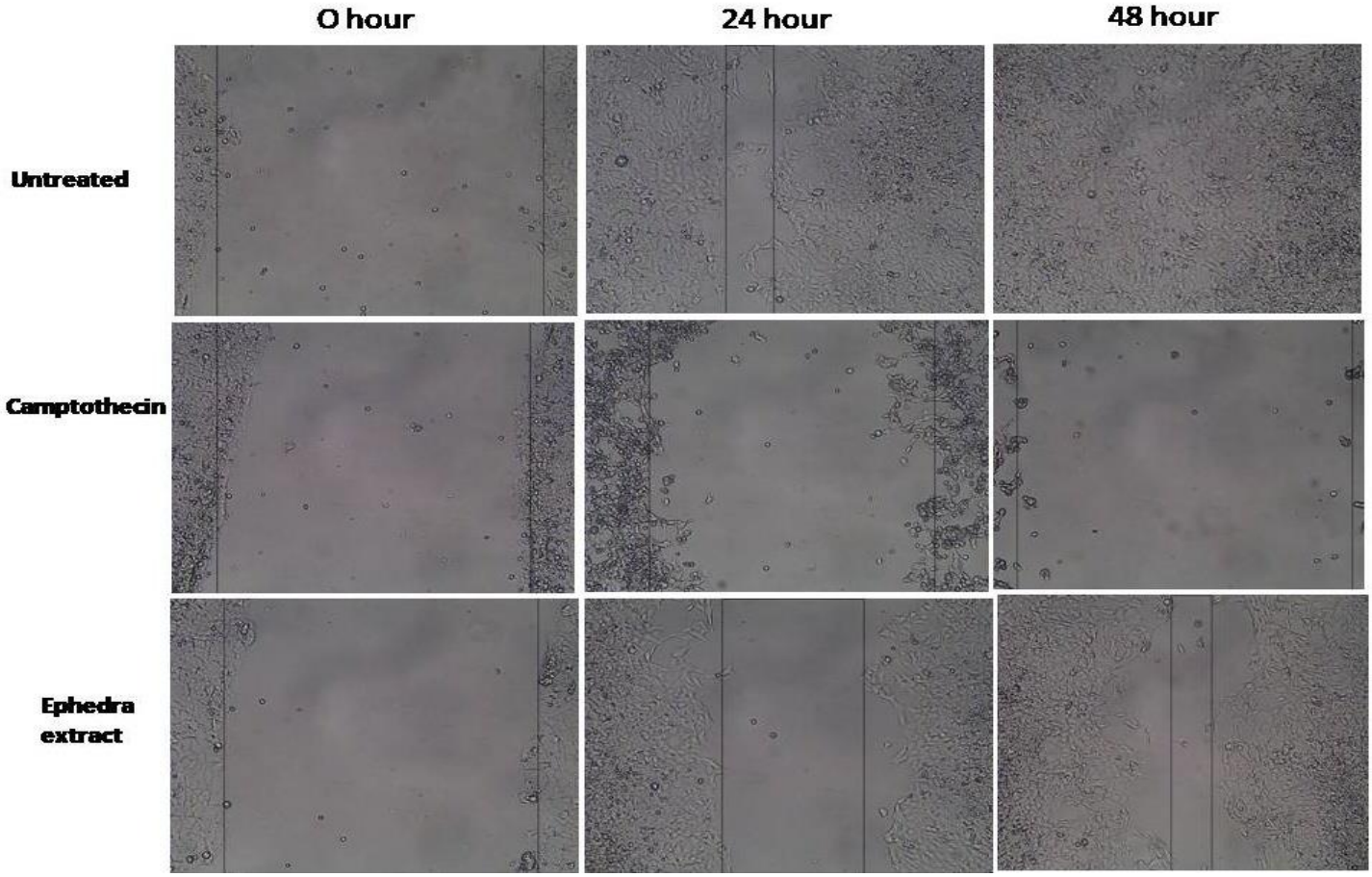
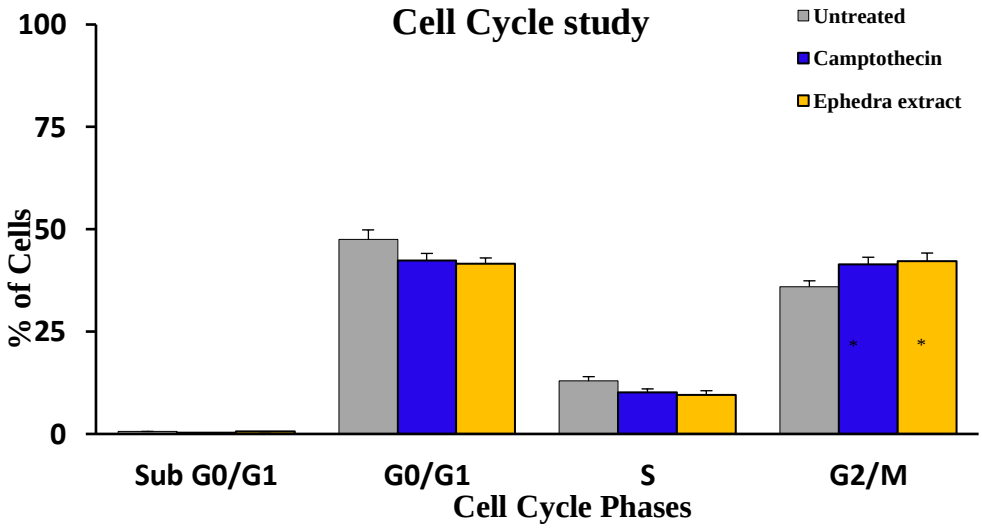


Figure 6: Percentage of 4T1 cells in different stages of cell cycle in cell cycle analysis**.**

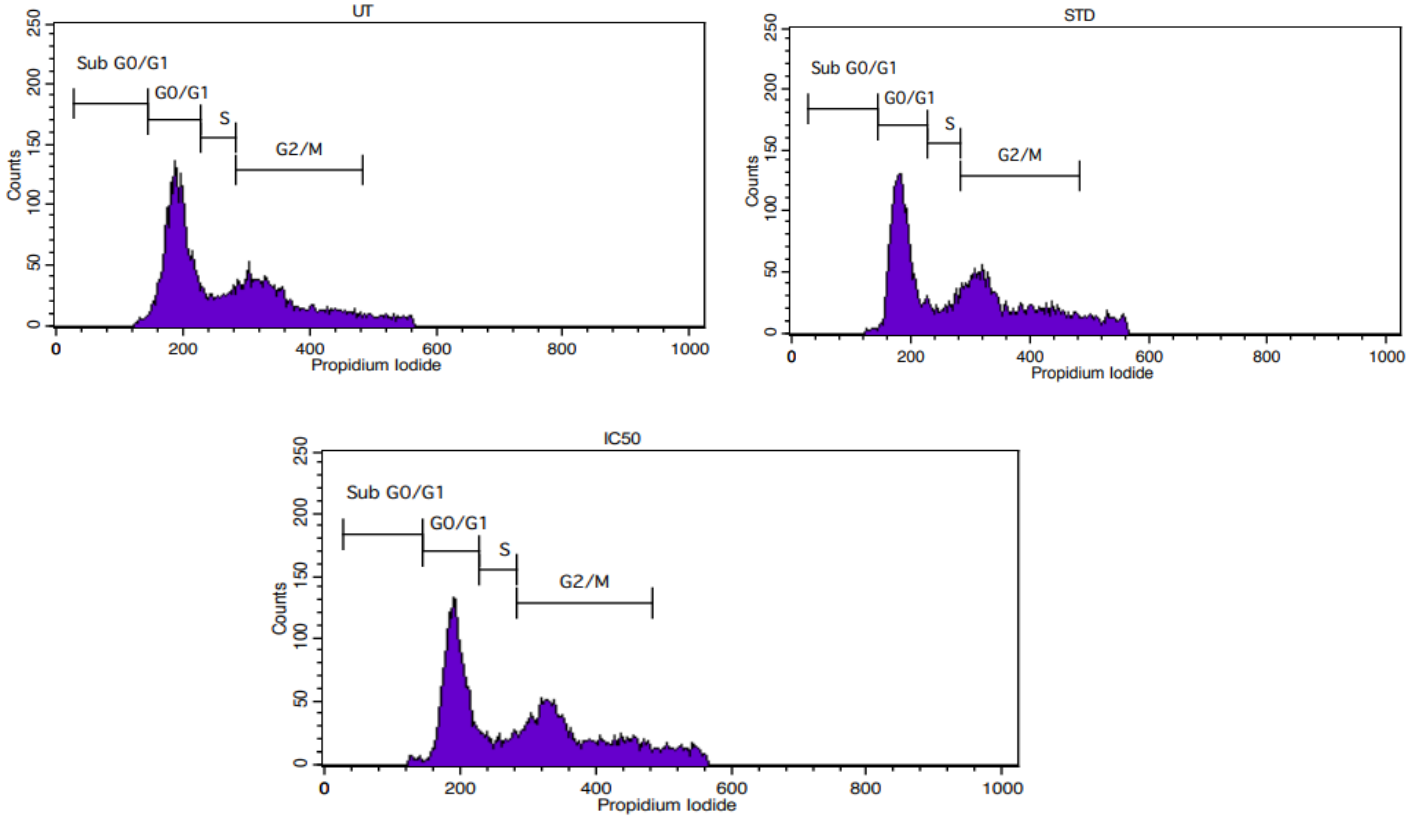


This cell cycle analysis was done using propidium iodide to determine the percentage of cells at different stages of the cell cycle. Propidium iodide intercalates DNA to stain cells. The flow cytometry was used to evaluate cell viability or DNA content. The cell cycle was arrested in G2/M by the ephedra extract, indicating its anti-cancer effect

The current study has a few limitations that can be overcome by further studies. One of the major limitations of the study is that it was carried out using *in-vitro* models only. Plants and their extracts are used by oral route. It is well known that many of the chemical constituents present in the extract undergo metabolism in the intestine or liver (first-pass effect) after oral administration [28]. Hence, some of the phyto constituents may be pro drugs that may get activated upon metabolism and may show anti-cancer effects *in-vivo*. On the contrary, it is also possible that the mild anti-cancer effect that was observed *in-vitro* may not be seen *in-vivo* due to the metabolism of active phyto constituents in the intestine and liver due to first-pass metabolism [29]. Hence, an *in-vivo* study should be done to determine its effect on breast

cancer in animals. The second limitation of the study is the place and time of collection of the plant. Though this plant is widely used by breast cancer patients, there is no clear information regarding the time, place and method of collection of the plant material as the plant was purchased from the local market? A detailed phytochemical analysis has to be done in the current study, and suspected molecules have to be identified and this has to be supported by more studies by collecting the plant in different seasons from different locations as the type and amount of phyto constituents may differ from place to place and in different seasons. This has to be carried out before completely ruling out the potent anti-cancer effect of *Ephedra foeminea* for the treatment of breast cancer. The current study utilized only one concentration of the extract at its IC_{50} value at single time interval. More detailed investigations using different concentrations of the extract and studying their effect at different time intervals may provide more information about the dose-response and the onset of the effect of the extract.

Figure 7: Flow cytometric histograms showing the phases of cell cycle distribution in 4T 1cells



CONCLUSION

The methanol extract of *E. foeminea* showed considerable anti-oxidant potential. Evaluation of cytotoxicity using MTT assay revealed a moderate cytotoxic effect with an IC_{50} value of 40.09 µg/ml. The extract showed stimulation of cell invasion and inhibited cell migration in the scratch assay. It also arrested cell division in the G2/M phase in the cell cycle assay. Additional research is needed to identify and evaluate chemical constituents responsible for the observed anti-oxidantand anti-cancer actions.

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