



Epilobium angustifolium L.: A medicinal plant with therapeutic properties

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Abstract

Epilobium angustifolium L. is a medicinal plant belonging to the Onagraceae family, which includes more than 200 different species from all over the world. Traditional medicinal applications include treatment of prostate, gastrointestinal, menstrual disorders and recently it has been used for its analgesic and anti-inflammatory activity. In this investigation *E. angustifolium* was collected in Ternopil region of Ukraine.

The obtained data demonstrated that *E. angustifolium* herb extract, rich in polyphenolic compounds such as flavonoids and tannins, display high antioxidant properties. In addition the potential anticancer activity has been investigated *in vitro* on human hepatocellular carcinoma cells (HepG2).

Furthermore the cytotoxic and genotoxic effects of *E. angustifolium* have been investigated respectively by MTT and Comet assay. Results showed that at low concentration, up to 25 µg/mL, the cytotoxic effect was not observed. Increasing concentration from 50 to 75 µg/mL reduced significantly cell viability and induced an important DNA damage in hepatocellular carcinoma. These promising data were also confirmed with mitochondrial potential test.

It is possible to conclude that *E. angustifolium* has beneficial properties in low concentration, in term of antioxidant activity, and it could be a potential antitumoral natural product if it will be used at high concentration.

Introduction

In the last decade, despite major advances in combinatorial and computational chemistries, natural products still represent a significant source of new drugs and lead compounds. About half of all prescription drugs used in cancer treatment are natural products or directly derived from these (1). Plant species represent a great source of biologically active compounds whose effects on heritable material are mostly unknown. Investigation of medicinal plants is valuable on two levels: as a measure of safety for the continued use of medicinal plants, and as a source of potential chemotherapeutic drugs. Although, plant extracts have been used in the treatment of diseases according to knowledge accumulated over centuries, scientific research has shown some substances present in these medicinal plants to be potentially toxic and carcinogenic (2,3). Due to widespread use in pharmacy and non-traditional medicine of extracts of medicinal plants, it could be necessary to investigate more on their safety.

The potential anticancer properties of *Epilobium angustifolium*, also known as *Chamerion angustifolium*, a plant rich in flavonoids and tannins, have not been sufficiently studied so far.

E. angustifolium is a medicinal plant that belongs to the Onagraceae family, which includes more than 200 different species from all over the world. This botanical family can be commonly found in large parts of Western Europe, Siberia, China, Japan, North America and also in Ukraine. Accumulation of bioactive compounds and its biological activity depends on its phenotype and more exactly on its geographic origin, climatic

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conditions (ecotype) or genotype. In our investigations we used *E. angustifolium* herb, collected in Ternopil, a region of Ukraine.

E. angustifolium is widely used in non-traditional medicine to treat gastrointestinal disorders, mucous membrane lesions, such as mouth ulcers, and to improve the speed of wounds healing, skin sores, swelling (4). In addition analgesic activity of *E. angustifolium* was fully described by Tita et al. (5) and this action is due to the anti-inflammatory flavonoids (6,7). Most recently, its anticancer properties were discovered in prostate cancer cells (8,9), and the mechanism through which induce apoptosis is related to activation of the mitochondrial pathway (10). These results open perspectives on the use of *E. angustifolium* preparations in prostate cancer prevention or a additional treatment to standard prostate cancer therapy.

The aim of this study is to assess the safety of *E. angustifolium* by evaluating its cytotoxicity and genotoxicity effects. Moreover, taking in consideration the application of *E. angustifolium* in the treatment of prostatic adenoma, we evaluated its potential pro-apoptotic properties *in vitro*. To this end the hepatocellular cell line HepG2 was used and mitochondrial potential assay together with DNA fragmentation assay were performed during this study.

Materials and Methods

Plant materials, extraction and samples preparation

The air-dried herb of *E. angustifolium* was collected in Ternopil region (Ukraine) in the period of flowering (July 2015).

Extraction of *E. angustifolium* was performed by modified percolation method (11). Briefely, 15 g crashed raw material was placed in the 500 mL flask, filled with 40% ethanol (up to 200 mL) and extracted with stirring for 24 h. After filtering, raw material was filled by hot water (60°C) and extracted on the water bath (60°C) for 1 h. Extraction with water was repeated twice. Then alcohol and water extracts were combined and lyophilized by SP Scientific VirTis Freeze-Drier/Lyophilizer.

To perform TPC (total phenolic content) and ORAC 0.5 g of freeze-dried *E. angustifolium* was solubilized with 10 mL of distilled water, heated in a water bath at 70°C for 1 h, centrifuged at 5000 g and the supernatant was used. Solubilization for MTT, Comet assay and Mitochondrial potential assay was carried out in DMSO at different concentrations.

Total phenolic content (TPC)

Total phenolic content (TPC) of *E. angustifolium* extract was determined using the Folin-Ciocalteu colorimetric method described by Rashidinejad et al. (12) with modifications (13). Gallic acid stock solution (5 mg/mL) and working standard concentrations of 0, 10, 25, 50, 100, 250, 500 µg/mL were prepared in deionized water. Folin-Ciocalteu procedure consisted of transferring 20 µL standard or sample into 4-5 mL borosilicate tube, followed by addition of water (1,58 mL) and Folin-Ciocalteu reagent (100 µL). After mixing the samples, 300 µL of 20% Na₂CO₃ were added and the samples mixtures

were kept for 30 minutes at 40°C.

Total phenols were determined at 765 nm and their values are expressed in terms of gallic acid equivalent (GAE), which is a common reference compound.

Antioxidant Assay by Oxygen Radical Absorbance Capacity (ORAC)

Antioxidant capacity of *E. angustifolium* extract was determined using the ORAC method based on the fluorescence decay (ORACFL) rate of a probe in presence of a radical oxygen species (ROO[·]) compared with that of a reference standard. Hydrophilic and lipophilic fractions were extracted according to Prior et al. (14). ORACFL assays were carried out on a FLUOstar OPTIMA microplate fluorescence reader (BMG LABTECH, Offenburg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The procedure was based on the method of Zulueta et al. (15) with slight modifications (16).

Briefly, 2,20-azobis (2-methylpropionamide) dihydrochloride (AAPH) was used as a peroxy radical generator, Trolox was used as a reference antioxidant standard, and fluorescein was used as a fluorescent probe. A 100 µL of diluted sample, blank or Trolox calibration solution (10–80 µmol) was mixed with 1 mL of fluorescein (80 nM); then, 200 µL of each mixture was placed in a well of the microplate. The microplate was placed in the reader and pre-incubated for 15 min at 37°C. To each well, 60 µL of AAPH was automatically added to initiate reaction. The fluorescence was measured every 60 sec. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample. The final ORACFL values were calculated by using a linear regression equation ($Y = a + bX$) to describe the relationship between Trolox concentration (Y) and net area under FL decay curve (X). Linear regression was used in range of 10–80 µM Trolox. Data are expressed as micromoles of Trolox equivalents (TE) per gram of sample (µmol TE g⁻¹).

Cell culture and treatments

HepG2 cells were chosen for their high degree of morphological and functional differentiation *in vitro*, and also because it is a suitable model to study drug and plant metabolites targeting *in vitro* (16). Cells were cultured in monolayer cultures in 25 cm² tissue flasks, with MEM supplemented with 10% heat-inactivated FBS, 1 mmol/L of sodium pyruvate, 2mM of L-glutamine and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). The cells were maintained at 37° C in a humidified atmosphere containing 5% CO₂. When the cells reached 80–90% of confluence, the routine culture medium was removed and the HepG2 cells were washed with PBS 1X. The cells were then harvested by 0.05% trypsin in 0.02% Na₄EDTA for 5 min at 37° C, and suspended in 1:3 supplemented growths medium to be maintained in the exponential growth phase. For the experiments, the cells were harvested and counted by using trypan blue dye exclusion assay, seeded and cultured

in absence or presence of *E. angustifolium* extract at different concentrations for different times.

Cytotoxicity assay

Cell viability was assessed by the MTT assay, a method based on the reduction of MTT to formazan (17). The cell suspension was seeded into a 96-well microplate, and incubated at 37° C with 5 % CO₂. Concentration of cells was 4×10⁴ for each well. Then, after 24 h, the medium was replaced with fresh complete medium, containing different concentrations (25, 50, 75, 100, 150, 200, 300, 400, 500 µg/mL) of *E. angustifolium* extract and incubated for additional 24 h. MEM was used as a negative control and 1% solution of DMSO as positive control. After 24h, MTT reagent was dissolved in PBS 1X, and added to the culture at 0.5 mg/ml final concentration. After 3 h incubation at 37° C, supernatants were carefully removed and formazan salt crystals were dissolved in 200 µl DMSO added to each well (18). The absorbance (OD) values were measured spectrophotometrically at 540 nm using an automatic microplate reader (Eliza MAT 2000, DRG Instruments, GmbH). Each experiment was performed twice in quadruplicate. Cell viability was expressed as a relative percentage to that of control cells set at 100%.

Comet assay

For the comet assay HepG2 cells were seeded into 6-well microtiter plates (5×10⁵ cells per well) and were incubated at 37° C in 5% CO₂ for 24 h. After incubation, the growth medium (MEM supplemented with 10% of FBS and antibiotics) was replaced with fresh medium and *C. angustifolium* extract in concentrations 25, 50, 75 µg/mL were added. These concentration were chosen on the basis of the results obtained in MTT assay. In each experiment negative (MEM) and a positive (100 mM of 1,2,4-benzenetriol) controls were included and cells were exposed for 4 h.

The comet assay was performed under alkaline conditions (alkaline unwinding/alkaline electrophoresis, pH > 13), basically following the original procedure (19,20). Briefly, after the treatments cells were trypsinized and about 1×10⁵ cells were re-suspended in 0,7% low melting agarose and spread onto pre-agarized microscope slide and kept at 4° C for 10 min. After that, another layer of agarose was spread over the slides. Slides were then immersed overnight in lysis buffer (Tris-HCl 10 mM, NaCl 2,5 M, Na₂EDTA 100 mM; triton X-100 1%) at 4° C. Then, slides were immersed in the electrophoresis tank with ice-cold electrophoresis buffer (NaOH 300 mM, Na₄EDTA 1 mM; pH>13) for 20 minutes, allowing the unwinding of DNA and for other 20 minutes applying a current of 300 mA and 1V/cm. After electrophoresis run, slides were rinsed with neutralization buffer (Tris-HCl 0.4M pH 7.5) and dehydrated with ethanol 70%. Slides were finally stained with ethidium bromide and examined with florescent microscope. For each experimental point, 100 randomly chosen cells were examined. DNA integrity in individual cell was scored with the "comet assay III" imaging analysis software (21, 22). Tail intensity,

that is the percentage of DNA in comet tails, was used as the parameter for DNA damage (23).

Mitochondrial potential assay

Mitochondrial potential assay was performed using the lipophilic cationic dye 1H-Benzimidazolium, 5,6-dichloro-2-[3-(5,6-dichloro-1,3-diethyl-1,3-dihydro-2H-benzimidazol-2-ylidene)-1-propenyl]-1,3-diethyl-, iodide (JC-1) (24). Samples were treated in 6-well microtiter plates with 25, 50, 75 µg/mL of *E. angustifolium* extract for 4h and 24 h. Then the cell cultures were trypsinized, transferred into tubes, centrifugated, washed and diluted with PBS to a final concentration of 1.5×10⁶ cells/L. Finally the samples were incubated with 12.5 mL of 200 mg/mL JC-1 solution for 10 minutes at 37°C. After incubation, samples underwent two washing steps and at the end of the second wash, samples were re-suspended in 250 mL of 1mg/mL of DAPI solution. Samples were analyzed with the NucleoCounter® NC-3000™ analysis system (25).

DNA fragmentation assay

In the final stage of apoptosis, occur a DNA fragmentation (26,27) that it is possible to determinate, in order to evaluate the late apoptosis event (28). The DNA fragmentation assay was performed using a NucleoCounter® NC-3000™ analysis system according to the manufacturer's instructions. Briefly, samples were treated in 6-well microtiter plates with 25, 50, 75 µg/mL of *E. angustifolium* extract for 4h and 24 h. After that cell cultures were trypsinized, moved into tubes, centrifugated and washed with PBS solution. Ethanol was added to every tube and cell suspensions were kept at 4°C for 12 h. After centrifugation ethanol was removed, cell pellets were suspended in PBS and centrifuged again after 5 minutes. Then cell pellets were suspended in a solution containing 1 µg/ml DAPI, 0.1% triton X-100 in PBS, and load on A8-ChemoMetec™ slides and DNA fragmentation was investigated with NucleoCounter® NC-3000™ analysis system (25).

Statistical Analysis

All the assays were carried out in triplicate. The results were expressed as mean values and standar deviation (SD). The statistical analysis was performed using the statistical software SPSS (SPSS Inc., Chicago, IL, USA).

Statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnet's post hoc analysis to examine where the differences actually occurred. The level of significance was set at *p < 0.05 for all statistical analyses.

Results and Discussion

The phenolic content of *E. angustifolium* was evaluated by TPC method and the results showed a very high value: 26.95 ± 2.16 gGAE/100g dry weight (DW), using 80% ethanol for the extraction (Fig. 1). In addition *E. angustifolium* extract exhibits an important antioxidant activity value: 179.57 ± 10.82 mmolTE/100g DW, measured by ORAC method. It is possible

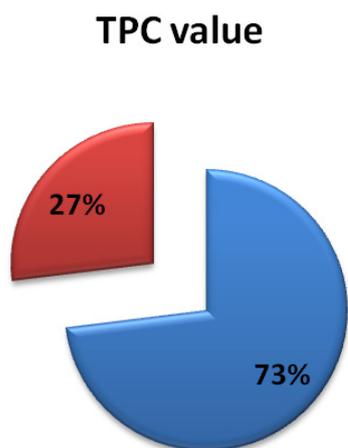


Figure 1. In red the % of total phenolic content (TPC) on 100 g of *E. angustifolium* extract.

to conclude that the total phenolic content and the antioxidant activity are both very high in *E. angustifolium*, according with literatures (29). In fact experimental studies have demonstrated that *E. angustifolium* posses a broad range of pharmacological activity and therapeutic effects, including antioxidant, anti-proliferative, anti-inflammatory, anti-bacterial and anti-aging thanks to polyphenolic compounds, such as flavonoids, ellagitannins derivatives, in particular oenothien B (10). This dimeric macrocyclic ellagitannin is responsible for the biological activities, such as antioxidant (30), anti-inflammatory (30), tumor cell cytotoxicity (31), enzyme inhibition (32-33) of *E. angustifolium* extract.

The therapeutic effects of polyphenols, biological active components, are directed towards multiple mechanisms, including selective induction of cell death in cancer cells and anti-microbial effects. To better understand and maximized the potential therapeutic use of *E. angustifolium*, cytotoxic and genotoxic effects were analyzed *in vitro* by two differ

approaches: MTT and Comet assay respectively.

The measurement of cell viability is the basis for numerous *in vitro* assays of cell responses to external factors and in this study was determined in the HepG2, a human hepatocellular carcinoma cells. This cell line has been chosen for its high degree of morphological and functional differentiation *in vitro*, and also because it is a suitable model to study drug and plant metabolites targeting *in vitro* (34-35). MTT assay has been used to test cell viability at different concentration of *E. angustifolium* extract (25, 50, 75, 100, 150, 200, 300, 400 and 500 µg/mL). Incubation of *E. angustifolium* extract after 24h resulted in a reduction of proliferation in a concentration-dependent manner (Fig. 2).

The subsequent objective was to test the potential genotoxic effect of *E. angustifolium* extract on HepG2 cell line. Based on the MTT results, we chose only three different concentrations of *E. angustifolium* extract (25, 50 and 75 µg/mL). Comet assay was performed after 4 h of treatment. Results of genotoxic assessment of *E. angustifolium* are shown in Fig. 3. Untreated cells, used as negative control, showed a tail intensity of 1.94 ± 0.55 % whereas the positive control, 100 mM BT, revealed significant high percentage of tail intensity of 21.85 ± 1.55 %. The value of tail intensity of samples are: 5.51 ± 2.22 % (25 µg/mL); 14.95 ± 1.02 % (50 µg/mL); 14.35 ± 1.46 % (75 µg/mL). These results indicates an important and severe genotoxic effect induced by *E. angustifolium* at the two highest concentrations.

This result is very important because it is the first time that genotoxicity of *E. angustifolium* has been tested *in vitro* and it allows a better understanding of *E. angustifolium* mechanisms of action.

Regarding apoptosis, results obtained in the mitochondrial potential assay shown no changes in mitochondrial potential induces by *E. angustifolium* at the tested concentrations, at 4 h, and 24 h of treatment. On the other hand the results obtained from the DNA fragmentation assay shown an increment dose-

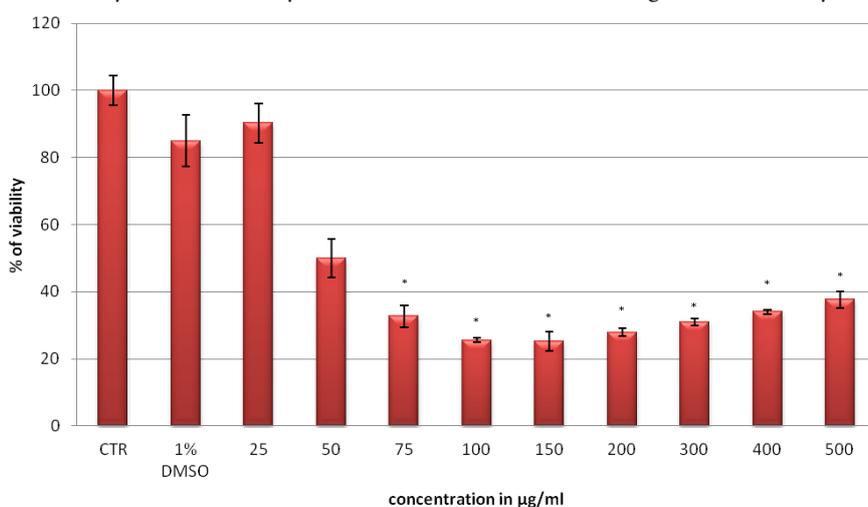


Figure 2. Cytotoxicity effect of *E. angustifolium* extract in HepG2 cell culture. CTR is the negative control (only MEM) and 1% DMSO is the positive control. The percentage of viability is showed in comparison with untreated control cells (CTR), for which was 100%. Results are expressed as mean \pm SD of three independent experiments.

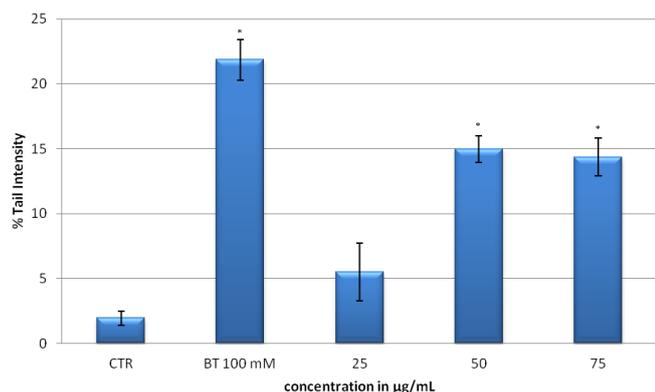


Figure 3. Percentage of Tail Intensity after treatment with *E. angustifolium* extract in HepG2 cell line. CTR is the negative control (only MEM) and BT 100 nM is the positive control. Results are expressed as mean \pm SD of three independent experiments.

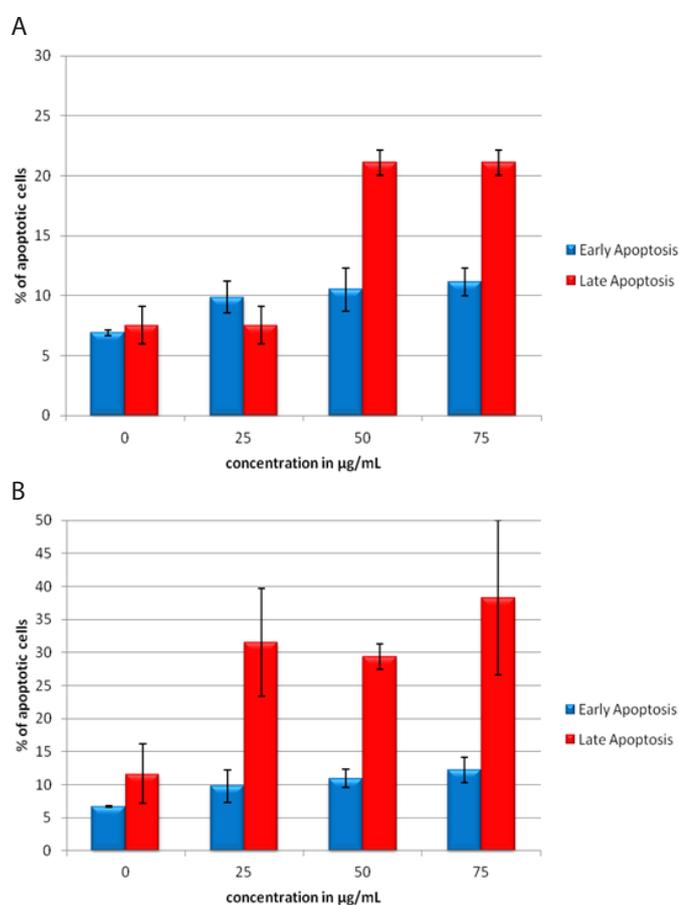


Figure 4. Percentage of apoptotic HepG2 cells after 4h (A) and 24h (B) of treatment with *E. angustifolium* extract.

response independent of percentage of cell with fragmented DNA in all concentrations tested at both time of exposure (Fig. 4). These results are similar to those observed in the comet assay, indicating that the DNA damage could be caused by a late apoptosis event. The final consideration was that apoptosis, and not necrosis, was responsible for cell death and the effect is visible after 4 h of treatment.

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Conflict of interest statement

The authors declare no commercial or financial conflict of interest.

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