Saudi Pharmaceutical Journal 32 (2024) 101960

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Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

# Assessment of the anti-cancer potential of *Ephedra foeminea* leaf extract on MDA-MB-231, MCF-7, 4 T1, and MCF-10 breast cancer cell lines: Cytotoxic, apoptotic and oxidative assays

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ARTICLE INFO

*Keywords:*
Alkaloids
Cancer
*Ephedra foeminea*
Flavonoids
LC-MS
MTT assay

ABSTRACT

*Ephedra foeminea* is traditionally used to treat breast cancer in several Arab countries. Scientific studies have reported different effects of this plant on some cancer cell lines. The current study determined the anti-cancer potential of the methanolic extract of *Ephedra foeminea* against four different types of breast cancer cell lines *in-vitro*. The extract was prepared by maceration and phytoconstituents were identified by LC-MS analysis. The $IC_{50}$ value was determined against MDA-MB-231, MCF-7, 4 T1, and MCF-10 cell lines using the MTT assay. Further investigations were carried out using $IC_{50}$ concentration of the extract (40.09 µg/ml) to determine live/dead cells by acridine orange/ethidium bromide staining. The effect on the expression of reactive oxygen species (ROS) was evaluated by flow cytometry. The results were analyzed using one-way ANOVA followed by Tukey's test. The LC-MS analysis revealed the presence of 34 and 30 phytoconstituents in positive and negative modes respectively. The *Ephedra foeminea* extract was most effective against 4 T1 cells in a dose-dependent manner ($P < 0.001$) with an $IC_{50}$ value of 40.09 µg/ml and showed negligible effect against MCF-10 cells. It increased apoptosis in 77.84 % of 4 T1 cells, as determined by acridine orange/ethidium bromide staining. The extract also increased the ROS expression in the 39.57 % of 4 T1 cells. The study results showed that Ephedra foeminea extract possesses an anti-cancer effect against 4 T1 cells by increasing the expression of ROS and inducing apoptosis in the 4 T1 cells. The result suggests *Ephedra foemenia* methanolic extract possesses a reasonable anti-cancer effect due to its effect on apoptosis and oxidative pathways. The results confirm the traditional belief that *Ephedra* is effective against breast cancer.

## 1. Introduction

The use of alternative and complementary medicines among cancer patients is common all over the world. Most of these patients develop anxiety because of the disease and explore other systems of medicine with the belief that it will provide a cure and lessen their suffering. The use of alternative systems of medicine varies, with some patients consuming alternative medicine along with modern drugs while others abandon their cancer chemotherapeutic course to use alternative medicines (Mao et al., 2022).

Numerous herbs and their formulations are used all over the world for the treatment of cancer. Some of these herbs have been tested scientifically, and it is believed that newer and more effective anti-cancer molecules may be isolated from the natural products (Talib et al., 2022). However, there are no studies on the therapeutic efficacy of many other natural products (Liu et al., 2015a). In some instances, the use of some herbal drugs in combination with anti-cancer drugs has led to increased mortality because of adverse reactions to herbal medicines (Pochet et al., 2022).

One of the controversial herbal medicine that is used widely among the Arab population for cancer treatment is *Ephedra foeminea* Forssk (Ephedraceae) commonly known in Arabic as 'Alanda' (Ben-Arye et al., 2016). It is a common traditional medicine, especially in the Mediterranean region and Palestine (Elhadef et al., 2020). It is also mentioned in Chinese medicine as 'Ma-Huang' (Dousari et al., 2022). Traditionally, the plant is used in the treatment of allergic skin reactions and asthma (Khalil et al., 2020). Earlier reports on the phytochemical analysis of *Ephedra foemenia* revealed the presence of phenolic compounds (0.68 %

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https://doi.org/10.1016/j.jsps.2024.101960
Received 15 July 2023; Accepted 17 January 2024
Available online 19 January 2024

**Table 1**
Gradient solvent system used in LC-MS.

| Time | Solvent A% (Acetonitrile) | Solvent B% (Ammonium formate) |
|---|---|---|
| 0 | 5 | 95 |
| 25 | 20 | 80 |
| 40 | 20 | 80 |
| 55 | 35 | 65 |
| 65 | 80 | 20 |

w/w), flavonoids (0.06 % w/w) and alkaloids (0.01 % w/w) (Ibragic and Sofić, 2015). The *Ephedra* species contain ephedrine as chemotype except *Ephedra foemenia*, which does not contain ephedrine(Abu Hajleh et al., 2022). Because of lack of ephedrine, the pharmacological effects of *Ephedra foemenia* are different from those observed with other *Ephedra* species (Ibragic and Sofić, 2015). An earlier study on the aqueous extract of *Ephedra foemenia* showed that 18 different flavonoids and phenolics are present in aqueous extract that includes kaempferol, quercetin, catechin to name a few. A total of 32 different terpenes, organic acids and other constituents were also reported in the same aqueous extract that includes limonene, stearic acid, vitamins and citric acid (Abu Hajleh et al., 2022). Though traditional medicine books do not mention this herb for the treatment of cancer, its use for cancer treatment has increased substantially in the last decade in Arab countries. A study reported that majority of Palestinian breast cancer patients (about 68 %) use herbal remedies containing *Ephedra foeminea* (Jaradat et al., 2016). An earlier study conducted in Saudi Arabia revealed that this herb is widely used among cancer patients in the kingdom (Alsharif, 2021). One of the reasons for its use is the widespread campaign about its efficacy in social media. The traditional method for the preparation of plant extract varies with location. Usually, 350 to 500 g of the plant stems are boiled in 2 to 7 L of water for two hours and the potion is consumed with doses ranging from 100 ml to 500 ml (Ben-Arye et al., 2016).

Earlier studies about the anti-cancer potential of this herb show contradictory reports. The ethanolic extract of *Ephedra foeminea* leaves was reported to be cytotoxic on MDA-MB-231, HCT116, and HaCaT cells through caspase-3 induced apoptosis *in-vitro* (Maayan et al., 2017), while another study reported it reduces viability and migration of human osteosarcoma U2OS *in-vitro* (Mpingirika et al., 2020). Another report among different cancer patients showed that it does not possess any anti-cancer action, and it actually reduces the efficacy of other anti-cancer drugs when administered simultaneously (Ben-Arye et al., 2016). Since the incidence of breast cancer is high in Arabian countries and Alanda is one of the herbs commonly used by cancer patients, the present study was undertaken to determine its efficacy through *in-vitro* evaluations. A methanolic extract of *Ephedra foeminea* leaves was prepared and its chemical constituents were determined using liquid chromatography-mass spectrometry analysis (LC-MS). The anti-cancer effect was evaluated using different breast cancer cell lines.

## 2. Methodology

### 2.1. Preparation of Ephedra foeminea extract

The plant material grown locally in Saudi Arabia was purchased from the market and it was identified by Prof. Hazem Mohammed Hassan in the Department of Clinical Laboratory Science, Shaqra University (Saudi Arabia). A voucher specimen (CAMS/CLS/2023/001) has been kept in the department of future reference. The methanolic extract of *Ephedra foeminea* leaves was prepared by maceration by soaking the coarse powder in the solvent for 72 h. The solvent was removed by filtration

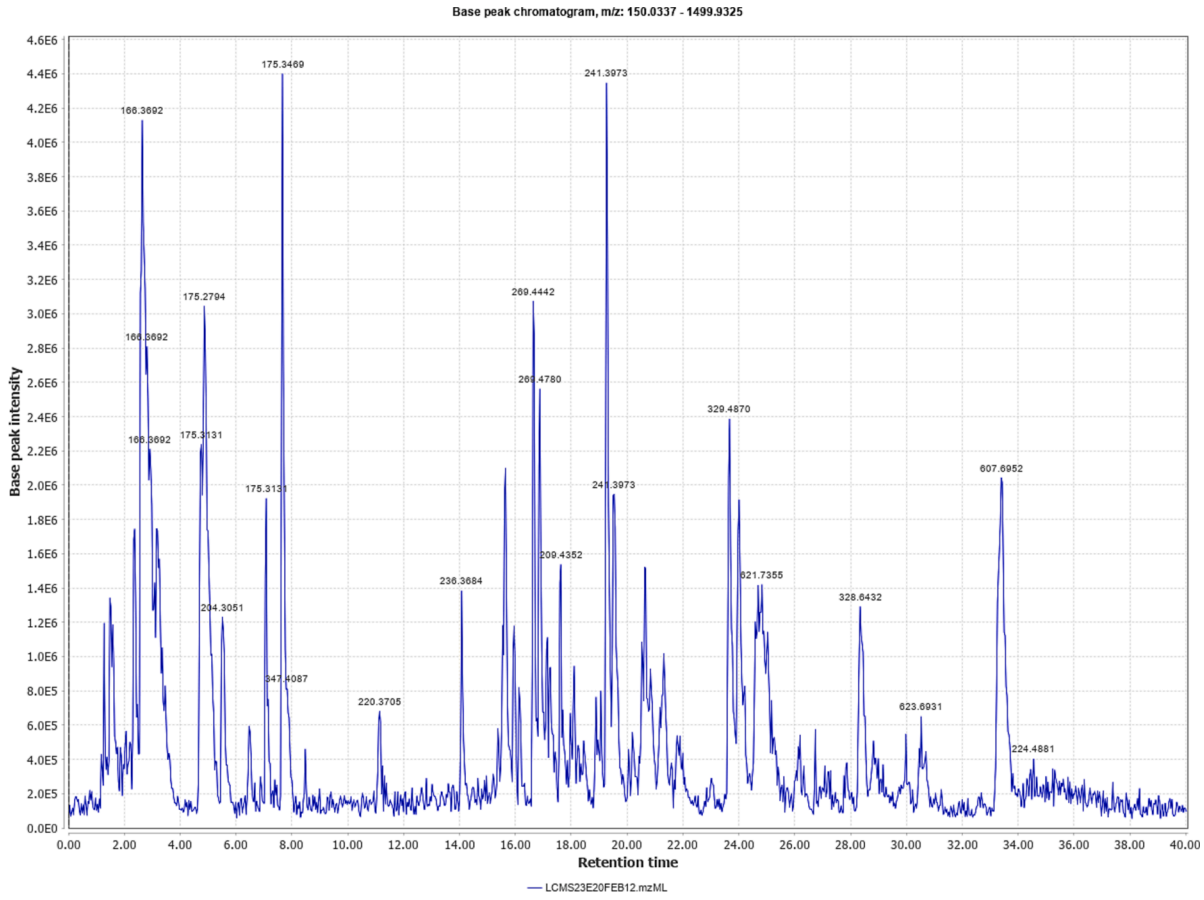


**Fig. 1.** Total ion chromatogram positive mode showing different peaks. The retention time is shown on the X-axis, and the base peak intensity is shown on the Y-axis. Data labels indicating base peak intensity are shown for prominent peaks.

**Table 2**
List of suspected compounds in positive mode.

| No. | Retention Time | Score | Compound Name | Ion | Formula | Exact Mass | Observed Mass | Mass Difference |
|---|---|---|---|---|---|---|---|---|
| | 1.28 | 0.619 | 2-Methyl-1,4-naphthoquinone | Positive | $C_{11}H_8O_2$ | 172.052 | 171.2630 | 0.789 |
| | 1.48 | 0.751 | Ferulic acid | Positive | $C_{10}H_{10}O_4$ | 194.057 | 190.3660 | 3.691 |
| | 1.59 | 0.905 | DL-alpha,epsilon-Diaminopimelic acid | Positive | $C_7H_{14}N_2O_4$ | 190.095 | 190.3323 | −0.2373 |
| | 2.34 | 0.749 | (-)-Nicotine | Positive | $C_{10}H_{14}N_2$ | 162.115 | 161.2728 | 0.8422 |
| | 2.37 | 0.706 | D-Carnitine hydrochloride salt | [M + H]+ | $C_7H_{15}NO_3$ | 161.2 | 161.2728 | −0.0728 |
| | 2.82 | 0.976 | 3-Methyl-L-histidine | Positive | $C_7H_{11}N_3O_2$ | 169.085 | 166.3692 | 2.7158 |
| | 2.92 | 0.986 | Benzocaine solution | Positive | $C_9H_{11}NO_2$ | 165.078 | 166.3692 | −1.2912 |
| | 3.26 | 0.921 | Caffeic acid | Positive | $C_9H_8O_4$ | 180.042 | 180.4095 | −0.3675 |
| | 4.73 | 0.819 | L(+)-Arginine | Positive | $C_6H_{14}N_4O_2$ | 174.111 | 175.3131 | −1.2021 |
| | 5.00 | 0.869 | 7-Hydroxy-4-methylcoumarin | Positive | $C_{10}H_8O_3$ | 176.047 | 175.3469 | 0.7001 |
| | 5.14 | 0.847 | L-Arginine monohydrochloride | Positive | $C_6H_{14}N_4O_2$ | 174.111 | 175.3131 | −1.2021 |
| | 5.51 | 0.963 | Etidronic acid | Positive | $C_2H_8O_7P_2$ | 205.974 | 204.3051 | 1.6689 |
| | 5.58 | 0.886 | O-Acety-L-carnitine hydrochloride | Positive | $C_9H_{18}NO_4$ | 204.123 | 204.3389 | −0.2159 |
| | 6.47 | 0.831 | Phytol,mixture of isomers | Positive | $C_{20}H_{40}O$ | 296.307 | 296.4112 | −0.1042 |
| | 7.08 | 0.768 | L-Canavanine | Positive | $C_5H_{12}N_4O_3$ | 176.09 | 175.3131 | 0.7769 |
| | 7.83 | 0.641 | Adenosine-5′-monophosphate sodium salt | M + H]+ | $C_{10}H_{14}N_5O_7P$ | 347.22 | 347.4087 | −0.1887 |
| | 11.14 | 0.776 | DL-Dihydrozeatin | Positive | $C_{10}H_{15}N_5O$ | 221.127 | 220.3705 | 0.7565 |
| | 14.08 | 0.752 | 1-Isothiocyanato-8-(methylsulfinyl)-octane | Positive | $C_{10}H_{19}NOS_2$ | 233.09 | 236.3684 | −3.2784 |
| | 15.55 | 0.728 | *sn*-Glycero-3-phosphocholine 1:1 cadmium chloride adduct | Positive | $C_8H_{21}NO_6P$ | 258.11 | 257.4289 | 0.6811 |
| | 15.65 | 0.715 | 1-Isothiocyanato-7-(methylsulfinyl)-heptane | Positive | $C_9H_{17}NOS_2$ | 219.075 | 217.3329 | 1.7421 |
| | 16.64 | 0.81 | Formononetin | Positive | $C_{16}H_{12}O_4$ | 268.073 | 269.4442 | −1.3712 |
| | 17.15 | 0.776 | N-Acetyl-Phytosphingosine | Positive | $C_{20}H_{41}NO_4$ | 359.303 | 357.5677 | 1.7353 |
| | 17.63 | 0.964 | Lipoic acid, reduced | Positive | $C_8H_{16}O_2S_2$ | 208.059 | 209.4352 | −1.3762 |
| | 18.10 | 0.883 | DSS (Chemical Shape Indicator) | Positive | $C_{16}H_{20}N_2O_8$ | 368.121 | 369.5830 | −1.462 |
| | 19.27 | 0.705 | L-Anserine nitrate salt | Positive | $C_{10}H_{16}N_4O_3$ | 240.122 | 241.3973 | −1.2753 |
| | 19.54 | 0.603 | L-Cystine | Positive | $C_6H_{12}N_2O_4S_2$ | 240.023 | 241.3973 | −1.3743 |
| | 20.63 | 0.852 | Sinapoyl malate | Positive | $C_{15}H_{16}O_9$ | 340.079 | 341.4685 | −1.3895 |
| | 21.31 | 0.766 | Kaempferide | M + H]+ | $C_{16}H_{12}O_6$ | 300.28 | 301.4738 | −1.1938 |
| | 24.01 | 0.8 | D-*erythro*-Dihydrosphingosine | Positive | $C_{18}H_{39}NO_2$ | 301.298 | 300.5963 | 0.7017 |
| | 25.03 | 0.218 | isorhamnetin-3-O-rutinoside | Positive | $C_{28}H_{32}O_{16}$ | 624.169 | 621.6680 | 2.501 |
| | 28.34 | 0.859 | Scoulerine | Positive | $C_{19}H_{21}NO_4$ | 327.147 | 328.6432 | −1.4962 |
| | 28.45 | 0.874 | Adenosine-3′,5′-cyclicmonophosphate | [M + H]+ | $C_{10}H_{12}N_5O_6P$ | 329.21 | 328.6095 | 0.6005 |
| | 33.39 | 0.821 | Neodiosmin | Positive | $C_{28}H_{32}O_{15}$ | 608.174 | 607.6952 | 0.4788 |
| | 33.53 | 0.869 | Guanosine-5′-diphosphoglucose sodium salt | M + H]+ | $C_{16}H_{25}N_5O_{16}P_2$ | 605.34 | 607.6614 | −2.3214 |

and the marc was extracted further using fresh solvent for 72 h twice as per WHO guidelines Annex 1 WHO guidelines on good herbal processing practices for herbal medicines). The solvent was evaporated using a rotavapor. The ratio in grams of herb to the solvent was 1:30 (Mukherjee, 2019). The percentage yield was recorded, and the extract obtained was subjected to LC-MS analysis.

The plant extract for anticancer studies was prepared by dissolving 10 mg of plant extract in 1000 µl of 100 % dimethylsulfoxide (SD fine, Mumbai, India) solution. From this 100 µl will contain 1 mg in 10 % DMSO of test solution, which was considered as the master stock solution. To that 1 mg (100 µl) solution, 900 µl of sterile cell culture media (Dulbecco's Modified Eagle Medium high glucose (#AL007A, Himedia, India) was added to make1:10 dilution for 1 % DMSO (contain 1 mg (1000 µl)). This master stock was further serially diluted to get working concentrations (12.5, 25, 50, 100, 200 µg/ml) of the test compound. Working concentrations: 12.5, 25, 50, 100, 200 µg/ml were used.

### 2.2. Liquid chromatography-mass spectrometry (LC-MS) analysis

A XEVO-TQD#QCA1232 instrument (Waters, USA) with a $C_{18}$ column (SUNFIRE $C_{18}$, 250 X 2.1, 2.6 mm) was used. Two solvents were used as the mobile phase; acetonitrile (SD fine, India), and ammonium formate (SD fine, India). The flow rate was 1.5 ml/min with a stop time of 5 min. The column temperature was maintained at 30 °C with a minimum pressure of 0 Bar and a maximum pressure of 300 bar. A gradient elution was followed, as shown in Table 1. Spectra were recorded in negative and positive ionization modes between *m/z* 150 and 2000 and the compounds were identified using ReSpect for phytochemicals (Sawada et al., 2012).

### 2.3. Cytotoxic effect against breast cancer cell lines in MTT assay

This was carried out using four different cell lines; MDA-MB-231 (Human breast cancer cell line, NCCS, Pune, India), MCF-7 (Human breast cancer cell line, NCCS, Pune, India), 4 T1 (Mouse breast cancer cell line, ATCC), MCF10 (Human breast epithelial cell line, ATCC).

The conventional MTT assay was employed (Kumar et al., 2018). Different concentrations of the *Ephedra foeminea* extract were added to cells that were grown overnight, followed by incubation at 37 °C in a 5 % $CO_2$ atmosphere for 24 h. Camptothecin (8.5 µM) (ChemExpress, Shanghai, China) was used as a standard anti-cancer agent. The spent media was removed, and MTT reagent (Himedia, India) was added and incubated, and the incubation time varied for different cell lines. After removing the MTT, dimethylsulfoxide was added to each well, and absorbance was read at 570 nm and 630 nm (MTT Cell Proliferation Assay | ATCC [WWW Document]).

The $IC_{50}$ value was determined by using a logarithmic regression equation, Y = mln(x) + C. Here, Y = 50, M and C values were derived from the viability graph.

### 2.4. Detection of live and dead cells (acridine orange/ethidium bromide staining) in 4 T1 cells by confocal microscopy

The 4 T1 cells were cultured cells in a 96-well plate and incubated in a CO2 incubator overnight at 37 °C for 24 h. The spent medium was aspirated, and cells were washed with phosphate buffer saline (PBS) (#TL1006, Himedia, India). After removing the PBS, *Ephedra foeminea* extract at its $IC_{50}$ values (40.09 µg/ml) or standard drug-camptothecin (8.5 µM) was added and incubated for 24 h. The cells were then stained with acridine orange (A1301, Thermo Fischer, USA) and ethidium bromide (17989, Thermo Fischer, USA) and incubated for 15

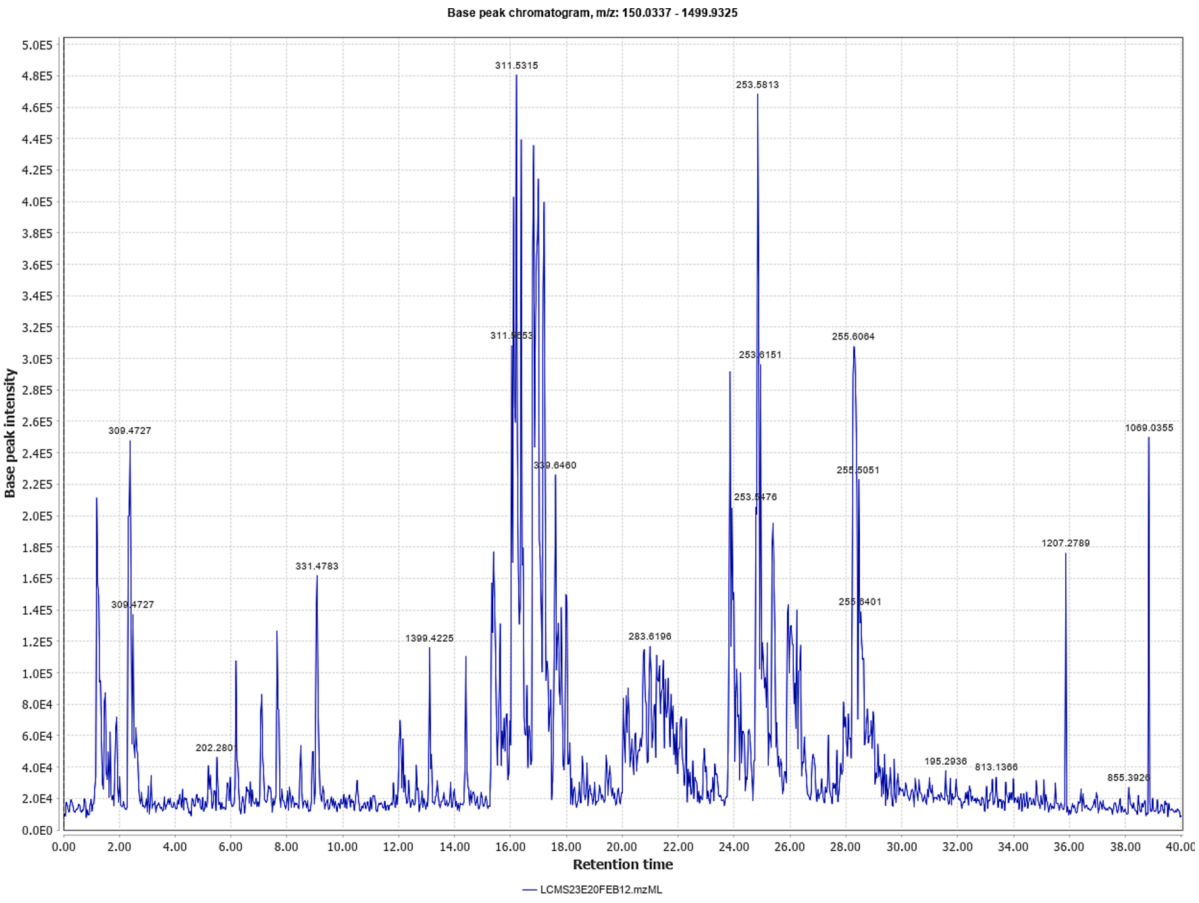


**Fig. 2.** Total ion chromatogram negative mode. The retention time is shown on the X-axis, and base peak intensity is shown on the Y-axis. Data labels indicating base peak intensity are shown for prominent peaks.

min at room temperature. Dual fluorescent staining solution (10 µl) containing 5 µg/ml AO and 5 µg/ml EtBr was used to find out live and dead cells. Five hundred cells were counted within 20 min and the morphology of apoptotic cells were examined to detect the percentage of the apoptotic cells (Liu et al., 2015b). The cells were imaged under confocal microscopy (Zeiss, Germany), and data analysis was done using ImageJ software.

### 2.5. Reactive oxygen species (ROS) expression in 4 T1 cells

The cells were cultured at a density of $1x10^7$ cells/2ml and incubated in a $CO_2$ incubator overnight at 37 °C for 24 h. The spent medium was aspirated, and cells were washed with PBS followed by treatment with different concentrations of the extract or standard for 24 h. The medium was removed, and cells were stained with 2′,7′-dichlorodihydro-fluorescein diacetate ($H_2$DCFDA) (LifeTechnologies,Invitrogen,#D-399) and incubated at 37 °C for 30 min protected from light. The ROS expression was analyzed by flow cytometry, and data analysis was done using cell QuestPro software (Zhang et al., 2019).

### 2.6. Apoptosis inducing effect by flow cytometry

The 4 T1 cells were cultured in a 6-well plate at a density of $0.5 \times 10^6$ cells/2 ml and incubated overnight in a $CO_2$ incubator. The spent medium was aspirated and cells were treated with $IC_{50}$ values of the extract or camptothecin (8 µM) in 2 ml of cultured medium and incubated for 24 h. At the end of the treatment, the medium was removed and cells were given a wash with PBS. The PBS was removed and trypsin-EDTA solution (200 µl) was added followed by incubation at 37 °C for 3–4 min. Culture medium (2 ml) was added and cells were harvested in 12x75 mm polystyrene tubes. The tubes were centrifuged at 300 g at 25 °C for 5 min. The cells were washed twice with PBS and FITC Annexin (5 µl) (#51-65874X,BDBiosciences) was added and vortexed for 15 min at room temperature in the dark. This was followed by addition of 5 µl propidium iodide (PI) (#51-66211E,BDBiosciences) and 400 µl of 1X binding buffer. Flow cytometry analysis (BDFACS Calibur) was carried out immediately after the addition of PI (FITC Annexin).

### 2.7. Statistical analysis

Values are triplicates of the measurements and are expressed as mean ± SEM. Statistical significance was measured using one-way ANOVA followed by Tukey's post test. SPSS-20 (version for Windows) software was used. $P < 0.05$ was used to indicate statistical significance.

## 3. Results

### 3.1. Extraction of Ephedra foeminea leaves and LC-MS analysis of the extract

The percentage yield obtained by maceration was 9.34 % w/w of the leaves. The methanolic extract of *Ephedra* showed a large number of molecules. A total of 34 molecules were identified in the positive mode (Fig. 1 and Table 2) while in the negative mode, another 30 molecules were identified (Fig. 2 and Table 3).

### 3.2. Anti-cancer activity against breast cancer

The results of the toxicity study (MTT assay) revealed that *Ephedra foeminea* extract has toxicity on MDA-MB-231, 4 T1, and MCF7 cell lines

**Table 3**
List of suspected compounds in negative mode.

| No. | Retention Time | Score | Compound Name | Ion | Formula | Exact Mass | Observed Mass | Mass Difference |
|---|---|---|---|---|---|---|---|---|
| | 1.19 | 0.566 | L-Iditol | Negative | $C_6H_{14}O_6$ | 182.079 | 181.2870 | 0.792 |
| | 1.26 | 0.662 | Dulcitol | Negative | $C_6H_{14}O_6$ | 182.079 | 181.3883 | 0.6907 |
| | 1.33 | 0.685 | D-(-)-Mannitol | Negative | $C_6H_{14}O_6$ | 182.079 | 181.3545 | 0.7245 |
| | 1.50 | 0.531 | 1-O-b-D-glucopyranosyl sinapate | Negative | $C_{17}H_{22}O_{10}$ | 386.121 | 187.2947 | 198.8263 |
| | 2.32 | 0.89 | N-acetylneuraminic acid | Negative | $C_{11}H_{19}NO_9$ | 309.105 | 309.4390 | −0.334 |
| | 2.49 | 0.858 | 2′-Deoxyuridine-5′-monophosphate disodium salt | Negative | $C_9H_{13}N_2O_8P$ | 308.04 | 309.4727 | −1.4327 |
| | 6.18 | 0.788 | Rhoifolin | Negative | $C_{27}H_{30}O_{14}$ | 578.163 | 577.6232 | 0.5398 |
| | 7.46 | 0.797 | Cytidine-3′-monophosphate | Negative | $C_9H_{14}N_3O_8P$ | 323.051 | 323.5806 | −0.5296 |
| | 9.08 | 0.886 | 2′-Deoxyadenosine-5′-monophosphate | Negative | $C_{10}H_{14}N_5O_6P$ | 331.068 | 331.4783 | −0.4103 |
| | 13.11 | 0.244 | Kaempferol-3-Rhamnoside-4-Rhamnoside,-7-Rhamnoside | [M−H]- | $C_{33}H_{40}O_{18}$ | 724.68 | 1399.4225 | −674.743 |
| | 14.40 | 0.897 | Pyridoxal-5′-phosphate hydrate | Negative | $C_8H_{10}NO_6P$ | 247.024 | 249.5987 | −2.5747 |
| | 15.32 | 0.92 | Kaempferide | Negative | $C_{16}H_{12}O_6$ | 300.063 | 297.5249 | 2.5381 |
| | 15.63 | 0.775 | Hesperetin | Negative | $C_{16}H_{14}O_6$ | 302.079 | 297.4912 | 4.5878 |
| | 16.04 | 0.923 | N-acetylneuraminic acid | Negative | $C_{11}H_{19}NO_9$ | 309.105 | 311.5653 | −2.4603 |
| | 16.83 | 0.921 | Uridine-5′-monophosphate | Negative | $C_9H_{13}N_2O_9P$ | 324.035 | 325.5381 | −1.5031 |
| | 17.20 | 0.949 | Cytidine-5′-monophosphate monohydrate | Negative | $C_9H_{14}N_3O_8P$ | 323.051 | 325.6057 | −2.5547 |
| | 17.61 | 0.906 | alpha-D-Glucose-1,6-diphosphate potassium salt hydrate | Negative | $C_6H_{14}O_{12}P_2$ | 339.996 | 339.6460 | 0.35 |
| | 17.81 | 0.872 | Esculinsesquihydrate | Negative | $C_{15}H_{16}O_9$ | 340.079 | 339.6122 | 0.4668 |
| | 17.99 | 0.899 | GalactinolDihydrate | Negative | $C_{12}H_{22}O_{11}$ | 342.116 | 339.6797 | 2.4363 |
| | 20.03 | 0.807 | Kaempferide | Negative | $C_{16}H_{12}O_6$ | 300.063 | 295.5336 | 4.5294 |
| | 20.78 | 0.886 | acacetin | Negative | $C_{16}H_{12}O_5$ | 284.068 | 283.6196 | 0.4484 |
| | 20.99 | 0.872 | Xanthosine | Negative | $C_{10}H_{12}N_4O_6$ | 284.075 | 283.6196 | 0.4554 |
| | 21.23 | 0.853 | Luteolin | Negative | $C_{15}H_{10}O_6$ | 286.047 | 283.6195 | 2.4275 |
| | 23.86 | 0.964 | 2′-Deoxycytidine | Negative | $C_9H_{13}N_3O_4$ | 227.09 | 227.4919 | −0.4019 |
| | 23.92 | 0.933 | L-Carnosine | Negative | $C_9H_{14}N_4O_3$ | 226.106 | 227.4919 | −1.3859 |
| | 24.78 | 0.885 | 2′-Deoxyinosine | Negative | $C_{10}H_{12}N_4O_4$ | 252.085 | 253.5476 | −1.4626 |
| | 25.39 | 0.857 | gamma-Linolenic acid | [M−H]- | $C_{18}H_{30}O_2$ | 278.43 | 279.5695 | −1.1395 |
| | 25.94 | 0.75 | L-Cystine | Negative | $C_6H_{12}N_2O_4S_2$ | 240.023 | 241.4310 | −1.408 |
| | 28.33 | 0.925 | D-Glucosamine-6-phosphate sodium salt | Negative | $C_6H_{14}NO_8P$ | 259.045 | 255.6064 | 3.4386 |
| | 28.63 | 0.631 | Kaempferol-3-O-alpha-L-rhamnoside | Negative | $C_{21}H_{20}O_{10}$ | 432.105 | 281.3563 | 150.7487 |

**Table 4**
$IC_{50}$ values of *Ephedra foeminea* extract against different cell lines.

| Cell line | $IC_{50}$ values *Ephedra foeminea* extract | % cell viability camptothecin (8.5 μM) |
|---|---|---|
| MDA-MB-231 | 47.20 ± 0.49 μg/ml | 44.60 ± 1.24 |
| 4 T1 | 40.09 ± 0.32 μg/ml | 57.60 ± 0.98 |
| MCF-7 | 88.83 ± 0.26 μg/ml | 49.31 ± 0.45 |
| MCF-10 | NA | 75.48 ± 1.32 |

All values are mean ± SEM. All experiments were triplicates, $IC_{50}$ value was determined by using a logarithmic regression equation.

after the treatment period of 24 h with $IC_{50}$ value of 47.20, 40.09 and 88.83 μg/ml (Table 4). However, it was non-toxic to the MCF10 cell line, and a significant inhibition of cell viability was observed at a very high concentration of 200 μg/ml (Fig. 3). A clear distinction was made on the relative cytotoxicity of the extract on different cell lines (Fig. 4). camptothecin (8.5 μM), a known anti-cancer agent, was most effective against MDA-MB-231 followed by MCF-7 and 4 T1 cell lines. Similar to the *Ephedra* extract, the least cytotoxic effect of camptothecin was observed on the MCF-7 cell lines (Table 4).

### 3.3. Detection of live and dead cells (ao/etbr staining) by confocal microscopy

The extract of *Ephedra foeminea* leaves induced apoptosis in 4 T1 mouse breast cancer cells, and the % of apoptosis induced by the extract and camptothecin was 77.84 % and 74.97 %, respectively (Fig. 5). Images showing cell morphology after treatments are shown in Fig. 6.

### 3.4. ROS expression in 4 T1 cells

The results showed that, as compared to untreated cells, a higher percentage of drug-treated cells were positive for $H_2$DCFDA, indicating the ROS expression-inducing property of the test compound (Fig. 7 and Fig. 8).

### 3.5. Apoptosis inducing effect by flow cytometry

The $IC_{50}$ concentrations of extract and camptothecin induced apoptosis and necrosis in the 4 T1 cells as indicated by an increase in percentage of necrotic cells, late apoptotic and early apoptotic cells as compared to untreated cells (Fig. 9).

## 4. Discussion

The results of the current study showed that *Ephedra* extract is effective in inhibiting breast cancer *in-vitro*. However, it is not a potent anti-cancer agent, as claimed by people using it traditionally for the treatment of breast cancer because the cytotoxic effect was observed with an $IC_{50}$ value of at least 40 μg/ml, which was more than minimum $IC_{50}$ of 20 μg/ml established by American National Cancer Institute (NCI) for cytotoxicity of crude plant extracts (Aoussar et al., 2020). The extract was most effective in inhibiting the mouse breast cancer 4 T1 cells as compared to other cell lines used in the study; the MDA-MD-231, MCF-7, and MCF-10 cell lines. The $IC_{50}$ value of the extract was determined using the conventional MTT assay. This $IC_{50}$ value was used to further study its effect on apoptosis and ROS generation using the 4 T1 cell line against which the extract was most effective. The current study was carried to confirm the traditional use of *Ephedra* in the treatment of breast cancer and a LC-MS analysis was carried out to identify suspected molecules present in the extract. Detailed phytochemical investigation

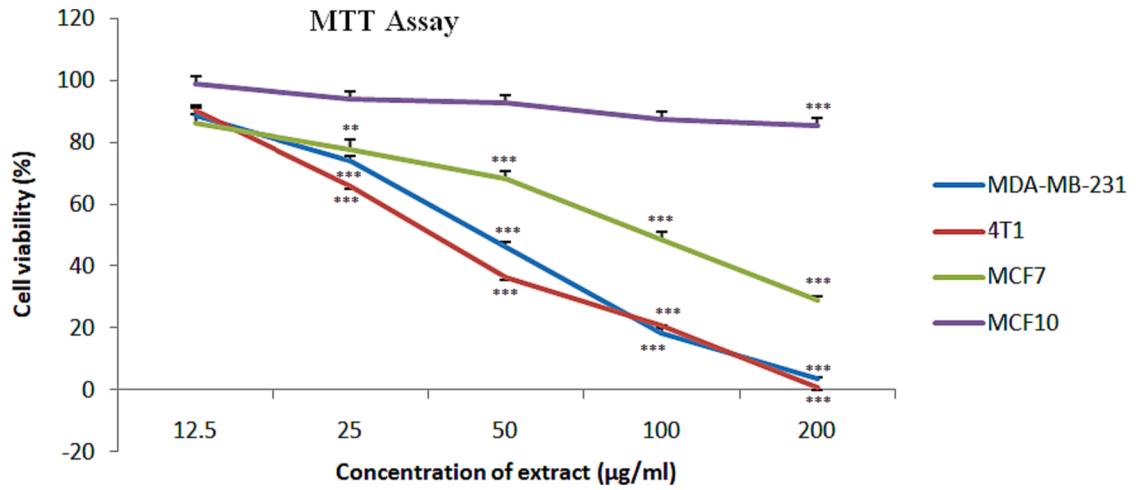


**Fig. 3.** Cytotoxic activity of *Ephedra foeminea* extract on different cell lines using MTT assay. Maximum anti-cancer activity was observed against mouse breast cancer cell line −4T1, while no effect was observed against human breast epithelial cell line-MCF-10 till the concentration of 100 µg/ml. All values are mean ± SEM, n = 3.

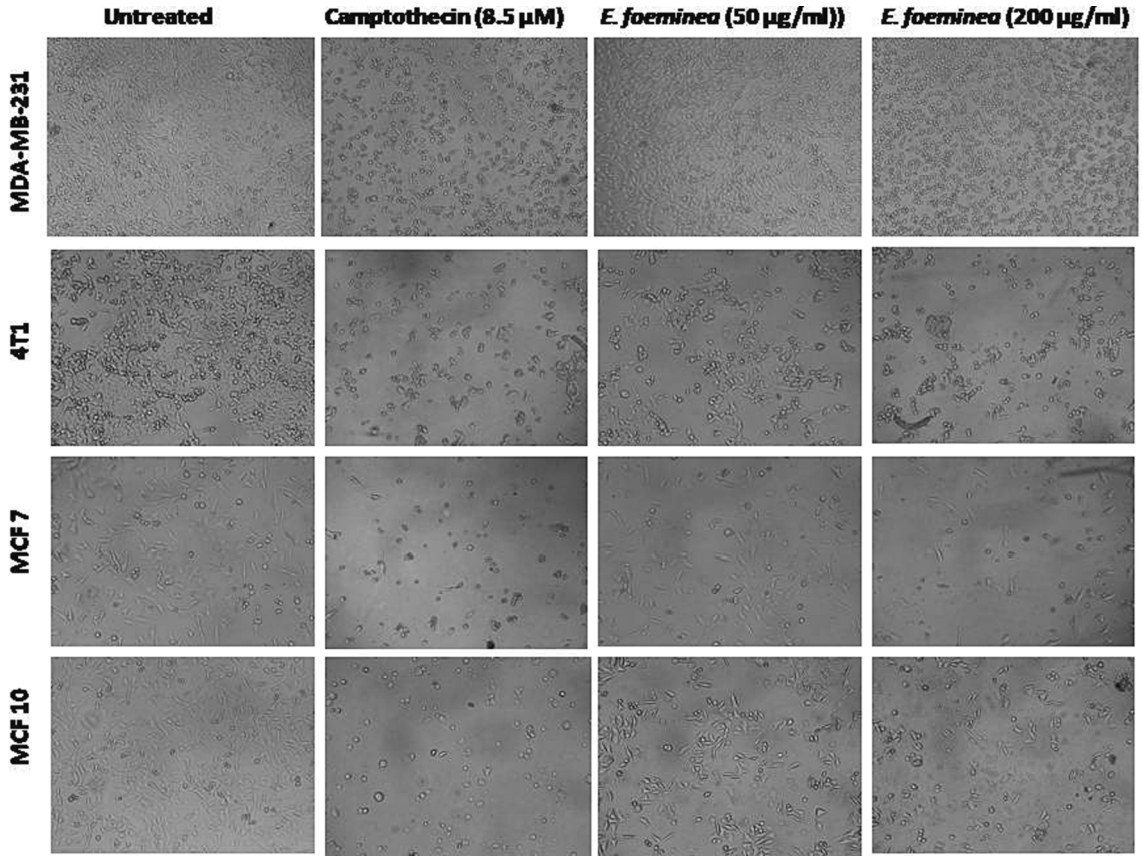


**Fig. 4.** Cell viability in MTT assay. The cell morphology shows cell shrinkage, rounding, and also a decrease in the cell population after different treatments. The relative effect of the camptothecin (8.5 µM) and *Ephedra foeminea* extract can be easily differentiated in the images.

using high-resolution mass spectrometry and NMR may provide more insight into the phytoconstituents present in the extract. Such studies are warranted for determination of constituent with anticancer activity.

The most commonly used *in-vitro* methods used for the evaluation of anti-cancer activity were employed in the present study. The MTT assay was used to determine the $IC_{50}$ value (Kumar et al., 2018). Acridine orange and ethidium bromide were used to study apopotosis (Liu et al., 2015b). Reactive oxygen species (ROS) refers to molecules with unpaired electrons. These highly reactive species interact with cellular components such as nucleic acids, proteins and lipids leading to cell death (Sahoo et al., 2022). Oxidative stress leads to generation of ROS such as superoxide, hydrogen peroxide and hydroxyl radicals. These induce apoptosis through activation of caspases (Chavda et al., 2022).

*Ephedra* is one of the oldest plant that is mentioned in the Chinese medicine and it is found abundantly in several countries in different continents (Dousari et al., 2022). In the folk medicine, a water decoction of *Ephedra foeminea* is used to treat cancer (Ben-Arye et al., 2016). Its use as anti-cancer herb has grown exponentially in the last decade due to

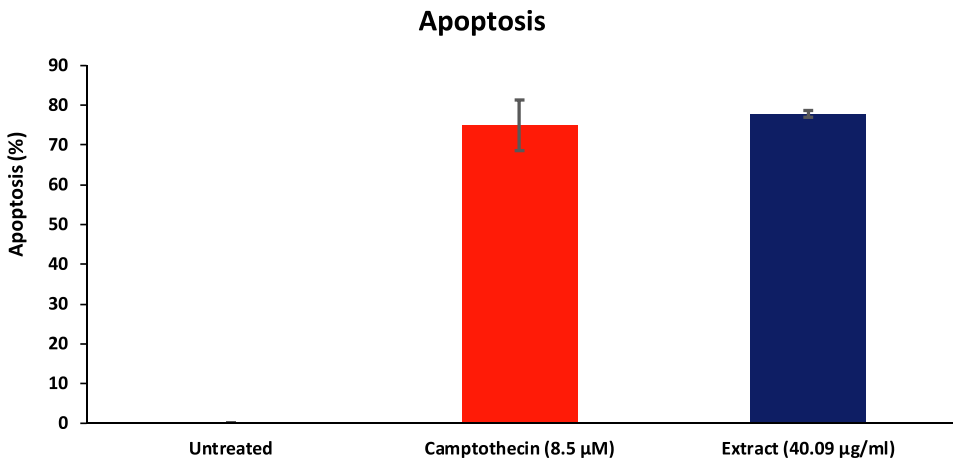


**Fig. 5.** Apoptosis cells (%) after treatment with $IC_{50}$ concentration of *Ephedra foeminea* extract and camptothecin (8.5 µM). Values are mean ± SEM, n = 3. The percentage increase in apoptosis between the two treatments was similar.

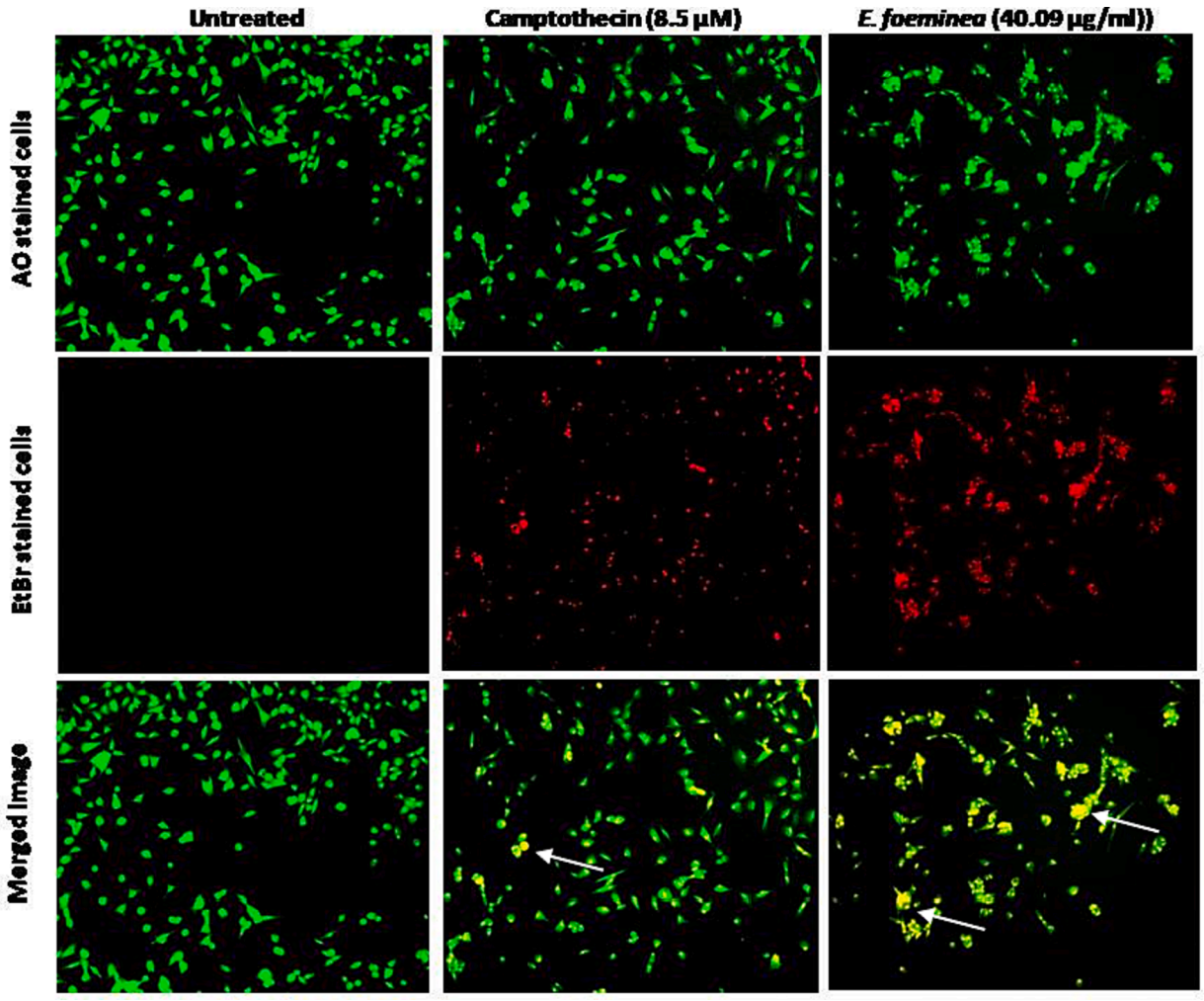


**Fig. 6.** Fluorescence microscopy images of 4 T1 cells treated with $IC_{50}$ concentrations of *Ephedra foeminea* extract and camptothecin. Untreated cells were used as negative controls. Green: Live cells; Orange nuclei: Early apoptosis; Red nuclei: late apoptosis. Apoptotic cells are stained green and because chromatin condensation and fragmentation, the nuclei have bright green dots; Ethidium bromide gives orange color to necrotic cells, these cells are similar to viable cells but do not have condensed chromatin. Apoptotic cell nuclei are shown by arrows yellowish green. The number of yellowish green nuclei was more in the extract treated group as compared to the standard while no apoptotic cells were observed in untreated control.

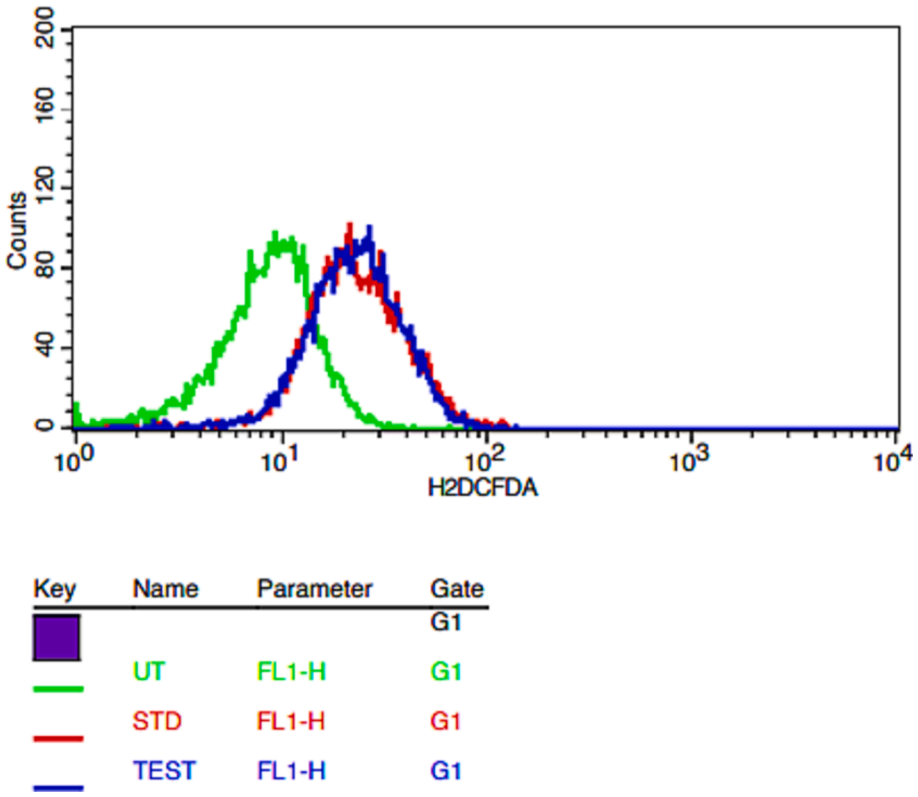


**Fig. 7.** Overlay of fluorescence intensities of $H_2$DCFDA in mouse breast cancer cell lines-4 T1 treated with $IC_{50}$ of *Ephedra foeminea* extract and camptothecin. Untreated cells were used as the negative control.

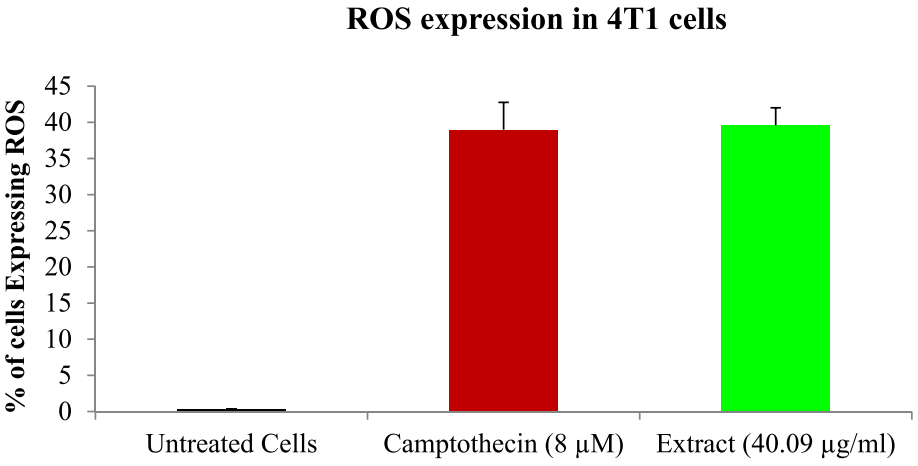


**Fig. 8.** Percentage of 4 T1 cells expressing H2DCFDA treated with $IC_{50}$ of *Ephedra foeminea* and camptothecin. Untreated cells were used as negative control.

widespread belief that it is an effective anti-cancer agent (Ali-Shtayeh et al., 2016). Earlier studies on the anticancer activities of *Ephedra* species revealed *Ephedra alata* Decne possess anticancer effects against 4 T1 cells by inducing apopotosis (Sioud et al., 2022). Another report on the anti-cancer and anti-oxidant effects of *Ephedra alata* and *Ephedra fragilis* showed that both these species are toxic to MCF-7 cells (Soumaya et al., 2020) while a study from Libya on *Ephedra alata* showed anti-cancer activity against MCF-7 cells with an $IC_{50}$ value of 38.7 µg/ml (Alshalmani et al., 2020). Another *Ephedra* species *Ephedra chilensis* is toxic to MCF-7 cells and PC-3 cancer cells *in-vitro* (Mellado et al., 2019). A preliminary study on the effect of methanolic extract of *Ephedra foeminea* on different cancer lines reported that it is toxic to breast cancer (MCF-7), lung (A549), colon (Caco-2), liver (HepG-2) and prostate (PC-3) cell lines with $IC_{50}$ values ranging from 52 µg/ml to 95 µg/ml with maximum activity observed against breast cancer cell lines (MCF-7) with an $IC_{50}$ of 52 µg/ml (Al-Saraireh et al., 2021). *Ephedra aphylla* has also been reported for anti-cancer against breast cancer cell lines (MCF-7 and T47D) with varying effects observed with different extracts (Al-Awaida et al., 2018). Many of the above studies were preliminary and determined only *in-vitro* cytotoxicity against cell lines without evaluation of probable mechanisms of action. The current study was carried out using *Ephedra foeminea* methanolic extract using only breast cancer cell lines and an attempt was also made to study the apoptotic and antioxidant effects of the extract.

A literature review to identify phytoconstituents that might have contributed to the observed anti-cancer effect revealed that several suspected molecules identified by the LC-MS analysis possess anti-cancer effects. The mass difference observed between the exact mass

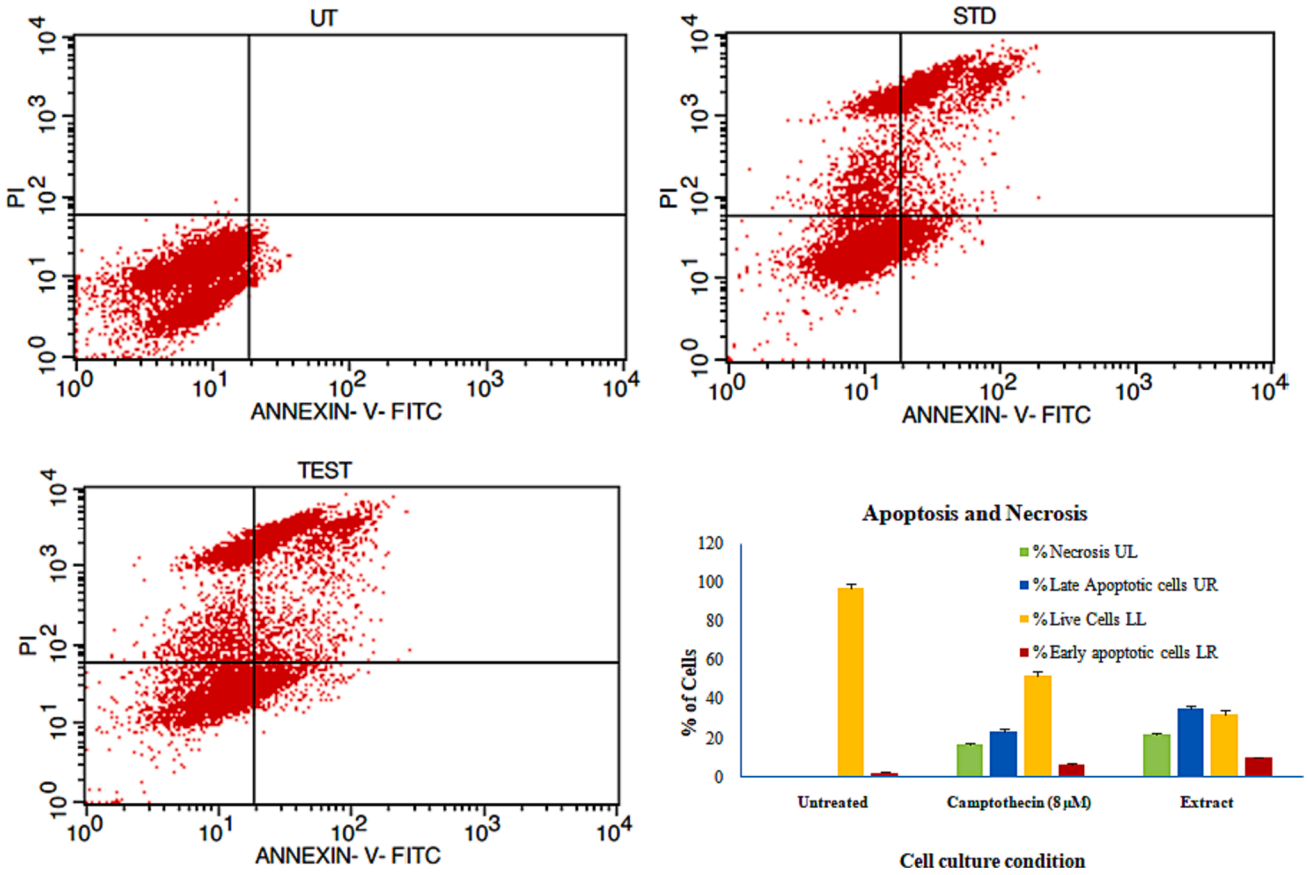


**Fig. 9.** Quadrangular plots representing the Annexin V/PI expression in 4 T1 cells upon culturing in the presence and absence of test compounds. Analysis was done by using BD FACS Calibur, Cell Quest Pro Software (Version:6.0). Here, AnnexinV-FITC-PrimaryMarker, PI- Propidium Iodide (Secondary fluorescence Marker). Camptothecin was used as the standard and test refers to extract at $IC_{50}$.

and observed mass was more in some cases. This could be due to unavailability of all the plant molecules in database and which are not reported yet. However, literature review of the suspected molecules shows that some of these may have anticancer properties. The 1,4-naphthoquinone derivatives have been reported to induce apoptosis in gastric cancer cells (Wang et al., 2019). Similarly, ferulic acid has been reported to induce cell cycle arrest and autophagy in cervical cancer cells (Gao et al., 2018). One of the most important phytoconstituent identified in the extract was caffeic acid. It has been reported for anti-cancer effects against breast, cervical, lung, and oral cancers. Its anti-cancer effect is reportedly mediated through the inhibition of cell migration and invasion (Alam et al., 2022). 7-Hydroxy-4-methylcoumarin is another phytoconstituent that is reported to possess an anti-cancer effect against skin cancer in mice (Kishk et al., 2022). Similarly, etidronic acid is cytotoxic to human breast cancer cells (Teixeira et al., 2021), and phytol is reported to be effective against sarcoma and human leukemic cancer cells (de Alencar et al., 2023). L-canavanine is reported to deprive cancer cells of L-arginine and has good anti-cancer action (Nurcahyanti and Wink, 2016). Scoulerene and formononetin also have anti-cancer actions that are mediated through multiple mechanisms (Habartova et al., 2018; Tay et al., 2019).

A comparison of phytoconstituents present in the leaf extract of *Ephedra foemenia* with an earlier report on LCMS analysis of aqueous extract of aerial parts of *Ephedra foemenia* collected in Jordan showed presence of kaemperol, and linoleic acid (Abu Hajleh et al., 2022). Other constituents reported were different and this could be due to plant part used, extraction solvent, extraction method, and geographical location.

Among the suspected molecules identified in the negative mode, dulcitol has been reported to suppress the proliferation and migration of hepatocellular carcinoma (Lin et al., 2020). Rhoifolin has anti-cancer effects against pancreatic cancer that is mediated through AKT/JNK pathways (Zheng et al., 2022). A kaempferol derivative, a known phytoconstitutent present in several plants and vegetables, was also identified in the present study. Among several activities reported for this phytochemical, it is also known to possess good anti-cancer effects (Amjad et al., 2022). Hesperetin, a phytoconstituent present in the extract, is a known anti-cancer agent, and its mechanism of anti-cancer effect has been studied in detail (Sohel et al., 2022). Similarly, N-acetylneuraminic acid is a potent anti-cancer agent that is considered as an emerging anti-cancer drug (Hwang et al., 2022).

The $IC_{50}$ value of the extract was 40.09 µg/ml against mouse 4 T1 cells, 47.20 µg/ml against MDA-MD-231, 88.83 µg/ml against MCF-7 cell lines, while it was very less effective in inhibiting the growth of MCF-10 cell lines. 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 h or 72 h is required to assign any crude plant extract or botanical as sufficiently cytotoxic (Aoussar et al., 2020). An upper $IC_{50}$ value of 30 µg/ml has been fixed for promising crude extracts for further purification (Massi et al., 2017). In the present study, the minimum $IC_{50}$ value observed was more than 40 µg/ml, and that too against the mouse breast cancer 4 T1 cell lines, and the extract was almost ineffective against MCF-10 cell lines. The reason for this difference in the effects cannot be explained with the present data. 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 (Alsharif, 2021; Jaradat

et al., 2016; Maayan et al., 2017; Mpingirika et al., 2020). 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, a known anti-cancer agent was used to check the accuracy of the protocol. The $IC_{50}$ of camptothecin (8.5 μM) determined as per in-house protocols was used for evaluation.

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 (Padmavathy and Saravanan, 2017). Hence, some of the phytoconstituents may be prodrugs 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 phytoconstituents in the intestine and liver due to first-pass metabolism (Alamgir, 2018). 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 was done in the current study, and suspected molecules were identified. However, this has to be supported by more studies by collecting the plant in different seasons from different locations as the type and amount of phytoconstituents 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 effect of the extract.

## 5. Conclusion

*Ephedra foeminea* methanolic extract showed an anti-cancer effect against MDA-MB-231, MCF-7, and 4 T1 cell lines, and it had the least anti-cancer effect against MCF-10 cells in the MTT assay. The maximum anti-cancer effect was observed against 4 T1 cells with an $IC_{50}$ value of 40.09 μg/ml. Further cytotoxic studies of the extract using the $IC_{50}$ concentration against 4 T1 cells by acridine orange/ethidium bromide staining and FITC Annexin V/PI showed that *Ephedra* extract induces apoptosis. The extract also increased the expression of ROS in the 4 T1 cells. The LC-MS analysis of the extract revealed the presence of 64 different suspected phytoconstituents. The study was conducted using *in-vitro* models only and results from *in to vivo* evaluation may provide more information about the anti-cancer effect of the *Ephedra* extract. Moreover, collecting plants at different seasons and more detailed phytochemical analysis using high-resolution mass spectrometry may further help to identify phytoconstituents contributing to the anti-cancer activity. Studies using holistic approach such as traditional preparation of *Ephedra* and consumption of adjuvants or other anti-cancer agents along with *Ephedra* may help to further confirm the anti-cancer potential of the *Ephedra foeminea* extract.

## Funding

The authors extend their appreciation to the Deanship of Scientific Research at Shaqra University for funding this research work through the project (SU-ANN-202230).

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

The authors extend their appreciation to the Deanship of Scientific Research at Shaqra University for funding this research work through the project (SU-ANN-202230).

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