Effect of *Ephedra foeminea* active compounds on cell viability and actin structures in cancer cell lines

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Received 19 August, 2017; Accepted 24 October, 2017

*Ephedra* is likely one of the oldest medicinal plants still currently in use. In folk medicine, extracts of *Ephedra foeminea* are commonly used to treat cancer patients. In relation to its traditional use, the aim of the present study was to determine the cytotoxic activity *in vitro* of *E. foeminea* extracts on cancer and non-cancer cells. Cell viability was determined using XTT assay, induction of apoptosis by cell sorting and caspase-3 inhibition, and the effects on cell cytoskeleton structure were detected using cell transfection utilizing different cytoskeleton markers. Chemical profiling, analysis of active extracts and identification of compounds was done using high pressure liquid chromatography (HPLC) and gas chromatography-mass spectroscopy (GCMS). *E. foeminea* leaf ethanol extract and *E. foeminea* fruit juice reduced cancer cell viability *in vitro*, whereas the water extract reduced cytotoxic activity in all cell lines. The extract's cytotoxic activity was conveyed at least partially via the induction of caspase 3-dependent cell apoptosis, and enhanced by the addition of Taxol. Both *E. foeminea* ethanol leaf extract and fruit juice affected actin-stained but not tubulin-stained filaments. Ethanol extract promoted the formation of invadopodia-like structures and fruit juice promoted the formation of large focal adhesion points in the treated cells. Active sub-fractions of *E. foeminea* extracts were found to contain several compounds including trans-sinapyl alcohol and trans-sinapaldehyde derivative.

**Key words:** *Ephedra foeminea*, plant extract, cancer cells, actin, invadopodia, apoptosis.

**INTRODUCTION**

*Ephedra* is a ubiquitous genus of gymnosperm shrubs that grow in temperate and subtropical regions usually on shores or in sandy soils under direct sunlight throughout North and Central America, Europe, Africa and Asia.
(Ickert-Bondmet et al., 2009). It is probably one of the oldest medicinal plants still currently in use, featured prominently in traditional Chinese medicine for over 2000 years (Abourashed et al., 2003). The main species used in Chinese medicine is *Ephedra sinica* Stapf, traditionally known in Chinese as ‘ma huang’ (Gurley et al., 1998). Premodern Chinese, Native Americans and Mormons boil the green leaves in water and serve this extract as tea to treat respiratory congestion and asthma (Normile, 2003).

The clinical interest in the folk use of *Ephedra* increased during the 20th century especially for the treatment of cancer. Importantly, an *Ephedra foeminea* decoction (that is, water extract) has been used widely by cancer patients to treat their ailments (Ben-Arye et al., 2016). Recently, in a research on the use of complementary and alternative medicines in Palestinian populations, it was reported that 68% of breast cancer patients were supplementing treatment with herbal remedies, with *E. foeminea* as the primary ingredient (45.7%) (Ali-Shtayeh et al., 2016). Moreover, *E. foeminea* only recently became one of the most commonly utilized plants by the Palestinian population-from 0.0% in 2011 to 55.2% in 2014, primarily due to local media reporting of *E. foeminea* decoctions as an effective herbal remedy for cancer patients (Ali-Shtayeh et al., 2016). These water extracts are generally prepared by boiling the leaves for an hour (Ben-Arye et al., 2016), although in some cases, the fruits are consumed as well. Reports show that some breast cancer patients even completely replace modern chemotherapy treatment with *E. foeminea* herbal extracts (Ben-Arye et al., 2016). Hence, it is extremely important to examine the effectiveness of *E. foeminea* against cancer in general, and breast cancer in particular.

In the last 15 to 20 years, the use of *Ephedra* has been restricted due to potentially hazardous effects (Abourashed et al., 2003). Indeed, the aerial parts of different *Ephedra* species contain active alkaloids (Yeung, 1980), including the phenylpropylamino alkaloids ephedrine and pseudoephedrine. Ephedrine is a sympathomimetic agent (Astrup et al., 1995; Kobayashi et al., 2003) and its consumption may lead to increased cardiac rate and contractility as well as overstimulation of the central nervous system (Abourashed et al., 2003; Krizevski et al., 2012). However, *E. foeminea* species specifically lacks both ephedrine and pseudoephedrine (Abourashed et al., 2003; Krizevski et al., 2012).

In this paper, the efficacy of *E. foeminea* extracts against cancer cells *in vitro* in relation to its folk-use was examined. The effect of different extracts of *E. foeminea* (leaf ethanol extract vs. water extract) and fruit juice on viability of cancer and non-cancer cells was evaluated and reported.

**MATERIALS AND METHODS**

**Water extraction (decoction)**

Leaves from male and female plants of *Ephedra foeminea* were collected in the Samaria Mountains (coordinates 32.087661° N, 35.274732° E WGS84) in Central Israel. The soils in the region are terra rossa; plants were collected during August 2016 (temperatures ranged from 22°C to 24°C) when plants were flowering. Botanical samples were verified by Dr. Jotham Ziffer-Berger, Director of the Herbarium of the National Natural History Collections, The Hebrew University of Jerusalem, Israel. *Ephedra* extracts were prepared according to instructions received from Y. Sharvit, a local herbalist. Leaves were weighed in a ratio of 43.7 g of male to female plants. To the leaves, 1 L of double distilled water (DDW) was added and the mixture was placed in a closed cooking pot. The mixture was brought to boil on a heating plate at medium heat; then reduced and left to simmer at a lower temperature for 2 h. After simmering, the pot was removed from the heating plate and left to cool at room temperature. The extract was then filtered using a 2.5 ml syringe with a 0.22 µm teflon filter and stored at -20°C. Diluted extract for cell treatment contained 30% of the extract and 70% of Dulbecco’s Modified Eagle Medium (DMEM, Biological Industries [BI] Ltd., Cat # 01-055-1A, Kibbutz Beit-Haemek, Israel).

**Fruit juice preparation**

*Ephedra* fruits were prepared according to the instructions received again from Y. Sharvit. Thirty grams of fruit were crushed using a mortar and pestle, producing 6 ml of *Ephedra* fruit juice. The juice and crushed material was transferred to 50 ml falcon tubes and placed in a centrifuge at 6500 rpm for 5 min. The supernatant was then stored at -20°C. The homogenous solution was filtered using a 2.5 ml syringe with a 0.45 µm teflon filter and diluted with 1 L of DDW (0.6% v/v). For higher concentrations, the volume of DDW was adjusted accordingly.

**Ethanol extraction**

Ethanol extraction was developed in the laboratory based on the protocol of Nagappan (2012). Two grams of *E. foeminea* leaves (from male and female plants at the same ratios as mentioned earlier) were crushed using a mechanical grinder and then transferred to a 50 ml falcon tube. 20 ml of 70% ethanol (EtOH) was added and the mixture was incubated overnight at 28°C with shaking at 180 rpm. The following day, the tube was centrifuged for 5 min at 2500 rpm and the supernatant was removed under vacuum in a rotary evaporator and stored at -20°C. The extract was reconstituted by adding 160 µl of 70% EtOH to the dried sample and incubated at room temperature for 10 to 15 min. One milliliter of DDW was added to the tube. Following vortex, the homogenized solution was filtered using a 2.5 ml syringe with a 0.45 µm teflon filter. The resulting filtrate was used for the experiments described below.

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Heat inactivation of *E. foelina* ethanol extract and fruit juice

Extracts (ethanol and fruit juice) were heated in order to examine the possible presence of heat-labile components. Heat treatment of the extracts was carried out by heating the filtered extract of each sample for 1 h at 90°C in a closed tube and left to cool for 2 h.

**Cell culture**

Human (*Homo sapiens*) cell lines used in this study included: MDA-MB-231-mammary gland/breast cells derived from metastatic site (ATCC® HTB-26™); A549 lung carcinomatous cells (ATCC® CRF- CCL-185™); HaCaT, keratinocytes from histologically normal skin (Creative Bioarray CSC-C8977H); HCT116, epithelial colorectal carcinoma cells (ATCC® CCL-247™). Cells were grown at 37°C in a humidified 5% CO₂ - 95% air atmosphere. All tissue culture media and serum were purchased from Biological Industries (BI), Ltd., Israel. MDA-MB-231, A549 and HaCaT were maintained in DMEM, and HCT116 were maintained in McCoy's 5A medium. Both media were supplemented with 10% FBS, 1% Penicillin-Streptomycin Solution, 1% L-glutamine and 0.002% plasmocin.

**XTT indirect viability and caspase-3 dependent assays**

Cells were seeded into 96-well plates at a concentration of 10,000 cells per well in triplicate in DMEM. The following day, DMEM was replaced with fresh medium containing either plant extracts, solvents used for extraction, or only fresh medium for control. Cells were incubated with the different treatments for 2 days and then 2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-5-[phenylamino]-carbonyl]-2H-tetrazolium inner salt (XTT) reduction was used to quantify viability according to the manufacturer’s instruction (Bii, Kributz Veit-Haemek, Israel). Cells were incubated with XTT reagent for 2 h at 37°C in a humidified 5% CO₂ - 95% air atmosphere. Absorbance was recorded by a photometer SPEKTRAFluor Plus (Tecan, Salzburg, Austria) at 490 nm with 650 nm reference wavelength. Cell survival was estimated from the equation: % cell survival = 100 × (At - Ac) / (At - Ac) (control), where At and Ac are the absorbencies (490 nm) of the XTT colorimetric reaction in treated and control cultures, respectively, minus non-specific absorption measured at 650 nm. Absorbance of medium alone was also deducted from specific readings. Dose-effect curves were determined for the extracts. For dose response assays, data points were connected by non-linear regression lines of the sigmoidal dose-response relation.

**RNA extraction, cDNA synthesis and quantitative PCR (qPCR)**

Cells were seeded into a 6-well plate at a concentration of 2,000,000 cells/ml per well. After 24 h incubation at 37°C in a humidified 5% CO₂ -95% air atmosphere, cells were treated with ethanol extract at a dilution of x5 for 1.5 and 3 h. Non-treated cells or cells treated only with ethanol served as controls. Cells were harvested and total RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer’s protocol. The RNA pellet was dissolved in nuclease-free sterile water and RNA was purified and concentrated using an RNasey MiniElute Cleanup Kit (Qiagen, USA) as per the manufacturer’s instructions. For cDNA synthesis, 2.5 µg of total RNA and 0.1 µM of random hexamer primers (Promega, USA) were heated for 5 min at 65°C and snap-chilled on ice. The following components were added to the reaction mixture: 0.2 mM dNTP mixture (Invitrogen, USA), 200 U of superscript II reverse transcriptase (RT) enzyme (Invitrogen, USA), 40 U of Ribonuclease inhibitor (Thermo Fisher-Scientific), reverse transcriptase (RT) buffer (1x final concentration), and nuclease-free sterile water (WWR, Amersco Life Sciences, OH, USA) to a reaction volume of 21 µl. The reaction was incubated at 42°C for 60 min.
followed by incubation at 70°C for 10 min. qPCR was used to determine the gene transcription of matrix metalloproteinase 9 (MMP9) in treated and non-treated MDA-MB-231 cells as described above. Expression of MMPs characterizes cells undergoing induction of invadopodia, whereas MMP9 is produced mainly by the MDA-MB-231 cells and contributes to metastatic progression (Mehner et al., 2014). The qPCR was performed using components supplied in the KAPA SYBR FAST qPCR kits (Kapa Biosystems, USA) and genes specific primers. For MMP9 (GenBank accession no. NM_004994.2), the following primers were used: (forward) 5'-TTGACAAGCAGACAAAGTGG-3' and (reverse) 5'-TCAGCTGTCCTTATGCAAG-3'. GAPDH served as the reference gene with forward (5'- CAGCCCTCAAGATCATCAGCA-3') and reverse (5'- TGTGTCATGAGTCTCTCA-3') primers. The reaction mixture consisted of the following components: 2x Master Mix with integrated antibody-mediated hot start, SYBR-Green I fluorescent dye, MgCl2, dNTPs, stabilizers, 2 μl of the template, and PCR-grade water to a final volume of 10 μl. The qPCR analysis was carried out on a Rotor-Gene 6000 instrument (Corbett-Giagen, Valencia, CA, USA) according to the following program: 3 min at 95°C, followed by 49 cycles of 95°C for 3 s, 60°C for 20 s and 72°C for 1 s. The threshold cycle (Ct) was calculated by the Rotor-Gene 6000 instrument software. The values of the steady-state level of gene transcripts were determined by the 2ΔΔCT method (Arocho et al., 2006), as a ratio between target gene (MMP9) versus the reference gene (GAPDH) and/or treated cell samples versus non-treated cells. A value above or below 1 represents an increase or decrease respectively in the steady-state level of gene transcripts for the examined conditions. Means ± SE (n = 3) were calculated for three biological replicates for each examined treatment.

**High pressure liquid chromatography (HPLC) and fractionation**

The concentrated Ephedra fruit juice, ethanol extract and water extract were filtered through 0.45 μm syringe filters. 350 μl of sample was loaded in HPLC for profiling and separation (Snyder et al., 2012). The separation of the sample was carried out with Varian Prostar HPLC system coupled with Varian 410 Autosampler, 210 pump, 320 UV/Vis detector. The separation was performed on a Purospher RP-18 endcapped column (250 mm × 4.6 mm I.D.; Merck KGaA, Darmstadt, Germany) with a guard column (4 mm × 4 mm I.D.). Solvent gradients were formed by varying the proportion of solvent A (0.1% acetic acid in DDW) to solvent B (methanol) with the flow rate of 1.0 ml min⁻¹. Initially, solvent B was maintained at 10% for 10 min and then increased to 55% in 5 min. From 55%, solvent B increased to 65% in 25 min and then maintained at 65% for 5 min and then reduced to 10% in 5 min and equilibrated for 5 min. Compound peaks were detected with two different wavelengths of 280 and 320 nm. Fractions were collected every 3 min from a total run of 30 min. Fractions were then dried using vacuum drying and lyophilization. After drying, fractions were taken for XTT assays as described above.

Sub-fractions from Fraction 7 of the ethanol extract were obtained using solvent gradients formed by isocratic proportion of 60% from solvent A and 40% of solvent B with a flow rate of 0.5 ml min⁻¹ for 25 min, and were collected based on peaks. Sub-fractions were then dried using vacuum drying and lyophilization. After drying, sub-fractions were taken for XTT assays as described above.

**Gas chromatography-mass spectroscopy (GCMS)**

Sub-fractions 7.1 to 7.9 of fraction 7 were dried using vacuum drying and lyophilization. After drying, samples were reconstituted in 100 μl of 100% EtOH and then loaded into the GCMS for analyses (Halke et al., 2004). The analysis was carried out using an Agilent 7890B gas chromatograph coupled to a 5977A mass spectrometer (electron multiplier potential 2 KV, filament current 0.35 mA, electron energy 70 eV, and the spectra were recorded over the range m/z 40 to 500). An Agilent 7893 autosampler was used for sample introduction. 1 μl of each sample was injected to the GCMS using a 1:10 split ratio injection mode. Helium was used as a carrier gas at a constant flow of 1.1 ml s⁻¹. An isothermal hold at 50°C was kept for 2 min, followed by a heating gradient of 6°C min⁻¹ to 300°C, with the final temperature held for 4 min. Solvent delay of 3 min was applied. A 30 m, 0.25 mm ID 5% cross-linked phenylmethyl siloxane capillary column (HP-5MS) with a 0.25 μm film thickness was used for separation and the injection port temperature was 200°C. The MS interface temperature was 280°C. Peak assignments were carried out with the aid of library spectra (NIST 14.0) and compared with published data and MS data obtained from the injection of standards purchased from Sigma-Aldrich.

**Statistical analysis**

Results are presented as mean ± SE of replicate analyses and are either representative of or include at least two independent experiments. Means of replicates were subjected to statistical analysis by Tukey-Kramer test (P ≤ 0.05) using the JMP statistical package and considered significant when P ≤ 0.05.

**RESULTS**

**Reduction of cell viability of cancer cells by E. foeminea extracts**

The *E. foeminea* leaf ethanol extract and fruit juice significantly reduced MDA-MB-231, HCT116 and HaCaT cell viability (Figure 1A, B and D, respectively). However, at these concentrations, they were not effective on A549 cell viability (Figure 1C). Water extract was the least active of the extracts on the different cell lines, and even at a relatively high concentration (30% v/v extract in XTT reaction mix) lacked significant activity on A549 and HaCaT cell viability (Figure 1C and D, respectively). Fruit juice was more effective on MDA-MB-231 than HaCaT cell lines with IC50 0.54 ± 0.07 and 0.69 ± 0.05%, respectively (Figure 2A). Ethanol extract was also more effective on MDA-MB-231 than HaCaT cell lines with IC50 0.57 ± 0.02 and 0.75 ± 0.01 mg/ml, respectively (Figure 2B).

**The activity of E. foeminea fruit juice and ethanol extract on cancer cell viability is heat sensitive**

Heat inactivation (90°C for 1 h) of *E. foeminea* fruit juice led to a significant reduction in activity in 1.5, 0.6 and 0.4% concentrations (v/v) (Figure 3A). Heat treatment of the ethanol extract affected activity to a much lesser extent; a significant reduction in activity was recorded in the 2.0 mg/ml concentration and to some extent, in the 0.8 mg/ml concentration (Figure 3B).
Figure 1. Effects of *E. foeminea* water and ethanol extracts and fruit juice on cell viability of (A) MDA-MB-231 breast cancer, (B) HCT 116 colon cancer, (C) A549 lung cancer and (D) HaCaT non-cancerous skin cells, determined by XTT viability assay. For each assay 10,000 cells per well were seeded in triplicate in normal growing media. Treatments were applied after 24 h and the cells were then incubated for 48 h. XTT reduction was used to quantify viability; absorbance was recorded by a photometer at 490 nm with 650 nm of reference wavelength. Cell survival was estimated from the equation: % cell survival = 100 × (At − Ac), where At and Ac are the absorbencies (490 nm) of the XTT colorimetric reaction in treated and control cultures, respectively, minus non-specific absorption measured at 650 nm. Ethanol extract (2 mg/ml extract in XTT reaction mix), fruit juice (0.6% v/v extract in XTT reaction mix) and water extract (30% v/v extract in XTT reaction mix). Levels not connected by same letter are significantly different. 70% EtOH, 70% ethanol extract of leaves; Fruit, fruit juice; Water, water extract of leaves.

The activity of *E. foeminea* ethanol extract and fruit juice on cancer cell viability as enhanced by Taxol

The addition of Taxol at a concentration of 10 nM (Mayzlish-Gati et al., 2015) to fruit juice at 0.6 and 0.4% concentration (v/v) showed reduced cell viability in comparison with activity of Taxol only or fruit juice only (in the corresponding concentrations) on cell viability (Figure 3A). Addition of Taxol at a concentration of 10 nM to the ethanol extract at concentration of 0.8 mg/ml showed reduced cell viability in comparison with the activity of Taxol only or ethanol extract only (in the corresponding concentration) on cell viability (Figure 3B).

Induction of cell death via apoptosis by *E. foeminea* extracts

Cell sorting by FACS based on Alexa Fluor® 488/Annexin V staining showed that treatment with high concentrations of the two different extracts, *E. foeminea* ethanol extract and fruit juice, lead respectively to a large proportion of cells that are in early (4.9 ± 0.7 and 43.3 ± 2.1) or late (68.2 ± 4.4 and 46.6 ± 3.8) apoptosis in comparison with the non-treated cells (FACS sorted at Q4 or Q2, respectively) (Figure 4A). The addition of Z-VAD-FMK caspase inhibitor I to *E. foeminea* ethanol extract and fruit juice led to a significant decrease in
activity on cancer cell viability (Figure 4B).

Effect of *E. foeminea* extracts on actin and tubulin filaments

Staining for actin and tubulin was carried out to determine the effect of *E. foeminea* ethanol extract, water extract and fruit juice on the organization of cytoskeleton filaments. Treatment of MDA-MB-231 cells with *E. foeminea* ethanol extracts (8.0 mg/ml for 90 min) caused reduction in focal adhesion points and induced formation of actin structure similar to invadopodia, characterized by bright points of actin staining distributed in MDA-MB-231 cells (Figure 5). Exposure of MDA-MB-231 cells to *E. foeminea* fruit juice (6% v/v, for 90 min) led to reduction in actin filaments and formation of what appears to be large focal adhesion points, whereas water extract did not show any effect on the actin filaments (Figure 5). No change in tubulin filaments were apparent following treatment with the *E. foeminea* extracts or fruit juice (Figure 5). Together, these results suggest that *E. foeminea* fruit juice and ethanol extract affect the organization of actin-stained but not tubulin-stained filaments. Despite their effect on actin, in a 2-D migration assay, *E. foeminea* extracts did not show significant effect on the cell migration of MDA-MB-231 breast cancer cells as compared to the control (Supplementary Figure 1).

Induction of matrix metalloproteinase 9 (MMP9) expression by *E. foeminea* ethanol extract

The steady state level of matrix metalloproteinases 9 (MMP9) mRNA in MDA-MB-231 cells was significantly increased by 6.96 ± 1.00 fold following treatment with *E. foeminea* ethanol extract.

Chemical profiling and analysis of *E. foeminea* extracts

Chemical profiling of the active *E. foeminea* ethanol extract and fruit juice was carried out by separating the chemical constituents of ethanol extracts and fruit juice in HPLC based on the multistep gradient program described in the materials and methods section. Subsequently, activity of the fractions on cell viability was tested using the XTT assay. The results demonstrated that Fraction 7 in both ethanol extract and fruit juice have high activity and significantly reduced MDA-MB-231 cell viability (Figure 6). However, the activity of other fractions was different between the two extracts. Fractions 6 and 8 had high activity in the ethanol extract, but only medium to low activity in the fruit juice. Fraction 2 also had high activity in the fruit juice, and medium activity in the ethanol extract (Figure 6).

Sub-fractionation of the active ethanol Fraction 7 in both *E. foeminea* ethanol extract and fruit juice was carried using the isocratic program described in the materials and methods section. Subsequently, activity of the sub-fractions on cell viability was tested using XTT assay. The results demonstrate that several sub-fractions, especially 7.2, 7.3 and 7.5, had high activity and significantly reduced MDA-MB-231 cell viability (Figure 7).

The active sub-fractions were analyzed for identification of compounds using GCMS. Results are presented in
Table 1. Only sinapyl-related compounds (trans-sinapyl alcohol, trans-sinapyl alcohol derivative and trans-sinapaldehyde derivative, respectively) were present in all the active fractions examined (Table 1); sugar present in all fractions were used as internal standard. Notably, sub-fraction 7.1, inactive for cell viability, did not contain sinapyl-related compounds.

DISCUSSION

The effect of different extracts of *E. foeminea* leaves and fruit juice on cancer and non-cancer cells, and on the cell cytoskeleton was examined. The results based on *in vitro* assays raise concerns that the folkloric use of *E. foeminea* decoction (water extract) does not have a significant effect on cancer cell viability. However, both *E. foeminea* leaf ethanol extract and fruit juice have a significant ability to reduce cell viability. It is possible that the water extraction method of boiling the leaves reduces its activity, and that some heat-labile compounds are being inactivated in the process. Indeed, some of the components in *E. foeminea* fruit juice and to a lesser extent, the ethanol extract, were found to be heat-labile; activity of the ethanol extract was reduced once heated. However, the lack of activity of the *E. foeminea* water extract stands in stark contrast to the recent sporadic reports of improvement in the medical condition of cancer patients following its use. The inefficacy of *E. foeminea* water extract was also reported in Ben-Arye et al. (2016).

The reduction in cancer cell viability by *E. foeminea* ethanol extract and fruit juice was shown on two different
Figure 4. Effect of E. foeminea extracts on cell death of MDA-MB-231 breast cancer cells. (A) Results of Annexin V/PI staining assay to assess apoptosis in MDA-MB-231. Cells were then stained using Alexa Fluor® 488 (Life Technologies, Cat # V13241, Eugene, Oregon, USA) or FITC (Invitrogen, Cat # V13242, Eugene, Oregon, USA) solution and propidium iodide (PI) working solution followed by incubation in room temperature for 15 min in the dark. Flow cytometry was performed using GALLIOS flow cytometer. Cells were considered to be apoptotic if they were Annexin V+/PI− (early apoptotic) and Annexin V+/PI+ (late apoptotic). Live cells were Annexin V−/PI−. Q4, early apoptosis. Q2, late apoptosis. (B) The effect of Z-VAD-FMK (N-benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethylketone, 100 µM, Abcam, Cat # ab120382, Cambridge, United Kingdom), caspase-3 inhibitor, on MDA-MB-231 cells. Levels not connected by same letter are significantly different. 70% EtOH, 70% ethanol extract of leaves; Fruit, fruit juice % (v/v); Z-VAD-FMK, N-benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethylketone treatment.

Figure 5. Effects of E. foeminea extracts on cell cytoskeleton. Representative fluorescent images of MDA-MB-231 cells transfected with markers for actin (red staining: Life Technologies, Cat # C10502, Eugene, Oregon, USA) and tubulin (green staining: Life Technologies, Cat # C10509, Eugene, Oregon, USA) following treatments with ethanol extract (8 mg/ml extract in the reaction mix), fruit juice (6% v/v extract in the reaction mix), water extract (30% v/v extract in the reaction mix), and non-treated control for 90 min. Images were taken using Leica SP8 laser scanning confocal microscope at 63x magnification.
types of cancer cell lines, MDA-MB-231 (breast cancer) and HCT116 (colon cancer), but not on A549 (lung cancer) cells. A somewhat reduced effect was also recorded on non-cancer skin cells (HaCaT). The fact that ethanol extract and fruit juice significantly reduced HaCaT viability may suggest that despite the potential beneficial activity of the E. foeminea ethanol extract and fruit juice on cancer cells, they may have adverse cytotoxic effects on non-cancer cells and tissues. The ineffectiveness of all extracts on A549 cell line may result from the insensitivity of this cell line to the cytotoxic compounds in E. foeminea extracts. However this needs to be further investigated.

The cytotoxic activity of E. foeminea ethanol extract and fruit juice is mediated, at least partially, by induction of apoptosis via a caspase 3-dependent pathway. This cytotoxic activity was enhanced by Taxol, and vice versa, that of Taxol was enhanced by the extract and juice, suggesting that they may act together to enhance cancer cell death.

E. foeminea ethanol extracts caused changes in the organization of actin filaments, leading to the formation of invadopodia-like structures. Invadopodia are actin-rich protrusions of the plasma membrane associated with cell migration and metastasis (Yamaguchi, 2012). Part of the activity of invadopodia in invading extracellular membranes is due to localized proteolytic activity of MMPs (Yamaguchi, 2012), involved in tumor metastasis (Foda and Zucker, 2001). In particular, MMP9 was found to be produced mainly by the MDA-MB-231 tumor cells and to significantly contribute to metastatic progression (Mehner et al., 2014). The fact that treatment with the E. foeminea ethanol extract leads to a marked increase in MMP9 expression suggests that this extract may indeed induce formation of invadopodia-like structures.

Fruit juice did not lead to the formation of invadopodia-like structures, but rather to the formation of what might be large focal adhesion points. The latter are also found as a result of treatment with Taxol. In human umbilical vein endothelial cells (HUVECs), it was shown that Taxol

![Figure 6. Chemical profiling of E. foeminea extracts and activity on cell viability. HPLC chromatogram and XTT of fractions from (A) E. foeminea fruit juice and (B) E. foeminea 70% ethanol leaves extract obtained from flow gradient with 0.1% acetic acid in water as Solvent A and methanol as Solvent B. The 1 ml min⁻¹ of flow started initially with 10% of Solvent B for 10 min and then increased gradually up to 65% from 10 to 40 min, followed by 65% of Solvent B from 40-45 min. Fractions 1-10 represents fractions collected every 3 min. XTT was performed using 3 cycles of HPLC for each fraction (each cycle is 50 µl of extract).](Image)
Figure 7. Chemical profiling of fraction 7 of *E. foeminea* ethanol extract and activity on cell viability. HPLC chromatogram and XTT of sub-fractions of fraction 7 of *E. foeminea* ethanol extract obtained from isocratic flow with 0.1% acetic acid in water as Solvent A and methanol as Solvent B. 0.5 ml min⁻¹ of flow with constant 40% methanol was used for 25 min. Sub-fractions 7.1 to 7.9 were collected and XTT was performed.

Table 1. Compounds identification and relative amount of sinapyl related compounds in sub-fractions of fraction 7 of *E. foeminea* ethanol extract. Compounds identification for each sub-fraction was obtained using GCMS. *Relative amount of sinapyl related compounds was normalized to D-Allose (sinapyl related compound:D-Allose area). **Relative amount of sinapyl related compounds was normalized to β-Ribose (sinapyl related compound:β-Ribose area).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Identified compounds</th>
<th>Relative amount of sinapyl related compounds*</th>
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<tr>
<td></td>
<td></td>
<td>trans-sinapyl alcohol</td>
</tr>
<tr>
<td>F7.1</td>
<td>D-Allose, Xylose</td>
<td>-</td>
</tr>
<tr>
<td>F7.2</td>
<td>β-Ribose, trans-Sinapaldehyde derivative</td>
<td>-</td>
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<tr>
<td>F7.3</td>
<td>D-Allose, trans-Sinapaldehyde derivative, trans-Sinapyl alcohol derivative</td>
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<tr>
<td>F7.4</td>
<td>D-Allose, β-Ribose, 5-(3-hydroxypropyl)-2,3-dimethoxyphenol, trans-Sinapyl alcohol derivative</td>
<td>-</td>
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<tr>
<td>F7.5</td>
<td>D-Allose, oxalic acid, benzendiol, benzaldehyde, phenol, β-Mannose, hydroxymethoxyphenol, Xylose, L-mannose, β-Ribose, 5-(3-hydroxypropyl)-2,3-dimethoxyphenol, C200I, trans-sinapyl alcohol, trans-Sinapaldehyde derivative, trans-sinapyl alcohol derivative, ketone</td>
<td>0.294</td>
</tr>
<tr>
<td>F7.6</td>
<td>D-Allose, trans-sinapaldehyde derivative, trans-sinapyl alcohol derivative</td>
<td>-</td>
</tr>
<tr>
<td>F7.7</td>
<td>D-Allose, β-mannose, C200I, trans-sinapaldehyde derivative, trans-sinapyl alcohol derivative</td>
<td>-</td>
</tr>
<tr>
<td>F7.8</td>
<td>D-Allose, phenol, hydroxymethoxyphenol, 5-(3-hydroxypropyl)-2,3-dimethoxyphenol, C200I, trans-sinapyl alcohol derivative</td>
<td>-</td>
</tr>
</tbody>
</table>

inhibits cell migration, at least in part, by disruption of the regulated formation and turnover of focal adhesions (Kamath et al., 2014). However, despite the effect on actin, and actin’s involvement in cell migration (Yamaguchi...
and Condeelis, 2007), there was no significant effect on cell migration by any of the *E. foeminea* extracts or fruit juice. Taxol affects tubulin by inhibiting the dynamic instability of microtubules (Schiff et al., 1979; Yvon et al., 1999). However, no changes in tubulin structure could be detected in MDA-MB-231 cells treated with *E. foeminea* extracts suggesting that the effect of the extracts are mainly on actin structures. Taken together, Taxol and *E. foeminea* extracts may differ from these components, that is, microtubules and actin, respectively, leading to enhanced aberrations of cell cytoskeleton.

When *E. foeminea* ethanol extract and fruit juice were fractioned, common active fractions and fractions with different activity levels were identified in the two *E. foeminea* extracts, suggesting differences in chemical composition. To further identify active compounds in *E. foeminea*, the active ethanol fraction (Fraction 7) was sub-fractionated and subjected to GCMS analysis, which revealed the presence of several compounds. Only sinapyl-related compounds (trans-sinapyl alcohol, trans-sinapyl alcohol derivative and trans-sinapaldehyde derivative) were present in all active fractions. Interestingly, derivatives of sinapyl alcohol were previously identified in *Ligularia nelumbifolia* (Zhao et al., 1994), a plant commonly used in the Chinese medicine for reducing inflammation, for the treatment of coughs, for curing apoplexy and for the treatment of tuberculosis (Zhao et al., 1994). Moreover, it was shown that derivatives of sinapyl alcohol are cytotoxic against several cancer cell lines (Zhao et al., 2002; Zou et al., 2006). It might be that these compounds are responsible for the cytotoxic activity against cancer cells of the *E. foeminea* ethanol extract. However, further research is needed to fully characterize the activity of sinapyl-related compounds, to exclude or include the activity of additional compounds in *E. foeminea* (e.g., those present in fruit juice) and to better understand mode of cytotoxic activity in *E. foeminea*.

**Conclusions**

The leaf ethanol extract and fruit juice of *E. foeminea* have a significant ability to reduce cancer cell viability. This is mediated at least partially via induction of caspase 3 dependent-cell apoptosis and may be induced by sinapyl-related compounds found in *E. foeminea* ethanol extract. However, cytotoxic activity was low in the water extract widely used in folk medicine. Importantly, the use of this plant for treating cancer may be dangerous, partially due to effects of the extracts on cell cytoskeleton and gene expression associated with cancer cells invasiveness and metastasis.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

**ABBREVIATIONS**

Z-VA-D-FMK, N-Benzylodoxycarbonyl-Val-Ala-Asp (OME)-fluoromethylketone; MMP9, matrix metallopeptidase 9; DDW, double distilled water; EtOH, ethanol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

**ACKNOWLEDGEMENTS**

M. M. was supported by a scholarship from The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel. R. N. and G. S. were supported by the ARO Postdoctoral Fellowship. The authors thank Dr. Jotham Ziffer-Berger of the Herbarium, The National Natural History Collections, Hebrew University of Jerusalem, Israel, for botanical authentication of the plants used in this study.

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Supplementary Figure 1. Effect of *E. foeminea* ethanol extract and fruit juice on recovered area of confluent monolayers of MDA-MB-231 cancer cell line. Cells were seeded into a 6-well plate at 400,000 cells per well in a volume of 1 ml of growth medium. After 48 h, medium was replaced with treatments including ethanol extract (1 mg/ml extract in the reaction mix), fruit juice (0.3% v/v juice in the reaction mix) and water extract (30% v/v extract in the reaction mix) and cells well incubated at 37°C in a humidified 5% CO₂–95% air atmosphere for 24 h. After incubation, the medium was removed and the cell monolayer was scratched with a 200 µl tip to create a straight line scratch. Three reference points along the scratch are labeled on the bottom of the well, and 1 ml medium containing 1% fetal bovine serum (FBS, to eliminate the possibility of cells filling the scratch by proliferation) was added to each well. Under a phase contrast microscope, images were taken at 0 h. After imaged, the medium was replaced with medium containing the specific treatment and 1% FBS. This procedure was repeated after 24 h. Images of same reference points were taken. The following equation is used to determined cell migration levels: 

\[
\left(\frac{\text{area of treat}}{\text{area of NT}}\right) \times 100.
\]

The values are means ± SE. The statistics was determined by Tukey’s HSD test (α =0.05).