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# Phytochemical Analysis and Evaluation of Anti-angiogenic and Anti-proliferative Activities of the Leaves of *Elaeagnus angustifolia L.* Grown in Jordan

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## Abstract

*Elaeagnus angustifolia L.* has a long history of use in ethnopharmacology. Only few studies examined the potential activities of the leaves. Furthermore, the leaves' chemical composition was not fully investigated. In this study, the chemical composition of *E. angustifolia* leaves extract was analysed and major compounds were isolated and identified. Extract obtained by maceration was further extracted with solvents differing in their polarity then submitted to open column chromatography, followed by isolation of major compounds. They were analysed using UV-Vis and/or NMR. One terpene (β-sitosterol) and four flavonoids (chrysin-7-glucoside, rutin, luteolin and kaempferol) were isolated and identified. For the biological activities, leaves were extracted using ethanol, ethyl acetate, chloroform and water. Anti-angiogenic activity was studied by rat aortic ring assay. Anti-proliferative activity was studied against MCF-7 and T-47D breast cancer cell lines. Ethyl acetate extract was found cytotoxic against T-47D breast cancer cell line ($IC_{50}$=23.05 µg/mL). Potent anti-angiogenic activity of ethanol-($IC_{50}$=3.039 µg/mL), ethyl acetate- ($IC_{50}$=6.289 µg/mL) and water-extract ($IC_{50}$=7.153 µg/mL) was reported for the first time.

**Keywords:** *Elaeagnus angustifolia*; Anti-angiogenesis; Breast cancer; Flavonoids; Terpenes; Jordan

## Introduction

Plant kingdom is a well-known source of useful drugs in many therapeutic fields. The plant investigated in the current study is *Elaeagnus angustifolia L.*, from the Elaeagnaceae family. *E. angustifolia* is a small tree or shrub that grows to 5-7 meters in height. It is native to western and central Asia, Afghanistan, from southern Russia and Kazakhstan to Turkey and Iran. It was also introduced to the United States and Canada [1].

*E. angustifolia* has a long history of use in ethnopharmacology [2]. It is used as an analgesic for rheumatoid arthritis in Iran. Fruits and flowers are also used in the treatment of nausea, vomiting, asthma and jaundice [3,4]. In addition, oil from the seeds is used with syrup as a paste to treat bronchial conditions [3]. In traditional Chinese medicine, the leaf extract is used in the treatment of asthma and chronic bronchitis, in addition to its anti-tussive properties [5]. In Turkey, the fruits of *E. angustifolia* are used as tonic and as anti-pyretic, as well as to treat kidney inflammation, kidney stones and diarrhoea [6]. Researchers have investigated the bioactivity of different parts of *E. angustifolia*, grown in different locations of Turkey and Iran. The seeds exerted muscle relaxant [7] and anti-nociceptive activity [8,9] in mice. Aqueous extract was found to have anti-inflammatory and pain reducing ability, probably by inhibiting cyclooxygenase type 1 and 2 [10]. It was also found to be effective in treatment of knee osteoarthritis [11]. The fruit aqueous extract was found to accelerate cutaneous wound healing [12]. Researchers also discovered that using film containing *E. angustifolia* helped reduce gag reflex; a problem that commonly occurs during dental procedures [13].

Cancer remains to be one of the leading causes of death worldwide [14,15]. Anti-proliferative activity was investigated in addition to the anti-angiogenic activity as tools for determining potential anti-cancer activity of the extract. Angiogenesis is the formation of new blood vessels from existing vessels [16]. Two types of angiogenesis can be distinguished; sprouting angiogenesis and splitting angiogenesis. Sprouting angiogenesis was the first type discovered in tumor growth by Judah Folkman in 1971 [17,18]. Angiogenesis is a hallmark of cancer which is involved in two main aspects. First, without angiogenesis a tumor cannot grow beyond a limited size, usually 1-2 $mm^3$ [19]. Formation of new blood vessels provides adequate supply of oxygen and nutrients to the rapidly growing cells. Furthermore, the new vessels serve as waste pathway for biological end products of the cancerous cells. Tumor cells trigger an "angiogenic switch" in their microenvironment [20]. This is achieved by upregulating the levels of their secretion of pro-angiogenic factors. At the same time, angiogenesis endogenous inhibitors are down regulated [21].

This study was designed to investigate and characterize the chemical composition of the leaves of *E. angustifolia* grown in Jordan and to study its potential anti-proliferative and anti-angiogenic activities.

## Materials and Methods

### Chemicals

Chemicals were obtained from Sigma (Dorset, UK), unless otherwise stated.

### Plant material and phytochemical analysis

*Elaeagnus angustifolia L.* leaves were collected from trees growing in The University of Jordan in late spring/ early summer time of 2014. Plant was taxonomically identified and authenticated by Prof. Barakat Abu Irmaileh, Faculty of Agriculture, The University of Jordan. Voucher specimens (ELEA-1FMJ) were deposited in the Department of Pharmaceutical Sciences, Faculty of Pharmacy, The University of Jordan. Fresh plant samples were air dried at room temperature and coarsely powdered. Maceration in 70% ethanol until exhaustion followed by solvent evaporation using rotary evaporator (Heidolph, Germany) at 40°C until a syrupy residue remained. All evaporated extracts were combined and dissolved in distilled water/ methanol mixture (1:1). It was washed with petroleum ether then submitted to exhaustive liquid-liquid extraction using chloroform and n-butanol. Each fraction was evaporated to dryness and crude extracts were obtained. Extracts were submitted to column chromatography (CC). Silica gel with 0.035-0.07 mm in diameter and pore diameter ca 6 nm (Acris Organics, USA) was used as stationary phase. Development of column was achieved with chloroform-methanol (100:0 to 0:100). Fractions of 100 mL were collected and evaporated to dryness using rotary evaporator. Fractions were evaluated by pre-coated TLC sheets (ALUGRAM S1L G/UV 254, Machery-Nagel GmbH and Co., Germany). Fractions that are similar in composition were collected together. Fractions that show great complexity in their composition were submitted to smaller sub-columns for further fractionation. Preparative TLC was used to isolate major compounds. To identify compounds, several methods were employed, in addition to $R_f$ value and color reaction comparison with reference substances. The absorbance spectra of the isolated compounds (UV-Vis) were determined and compared to reference compounds library and literature data. For structure determination, $^1$H NMR, $^{13}$C NMR and dept 90 and dept 135 NMR were used.

### Extracts preparation for biological activities screening

Ten grams of powdered leaves were weighed and gently boiled with 100 mL of the solvent for 10 minutes. Four different solvents were used; water, ethanol, chloroform and ethyl acetate. Extracts were covered and left overnight. Filtration followed by evaporation until dryness was performed the next day. To obtain stock solution, each 0.1 g plant extract was dissolved in 10 mL DMSO. Appropriate dilutions were prepared for each assay.

### Cell lines and cell culture

All procedures followed in the tissue culture to screen for anti-proliferative activity were conducted according to standard protocols established and verified by research group of the same laboratory [22]. All cell lines under study were purchased from the American Type Culture Collection (ATCC). Two breast cancer cell lines, MCF-7 and T-47D, and one normal fibroblasts cell line were investigated. They were all cultured in RPMI medium (PAA Laboratories GmbH, Austria) supplemented with of 10% fetal bovine serum (Biochrome, Germany), 1% of 2 mM L-glutamine (Biochrome, Germany), 50 IU/mL penicillin and 50 µg/mL streptomycin (Thermo Scientific, USA). All cells were maintained at 37°C, 5% $CO_2$ in a humidified incubator (Binder, Germany).

### *In-vitro* anti-proliferative and cytotoxicity assay

The four extracts prepared as described earlier were tested for their antiproliferative activity against two breast cancer cell lines; MCF-7 and T-47D. MCF-7 and T-47D cells were seeded at the density of 5,000 cells/ well and allowed to attach overnight. Plant extracts were screened on the following concentrations; 100, 50, 25, 12.5, 5, 2.5, 0.5, 0.25 and 0.05 µg/mL. Each concentration was added in triplicate. Control wells were filled with equal volume of 1% DMSO (Merck-Schuchardt, Germany). Doxorubicin s used as standard reference anti-cancer drug. After 72 hours, the cell viability was assessed using sulforhodamine B (SRB) assay. In addition, the extracts were tested for their cytotoxicity against normal human fibroblasts cell line. The same protocol was used, except that the cell density was 20,000 cells/ well and incubated for 7 days with regular media replacement.

### SRB assay

Cell monolayers were fixed with 200 µL of 10% w/v trichloroacetic acid (Reidel-de Haën, Germany) and incubated at 4°C for 60 minutes. Then plates were washed with cold water four times. Excess water was drained and plates were left to dry at room temperature. Then, 50 µL of SRB stain (Sigma Aldrich, USA) was added to each well and left for additional 30 minutes. The dye will bind to proteins in the living cells. Next, excess dye was removed by washing several times with 1% v/v acetic acid. The protein-bound dye was dissolved in 100 µL of 10 mM Tris base (Promega Corporation, USA) solution (pH=10.5). Plates were shaken gently for 15 minutes then absorbance of each well was read using ELISA plate reader (BioTek Instruments, USA) at 570 nm. The viability of the cells was expressed as the mean percentage of viable cells compared with control DMSO treated cells [22].

### Anti-angiogenic activity

The procedure of rat aortic ring assay was carried out according to the rules of the Animal Ethics Committee of The University of Jordan. Male Sprague Dawley rats weighing 200-250 grams were provided from the animal house of the Department of Biological Sciences, Faculty of Science, The University of Jordan. The rats were anesthetized gently with diethyl ether (Gainland Chemical Company, UK) and the thoracic aortae were excised, rinsed with phosphate buffer saline (Biowest, France), cleaned from the fibroadipose tissue and cross sectioned into thin rings of 1 mm thickness under the microscope. Each ring was embedded in 25 µL low growth factors (LGF) Matrigel™ (Corning, USA) in 48-well plate. The plate was incubated at 37°C for 30 minutes to allow for proper solidification. Then a volume of 250 µL of the desired concentration of the extract was applied in triplicate. The plate was then incubated at 37°C. On day 4, the media was aspired and fresh media containing extracts was added. On day 6, the rings were photographed under an inverted light microscope (Nikon, Japan). The angiogenic response was determined by measuring the length of blood vessels outgrowth from the primary ring explants on day 6 using ImageJ software (National Institute of Health, Bethesda, MD). The growth distance of at least 35 structures per ring selected at regular intervals around the ring was measured. Arbitrary units were used to express the actual length of the vessels. The following formula was used to calculate the inhibition of blood vessels formation:

Blood vessels inhibition=(1-(A°/A))*100, where:

A°: distance of blood vessels growth in treated rings in arbitrary units.

A: distance of blood vessels growth in control in arbitrary units.

A preliminary screening was done with the concentrations of 150, 100 and 50 µg/mL of each of the four extracts. The active extracts were then applied in serial dilution of different concentrations as follows: 25, 12.5, 6.25 and 3.125 µg/mL. For the negative control, DMSO was used at a concentration of 1%. Suramin was used as positive anti-angiogenic control. Statistical analysis was done using Oneway analysis of variance (ANOVA) with Dunnett's test using GraphPad Prism 6 was done. p-vlaue <0.05 was considered significant and NS indicates non-significance.

## Results and Discussion

### Phytochemical analysis

Two chemical classes of secondary metabolites were identified; terpenes and flavonoids. Terpenes were identified in all fractions obtained from the chloroform column and in early fractions eluting from n-butanol- and ethanol-columns. Preliminary identification of β-sitosterol was achieved by TLC by comparison of $R_f$ value and based on colour reactions using the reference using analytical TLC. The compound was then isolated and identified as β-sitosterol by spectral analysis using $^1H$, $^{13}C$ and dept NMR. $^1H$ NMR spectrum showed six methyl groups: $C_{29}$ (δ=0.694, 3H, s), $C_{24}$, $C_{26}$ and $C_{27}$ (δ=0.778-0.878, 9H, m), $C_{19}$ (δ=0.974, 3H, d) and $C_{28}$ (δ=1.022, 3H, s). Protons at $C_3$ (δ=3.505), $C_4$ (δ=2.285), $C_6$ (δ=5.371) and $C_7$ (δ=2.011) were clearly identified. However, the rest of protons appeared as multiples in the region δ=1.084 to 1.879. The $^{13}C$ NMR showed 29 carbons. Dept 135 and dept 90 indicated the presence of 3C, 9CH, $11CH_2$ and $6CH_3$.

The second group of secondary metabolites identified was flavonoids. The presence of flavonoids kaempferol, rutin and luteolin was established initially by comparison with standard references on TLC and further confirmed by measuring their UV-Vis spectra using different shifting reagents; NaOMe, $AlCl_3$, $AlCl_3$+HCl, NaOAc and $H_3BO_4$ with the samples isolated by preparative TLC. Comparison of the obtained data for these flavonoids from *E. angustifolia* with the spectra of reference substances confirmed their identity [23]. Data obtained through UV-Vis analysis is listed in Table 1.

| **UV-Vis Spectra (λ max)** | | | | | |
|---|---|---|---|---|---|
| **MeOH** | **MeOH+NAOMe** | **MeOH+$AlCl_3$** | **MeOH+$AlCl_3$+HCl** | **MeOH+NaOAc** | **MeOH+NaOAc+$H_3BO_4$** |
| Band I: 368<br>Band II: 266 | Band I: 417<br>Band II: 269 | Band I: 350, 426<br>Band II: 270 | Band I: 350, 426<br>Band II: 270 | Band I: 382<br>Band II: 274 | Band I: 372<br>Band II:272 |
| Band I: 362<br>Band II: 260 | Band I: 415<br>Band II: 278 | Band I: 440<br>Band II: 276 | Band I: 402<br>Band II: 274 | Band I: 415<br>Band II: 270 | Band I: 426<br>Band II: 274 |
| Band I: 350<br>Band II: 270, 291 sh | Band I: 416<br>Band II: 276 | Band I: 398<br>Band II: 276 | Band I: 356, 384<br>Band II: 276 | Band I: 422<br>Band II: 269 | Band I: 418<br>Band II: 270 |
| Band I: 314<br>Band II: 268 | Band I: 368<br>Band II: 280 | Band I: 304, 274<br>Band II: 230 | Band I: 302, 274<br>Band II: 232 | Band I: 308<br>Band II: 284 | Band I: 308<br>Band II: 288 |

**Table 1:** UV-Vis spectrophotometer data of isolated compounds. A: Kaempferol, B: Rutin, C: Luteolin and D: Chrysin-7-glucoside.

The major flavonoid compound that were present in most of polar fractions of both n-butanol and ethanol crude extracts was identified to be chrysin-7-glucoside based on UV-Vis and NMR spectra. Analysis of $^1H$ NMR spectrum showed the presence of one singlet peak at δ 6.22, which was assigned to $H_3$ of flavone. One doublet with integration of 2 protons appeared at δ 6.78 and assigned to H6 and H8. Aromatic ring has symmetrical protons as no substitution is observed. H3' and H5' were assigned to δ 7.31 (2H, d), while H4' was assigned to δ 7.47 (1H, t) and finally H2' and H6' were assigned to δ 7.95 (2H, t). Signals of the sugar's protons were found in the region δ 2.37 to 5.51 with the highest δ (5.51) assigned to H1". The $^{13}C$ NMR spectrum showed signals of flavone aglycone in addition to the signals for the sugar moiety. The signals for C-3 appeared at δ 104.9 (CH) confirming the presence of 2, 3 double bond of the flavone nucleus. Signals for C-6 and C-8 were assigned to δ 100 (CH) and 94.4 (CH) respectively. For the sugar moiety, signal for C-1" appeared at δ 103.1 (CH), while $CH_2$ signal for C-6" appeared at δ 63.0. The remaining signals of sugar carbons (C-2" to C-5") were found as CH's at δ 70.3, 74.3, 74.4 and 76.6. There are weak signals at the region 160-180, corresponding to C-2, C-7 and the carbonyl group at C-4. Together with UV-Vis spectra analysis (Table 1), the identity of the isolated flavonoid compound was confirmed as chrysin-7-glucoside.

### Anti-angiogenic activity

The anti-angiogenic potential of *E. angustifolia* leaves extracts prepared in four solvents differing in their polarity (ethanol, chloroform, ethyl acetate and water) was investigated. The angiogenic response of rat aortic rings was determined by measuring the length of vessels growing out of the primary ring and compared to 1% DMSO control. Screening was done first on all extracts using 150, 100 and 50 µg/mL. Based on the screening results, ethanol-, ethyl acetate- and water-extracts were further diluted and tested using concentration as follows: 25, 12.5, 6.25 and 3.125 µg/mL. Results obtained from both experiments are shown in Table 2, expressed as percentage of new blood vessels growth inhibition (mean ± SDEV). The percentage inhibition of vessels outgrowth of 1% DMSO control showed mean of 0.00 ± 27.43%. Figure 1 shows the percentage of new blood vessels growth inhibition for the ethanol, ethyl acetate and water extracts. Statistical analysis was done and p-value <0.05 was considered significant and NS indicates non-significance.

| Concentration | Ethanol extract | Chloroform extract | Ethyl acetate extract | Water extract |
|---|---|---|---|---|
| 150 µg/mL | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 100 µg/mL | 100 ± 0.00 | 94.36 ± 7.98 | 100 ± 0.00 | 100 ± 0.00 |
| 50 µg/mL | 98.55 ± 1.45 | 33.57 ± 3.33 | 100 ± 0.00 | 100 ± 0.00 |
| 25 µg/mL | 98.91 ± 1.54 | NA* | 100 ± 0.00 | 96.86 ± 2.39 |
| 12.5 µg/mL | 100 ± 0.00 | NA* | 69.48 ± 43.16 | 85.20 ± 13.46 |
| 6.25 µg/mL | 100 ± 0.00 | NA* | 54.33 ± 28.84 | 40.44 ± 32.59 |
| 3.125 µg/mL | 66.54 ± 24.50 | NA* | 35.17 ± 22.18 | 14.58 ± 0.14 |
| NA*: not available | | | | |

**Table 2:** Percentage inhibition of angiogenesis exhibited by four extracts of *E. angustifolia* leaves (mean ± SDEV).

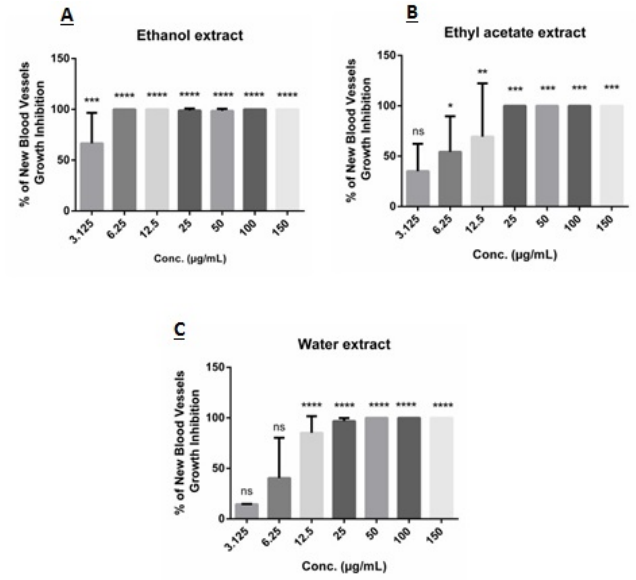


**Figure 1:** The anti-angiogenic effect determined by % of new blood vessel growth inhibition from the primary ring. A. ethanol extract B. ethyl acetate extract C. water extract, where * signifies p<0.05, ** signifies p<0.01, *** signifies p<0.001 and **** signifies p<0.0001.

GraphPad Prism 6 software was used to generate sigmoidal model and calculate $IC_{50}$ values for the three extracts. Figure 2 shows the sigmoidal model obtained by nonlinear fit. All $R_2$ values were excellent (>0.98) and indicated sufficient fitness of the model. $IC_{50}$ values of ethanol, ethyl acetate and water extracts were found to be 3.039, 6.289 and 7.153 µg/mL respectively.

The angiogenic response of the rat aortic rings to the control and the most potent extract (ethanol) at various concentrations are shown in Figure 3 as images captured of the rings at day 6 of the experiment.

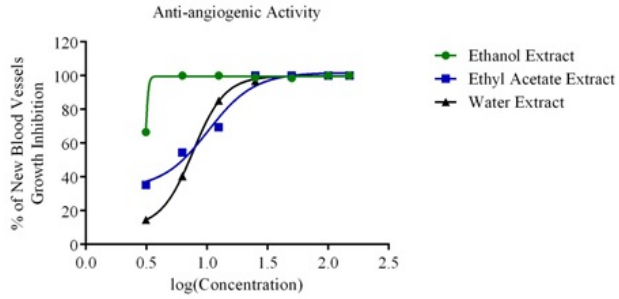


**Figure 2:** Anti-angiogenic activity of ethanol- (●), ethyl acetate- (▪) and water- (▲) extracts of *E. angustifolia* leaves.

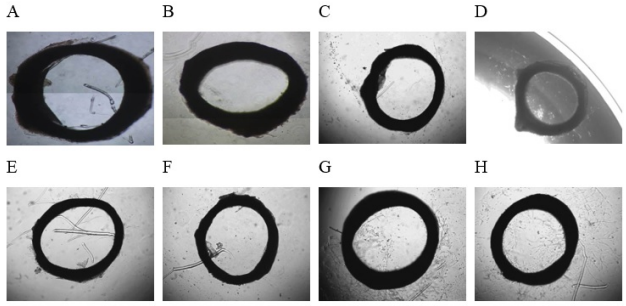


**Figure 3:** Anti-angiogenic response of rat aortic rings to ethanol extract at concentration of 150 µg/mL (A), 100 µg/mL (B), 50 µg/mL (C), 25 µg/mL (D), 12.5 µg/mL (E), 6.25 µg/mL (F), 3.125 µg/mL (G) and control (H).

## Anti-proliferative activity

In the present study, the anti-proliferative properties of four extracts of *E. angustifolia* leaves were evaluated using SRB assay. The assay was conducted on two breast cancer cell lines; MCF-7 and T-47D. The experiment was done in duplicate on two different passages of the cell lines. Nine concentrations were used, each applied in triplicate. The percentage proliferation of each concentration was calculated by dividing the absorbance of the test well on the average of control wells (1% DMSO). Results expressed as a curve of percent proliferation against logarithm of concentration, using GraphPad Prism 6 software, are shown in Figure 4.

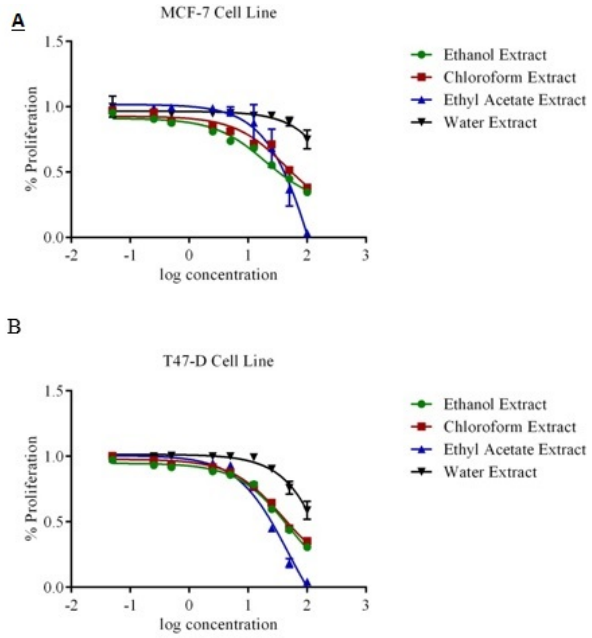


**Figure 4:** Anti-proliferative activity of ethanol- (●), chloroform- (▪), ethyl acetate- (▲) and water- (▼) extracts of *E. angustifolia* leaves against MCF-7 (A) and T-47D (B) cell lines.

The $IC_{50}$ values were calculated and listed in Table 3. Doxorubicin control had $IC_{50}$ values of 0.2498 ± 0.0765 μM for MCF-7 cell line and 0.1 ± 0.01 μM for T-47D cell line. Ideally, $IC_{50}$ values should be calculated from perfect sigmoidal curves. Although the curves obtained in this study were not perfect sigmoidal curves, we couldn't increase the concentration of the extracts beyond 100 μg/mL due to the cytotoxic activity of the solvent used. The resulting effects on cell cytotoxicity will be mixed between the solvent and extract chemical constituents. More accurate calculation of $IC_{50}$ will be studied in more advanced animal studies.

| *E. angustifolia* leaves extract | $IC_{50}$ (μg/mL) ± SDEV | | p-value | Significance |
|---|---|---|---|---|
| | MCF-7 cell line | T-47D cell line | | |
| Ethanol | 39.03 ± 0.86 | 39.26 ± 0.75 | 0.8018 | No |
| Chloroform | 60.63 ± 0.31 | 44.53 ± 2.46 | <0.0001 | Yes |
| Ethyl acetate | 38.41 ± 6.08 | 23.05 ± 0.90 | <0.0001 | Yes |
| Water | 188.76 ± 34.89 | 270.14 ± 16.27 | <0.0001 | Yes |

**Table 3:** $IC_{50}$ values of ethanol-, chloroform-, ethyl acetate- and water-extracts of *E. angustifolia* leaves on MCF-7 and T-47D cell lines (mean ± SDEV).

According to the NCI guidelines, crude extracts that have $IC_{50}$<100 μg/mL in preliminary assay are considered to be active. However, only crude extracts with $IC_{50}$ value <30 μg/mL are considered to be cytotoxic [24,25]. As a result, activity was demonstrated for ethanol, chloroform and ethyl acetate extracts of *E. angustifolia* leaves against both MCF-7 and T-47D cell lines. Nevertheless, only ethyl acetate extract was found cytotoxic against T-47D cell line with $IC_{50}$=23.05 μg/mL, which was significantly different than $IC_{50}$ values of other extracts against same cell line.

In order to study the selective cytotoxic effect of *E. angustifolia* leaves extracts, a cell proliferation assay against normal fibroblasts cell line was done. Figure 5 exhibits the obtained data. The $IC_{50}$ values for the extracts against fibroblasts could not be determined by the software, because interpolated values were way outside the output range for the fitted curve, indicating that extracts under study showed very low toxicity against normal fibroblast cells. This indicates a selective activity against cancer cells, which is advantageous in the search for new anticancer compounds. However, further detailed toxicity studies, both *in-vitro* and *in-vivo*, are needed to confirm the safety of the extracts and their active constituents.

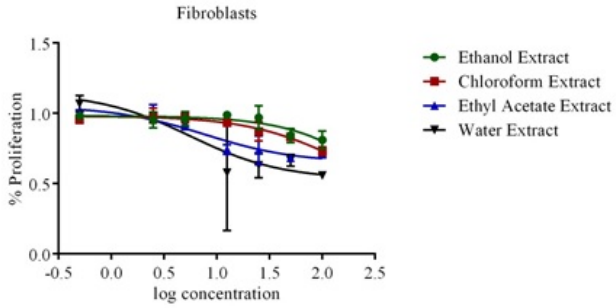


**Figure 5:** Anti-proliferative activity of ethanol- (●), chloroform- (▪), ethyl acetate- (▲) and water- (▼) extracts of *E. angustifolia* leaves against normal fibroblasts cell line.

## Conclusion

The present study reported the chemical analysis of *E. angustifolia L.* leaves, grown in Jordan. Chemical classes of secondary metabolites identified were terpenes and flavonoids. Using NMR, β-sitosterol and chrysin-7-glucoside were identified. Using UV-Vis spectra along with shifting reagents, kaempferol, rutin and luteolin were identified. To the best of our knowledge, these compounds were isolated for the first time from *E. angustifolia* leaves.

Four extracts of *E. angustifolia* leaves, grown locally in Jordan, were obtained using solvents differing in their polarity; ethanol, chloroform, ethyl acetate and water. They were assessed for their anti-angiogenic activity using rat aortic ring assay and anti-proliferative activity against two breast cancer cell lines (MCF-7 and T-47D). Results indicated that ethanol, ethyl acetate and chloroform extracts were active inhibitors of cell proliferation of both MCF-7 and T-47D, while water extract was found to be inactive against both cell lines. Furthermore, ethyl acetate extract was found cytotoxic against T-47D cell line with $IC_{50}$=23.05 μg/mL. In addition, potent anti-angiogenic activity of ethanol extract ($IC_{50}$=3.039 μg/mL), ethyl acetate extract ($IC_{50}$=6.289 μg/mL) and water extract ($IC_{50}$=7.153 μg/mL) was reported for the first time for *E. angustifolia* leaves. This plant should be further assessed for its anti-proliferative activity using additional cancer cell lines. The anti-angiogenic activity also needs further evaluation using CAM assay.

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