



In vitro bioactivity and phytochemical evaluation of extracts from aerial parts of *Eremostachys macrophylla* Montbr. & Auch. growing in Iran

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Abstract

Background and objectives: The aerial part extracts of *Eremostachys macrophylla* from Labiatae family, which has been traditionally used in wound healing, snake bites, rheumatism and joint pains, were investigated for general toxicity, anti-proliferative, free radical scavenging and anti-bacterial effects. Moreover, preliminary phytochemical investigations were carried out on the extracts.

Methods: Extracts were prepared using a soxhlet apparatus with *n*-hexane, dichloromethane (DCM) and methanol (MeOH), respectively. Brine shrimp lethality test (BSLT) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay were performed to evaluate general toxicity and free radical scavenging properties, respectively. Anti-proliferative and anti-bacterial activities were assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and disc diffusion methods, respectively. MTT assay was carried out on one normal and two cancer cell lines including human umbilical vein endothelial cells (HUVEC), human colorectal adenocarcinoma (HT-29) and human lung carcinoma (A-549), respectively. Additionally, all the extracts were tested for the presence of various phytoconstituents by different reagents. **Results:** The *n*-hexane extract was the most active fraction in BSLT whereas the MeOH extract showed significant free-radical-scavenging activity. The results indicated that the *n*-hexane and MeOH extracts possessed anti-proliferative effects against HT-29 cells while all the three extracts were effective against A-549 cell line. None of these three extracts showed any significant effect against HUVEC. The extracts didn't show any antimicrobial effects against Gram positive, Gram negative and *Candida albicans* species. **Conclusion:** Considering the results, the species might be a good candidate for further phytochemical and biological studies for the isolation of active and pure ingredients and clarification of anti-neoplastic mechanism.

Keywords: antimicrobial, antioxidant, cytotoxicity, *Eremostachys macrophylla*, general toxicity

Introduction

Nowadays, medicinal plants as sources of natural compounds play a remarkable role in the

treatment of various diseases which have given new visions to researchers both in their natural

forms and as templates for synthetic modifications [1]. Herbal medicines have demonstrated potential anti-bacterial, antiprotozoal and antiviral activities [2-4]. Moreover, medicinal herbs have a vital role in the prevention and treatment of cancer [5]. As an illustration, nearly 100 plant-based new drugs including vinblastine and vincristine have been introduced against cancer in USA drug market during 1950 to 1970. Furthermore, other plant-based drugs have been present all over the world including ectoposide from 1971 to 1990 along with paclitaxel and topotecan during 1991 to 1995. Therefore, one of the recent aims of medical research has been to screen the phytochemicals and their derivatives against cancer [6].

Eremostachys macrophylla Montbr. & Auch. (Lamiaceae) is one of the 15 species of the *Eremostachys* genus, which have been distributed in Iran. The plants of this genus are perennial herbs with thick roots which have grown in the countries of the Middle-East Asia, West Asia and Caucasus [7]. Based on the previous reports, a number of *Eremostachys* species such as *Eremostachys laciniata* have been orally used for the treatment of allergies, headaches and liver disorders [8]. Other studies have demonstrated various effects from the plants of this genus such as local analgesic, anti-inflammatory, antinociceptive, antidepressant, antioxidant and antibacterial properties [9-12].

Medicinal uses of *E. macrophylla* in folk medicine comprise wound healing, using for snake bites, rheumatism and joint pains while our previous findings suggested antimalarial effect from the aerial parts and rhizomes of this species [13,14].

Previous phytochemical investigations on just a few species of this genus have revealed the presence of different natural compounds. The rhizomes of *E. laciniata* have been identified as a rich source of phytosterols, flavonoids and iridoid glycosides [10-12]. Loasifolin and loasins A and B flavonoids and eremosides A to C iridoid glycosides have been isolated from *Eremostachys loasifolia* [15-17]. Ferulic acid derivatives, furanolabdane diterpene glycosides, iridoid

glycosides and phenylethanoid glycosides have been identified in the rhizomes of *Eremostachys glabra* [18-20]. Iridoid glycosides and an isoflavone compound have been found in *Eremostachys moluccelloides* and *Eremostachys vicaryi*, respectively [21,22]. The presence of iridoid glycosides, phenylethanoid glycosides and flavonoid derivatives have been demonstrated in *Eremostachys azerbaijanica* [23].

The objectives of this study were to evaluate some biological effects such as general toxicity, antioxidant, anti-proliferative and anti-bacterial activities of *E. macrophylla* extracts and also preliminary phytochemical investigations of these extracts.

Experimental

Plant material

The aerial parts of *E. macrophylla* Montbr. & Auch. were collected during July 2012 from Sahand mountains in East Azarbaijan province in Iran 37.759 (37° 45' 32.4" N) latitude 45.9783 (45° 58' 41.9" E) longitude and altitude 1950 m above sea level. A voucher specimen (TBZ-fph-739) has been retained at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Extraction

Air-dried and ground *E. macrophylla* aerial parts (100 g) were Soxhlet extracted continually with *n*-hexane, dichloromethane (DCM) and methanol (MeOH) (1.1 L each, Caledon company, Canada). All these three extracts were separately concentrated using a rotary evaporator (Heidolph, Germany) at a maximum temperature of 45 °C.

Brine shrimp lethality test (BSLT)

The general toxicity of the three extracts from the aerial parts of *E. macrophylla* was monitored by BSLT method [10]. The *Artemia salina* eggs (Sera brand, Turkey) were placed and hatched in a conical flask with 300 mL artificial sea water (Aqua Marine brand, Thailand). The flask was well ventilated with an air pump, and preserved in a water bath at 29-30 °C. A light source was left on the top of the flask. The nauplii

appeared after 48 h. Three extracts were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) to get the 1 mg/mL concentration and then the stock solutions were diluted with artificial sea water. Seven different concentrations of extracts were prepared by serial dilution. Solution of each concentration (1 mL) was transferred into clean sterile universal vials and then aerated sea water (10 mL) was added. Almost 10 nauplii were counted and transferred into each vial. Surviving nauplii were counted after 24 h and the mortality rate was calculated at each extract doses via the best-fit line plotted concentration versus percentage of lethality. The controls were DMSO, normal saline and podophyllotoxin. The lethal concentration 50% (LC₅₀) value was estimated using linear regression analysis by Excel software.

Free radical scavenging activity test

Antioxidant effects of the extracts were assessed using DPPH reagent (Sigma-Aldrich, Germany) [19]. DPPH solutions were prepared (0.08 mg/mL) in chloroform (CHCl₃) for assessing the *n*-hexane and DCM extracts and in methanol for evaluating the MeOH extract.

The extracts were dissolved in CHCl₃ or MeOH to obtain the stock concentration of 4 mg/mL. Serial dilutions were made to obtain concentrations of 1, 5×10⁻¹, 2.5×10⁻¹, 1.25×10⁻¹, 6.25×10⁻², 3.13×10⁻² and 1.56×10⁻² mg/mL. Diluted solutions (5 mL each) were mixed with DPPH solution (5 mL) and allowed to stand for 30 min to complete the reaction. The UV/Visible absorbance was recorded at 517 nm. The percentage of reduction capacity was calculated as $R\% = ((A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}) \times 100$ where A_{blank} was the absorbance of the control, and A_{sample} was the absorbance of the extract/standard. Reduction capacity, 50% (RC₅₀) value was defined as the extract concentration providing 50% loss of DPPH activity. The test was done in triplicate and the same manner was followed for the positive control, trolox or quercetin.

MTT assay

A-549 (human lung carcinoma), HT-29 (human colorectal adenocarcinoma), and HUVEC

(human umbilical vein endothelial) cells were cultured in RPMI 1640 (Roswell Park Memorial Institute) medium with essential additives including 100 µg/mL streptomycin and 100 IU/mL penicillin augmented with 10% fetal bovine serum (FBS). The cells were kept in a humidified atmosphere of a 5% CO₂ (37 °C). MTT colorimetric assay was employed to determine the anti-proliferative effect of medicinal plants [24]. MTT (Sigma-Aldrich, USA) was dissolved in phosphate buffered serum (5 mg/mL PBS); 1 × 10⁴ cells/well were seeded into 96-well plates and hatched in 24 h. Then the cells were treated with different concentration of the test samples and incubated for 3 days in a moistened atmosphere at 37 °C in presence of 5% CO₂. Different dilutions of *n*-hexane, DCM and MeOH extracts (including: 1, 10, 100, 1000 µg/mL), which were dissolved in DMSO and were diluted with cell culture medium, were added to the cells and transferred to incubator. After 72 h of incubation, 20 µL of MTT reagent was added to each well. The plates were incubated at 37 °C for 4 h. After that, the medium was removed and pure DMSO (100 µL) was added to each well. Finally, the metabolized MTT product was measured by reading the absorbance at 570 nm with a microplate reader (ELISA plate reader, Bio teck, Germany). Paclitaxel and DMSO were considered as positive and negative controls, respectively. The cell survival was calculated by the following formula:

$$\text{Relative viability (\%)} = (A_{\text{test}} / A_{\text{control}}) \times 100$$

Where A_{control} is the absorbance of control reaction (including all reagents excluding the plant extracts) and A_{test} is the absorbance of the sample. The results were generated from three independent experiments. The IC₅₀ (the concentration which caused 50% inhibition of cell growth) was calculated from a dose response curve plotted with the Sigma Plot 10 software [25,26].

Antimicrobial assay

Microbial strains

Examined organisms included two species of

Gram negative bacteria, *Pseudomonas aeruginosa* (PTCC1074) and *Escherichia coli* (PTCC1330), two strains of Gram positive species, *Staphylococcus epidermidis* (PTCC1114) and *Staphylococcus aureus* (PTCC1112) and a fungus, *Candida albicans* (PTCC5027) which were purchased in lyophilized culture from Persian Type Culture Collection (Iran).

Disc diffusion test

Activated microorganisms were transferred to Muller Hinton Broth medium (Merck, Germany) and incubated overnight at 37 °C. Saline solution was twice applied to provide the turbidity for the centrifuged pallets at 3000 rpm for 15 min (equal to 0.5 Mc Farland, 10⁸ CFU/mL as a standard optical density). The final concentration of inoculums was adjusted to about 10⁶ CFU/mL with sterile saline solution. To get a uniform microbial growth, 10 mL of prepared inoculum suspensions were spread over the autoclaved Muller Hinton agar medium and then the sterile discs of Whatman paper with 6 millimeters diameter that were impregnated with 50 µL of different concentrations of extracts in 50% aqueous DMSO (1:1, 1:5, 1:10), were placed on the surface of the media. The plates were incubated for 30 min in refrigerator to let the diffusion of the extracts and they were then incubated at 37°C for 24 h. Finally, the inhibition zones obtained around sterile discs were measured.

In order to compare the potency of the antimicrobial effect of the extracts, two control groups were considered including aqueous DMSO as a negative control and a standard disc of amikacin as a positive control. All tests were performed three times and the mean value was calculated [10, 27].

Phytochemical screening

Different methods were used for preliminary phytochemical screening of the three extracts; Alkaloids by Dragendroff and Hager tests, reducing sugars with Benedict reagent, sterols and triterpenoids by Libermann- Buchard test,

proteins and amino acids by Ninhydrin test, cardiac glycosides by Kedde and Keller killiani tests, tannins and phenolic compounds by ferric chloride test, flavonoids by Shinoda test and iridoids with Trim-Hill reagent [28-32].

Total phenol content (TPC)

Total phenolics content was determined using Folin-Ciocalteu assay. Gallic acid (GAE) (Merck, Germany) was used as the standard. One mL of samples with concentration of 5 mg/mL in acetone: water (60:40) was mixed with 0.2 mL Folin- Ciocalteu reagent (Merck, Germany) (1:2 diluted with water) and 1 mL 2% Na₂CO₃. Control sample didn't contain any extract. The samples were incubated for 30 min in room temperature and then the absorbances were quantified at 750 nm (Pharmacia Biotech, England). Different concentrations of gallic acid were used to draw the calibration curve with the same method. All measurements were done in triplicate [29].

Total flavonoid content (TFC)

Total flavonoids content was estimated using AlCl₃ (Merck, Germany) method. The solution containing samples was prepared in 80% methanol. Moreover, 133 mg crystalline aluminum chloride and 400 mg crystalline sodium acetate (Merck, Germany) were dissolved in 100 mL 80% methanol for preparing the AlCl₃ reagent. To estimate the flavonoid content, 2 mL of sample, 400 µL of water and 1 mL of AlCl₃ reagent were added.

The sample absorbance was recorded at 430 nm against blank containing no AlCl₃ reagent. The stock solution of quercetin in 80% methanol (1 mg/mL) was used to prepare the various dilutions of quercetin (5-25 µg/mL) for drawing the standard curve and the amount of flavonoids was calculated as quercetin equivalent from the calibration curve of quercetin (5-25 µg/mL) [31].

Statistical Analysis

The data were expressed as mean ± standard deviation (SD) for at least three independent determinations for each experimental point.

Results and Discussion

In the present study, general toxicity, anti-proliferative, antimicrobial and free radical scavenging activities of three extracts from the aerial parts of *E. macrophylla* with different polarity were determined and the results have been shown in table 1. Based on mentioned results, all of the test samples showed lethal effect on brine shrimp nauplii. The *n*-hexane extract was the most potent one with LC₅₀ value of 26 ± 1 µg/mL. The LC₅₀ values for DCM and MeOH extracts were 103 ± 2 µg/mL and 649 ± 20 µg/mL, respectively. The positive control of this assay was podophyllotoxin (LC₅₀=2.8 ± 0.1 µg/mL), as a well-known cytotoxic lignan. In addition, the free radical scavenging activity of the three extracts were assessed using DPPH method and the MeOH extract demonstrated the highest antioxidant activity with RC₅₀ value of 93 ± 1 µg/mL, whereas quercetin as the positive control showed RC₅₀ value of 3.9 ± 0.1 µg/mL.

The anti-proliferative activity of the *n*-hexane, DCM and MeOH extracts were evaluated on three cell lines. The results showed that the MeOH and *n*-hexane extracts inhibited the growth of HT-29 cell line with IC₅₀ value of 133 ± 63 µg/mL and 396 ± 48 µg/mL, respectively. Moreover, all extracts (*n*-hexane, DCM and MeOH) inhibited A-549 cell growth with IC₅₀ values of 353 ± 41 µg/mL, 380 ± 104 µg/mL and 624 ± 182 µg/mL, respectively. None of these extracts exhibited significant anti-proliferative activity against HUVEC cell line.

Furthermore, the antimicrobial effect of the three extracts was subjected to screen by agar disc diffusion method; however, no antimicrobial activity was observed against examined microorganisms. For more clarification, the

preliminary phytochemical screenings were performed for the identification of the content in three extracts and the results have been shown in table 2. The obtained results from the determination of total phenolics and flavonoid contents showed that the MeOH extract contained more phenolic content in comparison to the other extracts and subsequently possessed more flavonoids with 48.674 ± 0.0420 mg GAE/100g (gallic acid equivalent /100g) of extracts and 7.844 ± 0.03 mg quercetin per 100 g dry extract, respectively.

The brine shrimp lethality test and the free radical scavenging activity are two efficient general bioassay methods for determining the broad spectrum of bioactivity in crude extracts. Both techniques are easily mastered, low cost, and utilized in low amounts of test materials [33]. According to the table 1 although all of the extracts with different polarities were effective in brine shrimp lethality test, the *n*-hexane extract showed the most potent activity in comparison with podophyllotoxin, as a well-known cytotoxic lignan.

In our previous studies on pure iridoid glycosides of *E. laciniata*, we have not observed any significant level of toxicity in brine shrimp lethality test, and the LC₅₀ values were higher than 1.0 mg/mL [11]; while the standard brine shrimp lethality bioassay stipulates that LC₅₀ values lower than 1 mg/mL are considered bioactive in general toxicity evaluation of plant extracts [34]. Moreover, the mentioned test has been used routinely in the primary lethal screening of the crude extracts and can also provide an indicator for possible cytotoxic properties of the test materials.

Table 1. General toxicity, free radical scavenging and antiproliferative activities of the *n*-hexane, DCM and MeOH extracts of *Eremostachys macrophylla* aerial parts

Extracts	General toxicity ¹	Free radical scavenging activity ²	antiproliferative activity (IC ₅₀ µg/mL)		
	LC ₅₀ (µg/mL)	RC ₅₀ (µg/mL)	HT-29	A-549	HUVEC
<i>n</i> -hexane	26 ± 1	518 ± 16	396±48	353±41	>1000
DCM	103 ± 2	>1000	>1000	380±104	>1000
MeOH	649 ± 20	93 ± 1	133±63	624±182	>1000

¹The LC₅₀ value of podophyllotoxin as the positive control was 2.8 ± 0.1 µg/mL.

²The RC₅₀ value for quercetin as the positive control was 3.9 ± 0.1 µg/mL.

Table 2. Phytochemical evaluation for various extracts of *Eremostachys macrophylla* aerial part

Phytochemicals		<i>n</i> -hexane extract	DCM extract	MeOH extract
Alkaloids	Dragendroff's test	-	-	-
	Hager's test	-	-	-
Reducing sugars	Benedict, s test	-	-	+++
Sterol and triterpenoid	Libermann-	+	+++	+
	Buchard test			
Proteins and Amino acids	Ninhydrin test	-	-	-
	Kedd test	+++	+++	-
Cardiac glycosides	Keller- Killiani	+	-	-
	test			
Tannins & phenolic compounds	Ferric Chloride test	-	-	+
Flavonoids	Shinoda test	-	-	+
Iridoids	Trim-Hill test	-	-	+++

(+): test positive, (++): present in relatively moderate larger amount, (+++): relatively larger amount, (-): test negative

It has been established that the compounds with cytotoxic effects usually show significant activities in the brine shrimp lethality bioassay, so this assay can be recommended as a preliminary method for detecting of antitumor compounds [33]. The free radical scavenging activity of these extracts, as a mechanism for cancer prevention, was evaluated by means of DPPH method [19]. The obtained data revealed a high level of the antioxidant activity in MeOH extract; moreover, TPC and TFC values of the MeOH extract demonstrated the presence of high level of phenols and flavonoids as powerful antioxidant substances. Hence, a good correlation was observed between the free radical scavenging property and the total phenolic and flavonoid contents [29]. These data are in agreement with our previous studies on other species of *Eremostachys* including *E. laciniata* and *E. glabra* that showed the presence of antioxidant compounds like phenylethanoide glycosides and flavonoids [11,19,20].

The anti-proliferative activity (IC_{50}) of the aerial part extracts of *E. macrophylla* extracts against two cancer cell lines and one normal cell line was evaluated and the results showed that the MeOH and *n*-hexane extracts possessed anti-proliferative effects against human colon adenocarcinoma cell line (HT-29), whereas the DCM extract had no significant activity. In contrast, all examined extracts showed anti-proliferative activity on human lung carcinoma cell line (A-549). The weakest effect was obtained by MeOH extract.

The three extracts of *E. macrophylla* didn't show any significant effect against HUVEC as a normal cell line; therefore, based on these results (especially the anti-proliferative effects on HT-29 cells of the MeOH extract), this plant could be suggested for isolation of its pure compounds and examination of their antitumor properties in the future. As shown in table 2, the preliminary phytochemical screening indicated that the DCM and *n*-hexane extracts contained sterols, triterpenoids and cardiac glycosides which might cause the observed anti-proliferative activity. These findings were consistent with those acquired in previous investigations [35,36].

In the case of the MeOH extract, iridoids, reducing sugars, phenolic compounds (tannins and flavonoids), sterols and triterpenoids were detected. Comparing our results with proceeding published data, the presence of iridoid glycosides were reported in *E. laciniata*, *E. lasifolia*, *E. moluccelloides*, *E. glabra* and *E. azerbaijanica* species [10,14,17,19,21]. Moreover, anti-proliferative activity of these compounds have been demonstrated in the previous literatures which have showed the potent activity of iridoids on various cell lines like breast cancer MCF-7 cells, human cervical carcinoma HeLa cells, gastric carcinoma MNK-45 cells and prostatic cancer cells [37-40].

Thus, the observed potent cytotoxic effect of MeOH extracts on HT-29 cell line may be related to the presence or synergistic correlation between iridoids, reducing sugars, phenolic compounds

(tannins, flavonoids), sterols and triterpenoids that have been found in phytochemical screenings [41,42].

Regard to A-549 cell lines, It is interesting to note that a parallel relationship was established between brine shrimp lethality test as a primary cytotoxic test and anti-proliferative activity. Moreover, the antimicrobial activities of the three extracts were screened by disc diffusion method and although in previous studies the pure iridoid glycosides isolated from *E. laciniata* had displayed low to moderate levels of activity, none of the examined microorganisms showed sensitivity to the extracts [10].

Our studies for the first time demonstrated the anti-proliferative activities of the *n*-hexane, DCM and MeOH extracts of *E. macrophylla* on three cell lines. The MeOH extract possessed the most potent free radical scavenging activity and cytotoxicity against HT-29 cell line. The TPC, TFC and antioxidant activity of the MeOH extract were in agreement with each other. Moreover, the *n*-hexane extract showed the most cytotoxicity against *A. salina* and A-549 cell line. In the future, more studies should be focused on isolation of active and pure ingredients and clarification of anti-neoplastic mechanism of this plant.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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