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Research Article

Bioactive Properties of *Eremostachys macrophylla* Montbr. & Auch. Rhizomes Growing in Iran

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ABSTRACT

Background: The current study was assigned to evaluate the antioxidant, general toxicity, anti-proliferative and antimicrobial activities of different extracts obtained from rhizomes of *Eremostachys macrophylla* (Lamiaceae).
Methods: All activities were evaluated by obtaining extracts of *E. macrophylla* in n-hexane, DCM (dichloromethane) and MeOH (methanol) by soxhlet apparatus. The antioxidant activity of the extracts was evaluated in terms of FRST (free radical scavenging activity test) by DPPH (2, 2-diphenyl-1-picrylhydrazyl). BSLT (Brine shrimp lethality tests), MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay and disc diffusion method were carried out to determine the general toxicity, anti-proliferative and antibacterial activities of the different extracts, respectively.
Results: The findings of the study for antioxidant, anti-proliferative and antibacterial effects showed that DCM extract was the most active fraction, but n-hexane extract indicated the most potent effect against *Artemia salina*.
Conclusion: The results revealed strong bioactive effects of nonpolar fractions of *E. macrophylla* rhizomes. Thus, it is possible to suggest some new potential antioxidant, cytotoxic and antibacterial agents with no harmful effects on noncancerous cells.

Introduction

Eremostachys or desert rod (Family: Lamiaceae; subfamily: Lamioideae) is a genus of 60 known species that are distributed mainly in the Middle-East, Central and Western Asia. The genus contains 15 species of perennial herbs in Iran.[1,2] Based On previous investigations, a number of species such as *Eremostachys laciniata* have been used orally for the treatment of allergies, headache and liver disorders.[3] Other studies have reported various effects of *E. laciniata* such as local analgesic, anti-inflammatory, antinociceptive, antibacterial, antidepressant and antioxidant properties,[4-8] it is also can be effective in the treatment of mild and moderate Carpal Tunnel Syndrome (CTS).[9]

Phytochemical studies on just a few species of *Eremostachys* genus revealed the presence of different natural compounds in various parts of plant. For example, the rhizomes of *E. laciniata* have been identified as a rich source of phytosterols, phenylethanoids, flavonoids and iridoid glycosides.[6-8] Furanolabdane diterpene glycoside, iridoid glycosides and ferulic acid derivatives have been reported from rhizomes of *E. glabra.*[1,10,11] Flavonoids and iridoid glycosides have been isolated from *E. loasifolia.*[12-14] Moreover, iridoid glycosides from *E. moluccelloides* aerial parts, flavonoids from *E.vicaryi* have been found. Phytochemical evaluations of *E. azerbaijanica* rhizomes and aerial parts showed the presence of iridoid glycosides, phenylethanoid glycosides and flavonoid derivatives.[15-17]

E. macrophylla Montbr. & Auch is another wild species growing in Iran. Medicinal uses of *E. macrophylla* in folk medicine comprise wound healing, snake bites, rheumatism and joint pains and our previous findings suggested antimalarial effect from the aerial parts and rhizomes of this species.[18,19]

The objectives of this study was evaluation of some biological properties such as antioxidant, general toxicity, anti-proliferative and antibacterial effects of *E. macrophylla* rhizomes as a wild species growing in East Azarbaijan province of Iran.

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Material and Methods

Plant material

The rhizomes 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 in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Extraction

Air-dried and ground rhizomes of *E. macrophylla* (100 g) were Soxhlet extracted respectively with n-hexane, dichloromethane (DCM) and methanol (MeOH) (1 L each, Caledon Company, Canada). All these extracts were separately concentrated using a rotary evaporator at a maximum temperature of 45 °C.

Free radical scavenging activity test (FRST)

Antioxidant activity of the three extracts was assessed using DPPH reagent (Sigma-Aldrich, Germany).[1,17] DPPH solutions were prepared (0.08 mg/mL) in chloroform ($CHCl_3$) for assessing the n-hexane and DCM extracts and in MeOH for evaluating the MeOH extract.

The extracts were dissolved in $CHCl_3$ or MeOH to obtain the stock concentration of 1 mg/mL. Serial dilutions were made to obtain concentrations of 0.5, 0.25, 0.125, 0.0625, 0.0312 and 0.0156 mg/mL. Diluted solutions (2 mL each) were mixed with DPPH solution (2 mL) and allowed to stand for 30 min for occurring any reaction. The UV/Visible absorbance was recorded at 517 nm. The percentage of reduction capacity was calculated as:

$$R\% = \left(\frac{A_{blank} - A_{Sample}}{A_{blank}}\right) \times 100 \qquad \text{Eq. (1)}$$

where A_{blank} was the absorbance of the control, and A_{sample} was the absorbance of the extract/standard. Reduction capacity, 50% (RC_{50}) value was defined as the extract concentration providing 50% loss of DPPH activity. The experiment was done in triplicate and the same manner was followed for the positive control, trolox or quercetin.

Brine shrimp lethality test (BSLT)

The general toxicity of different extracts from rhizomes of *E. macrophylla* was monitored by BSLT method.[5] The *Artemia salina* eggs (Sera brand, Turkey) were hatched in a conical flask containing 300 mL artificial seawater (Aqua Marine brand, Thailand). The flasks were well aerated with an air pump, and kept in a water bath at 29-30 °C. A bright light source was left on. The naupliies hatched within 48 h. The extracts were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) to obtain a concentration of 1 mg/mL and diluted with artificial see 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 seawater (10 mL) was added. About 10 naupliies were counted and transferred into each vial. Surviving naupliies were counted after 24 h and the mortality rate was calculated at each extract dose via the best-fit line plotted concentration versus percentage lethality. The controls were DMSO, normal saline and podophyllotoxin. The lethal concentration, 50% (LC_{50}) value was estimated using linear regression analysis by Excel software.

MTT assay

HT29 (human colorectal adenocarcinoma), A549 (human lung carcinoma) and HUVEC (human umbilical vein endothelial) cell lines were cultured in *RPMI 1640 (Roswell Park Memorial Institute)* medium with essential additives including 100 µg/mL streptomycin and 100 IU/mL penicillin supplemented with 10% fetal bovine serum (FBS). The cells were kept in a humidified atmosphere of a 5% CO_2 (37 °C). (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) colorimetric assay was employed to determine the anti-proliferative activity of the extracts.[20] MTT was dissolved in phosphate buffered serum (5 mg/mL PBS). In MTT assay, 1×10^4 cells/well were seeded into 96-well plates and incubated for 24 h. Then cells were treated with different concentration of extracts and incubated for 3 days in a humidified atmosphere at 37 °C in presence of 5% CO_2. 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 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 quantified by reading the absorbance at 570 nm on a microplate reader (ELISA plate reader, Bio teck, Bad Friedrichshall, Germany). For comparing the anti-proliferative activity of extracts, Paclitaxol and DMSO were considered as positive and negative controls. The cell survival was calculated by the following formula:

$$Relative\ viability(\%) = \left(\frac{A_{test}}{A_{control}}\right) \times 100 \qquad \text{Eq. (2)}$$

Where $A_{control}$ is the absorbance of the control reaction (including all reagents except the plant extracts) and A_{test} is the absorbance of the sample. The results were generated from three independent experiments; each experiment was performed in triplicate. The IC_{50} (The concentration causing 50% growth inhibition) was calculated from a dose

response curve plotted in the Sigma Plot 10 software.[21,22]

Antimicrobial assay

Microbial strains

Examined organisms included two species of Gram negative bacteria, *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (ATCC 8739), two strains of Gram positive species, *Staphylococcus epidermidis* (ATCC 12228) and *Staphylococcus aureus* (ATCC 6538) and a fungus, *Candida albicans* (ATCC 10231) which were purchased in lyophilized culture from the 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. A saline solution was twice applied to provide the turbidity for the centrifuged pallets at 3000 rpm for 15 minutes (equal to 0.5 Mc Farland, 10^8 CFU/mL as a standard optical density). The final concentration of inoculums was adjusted to about 10^6 CFU/mL with sterile saline solution. To get a uniform microbial growth, 10 mL of prepared inoculums suspensions was 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) , placed on the surface of the media. The plates were incubated for 30 min in refrigerator to allow the diffusion of extract, and then they were 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 activity 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 experiments were performed in triplicate and the mean value was calculated.

The extracts which were illustrated significant antibacterial activity, were selected for further assaying for their minimum inhibitory concentration. Serial twofold dilutions of extracts were prepared in broth. To each test tube an equal volume of the adjusted inoculums was added. After incubation at 37 ºC for 24 h the MIC was read. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of an extract which was able to completely inhibit the growth of each bacterial strain .[5,23]

GC-MS Analysis of potent Fractions

GC–MS analyses were carried out on a Shimadzu QP-5050A GC–MS system equipped with a DB-1 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 µm). Oven temperature, rising from 50 °C to 230 °C at a rate of 4°C/min and then rising from 230 °C to 310°C at a rate of 1.5°C/Min; injector temperature, 280 °C carrier gas, helium at a flow rate of 1.3 ml/min; split ratio, 1:10; ionization energy, 70 eV; scan time, 1 s; mass range, 30–600 amu.

Identification of Components

Identification of the constituents was based on direct comparison of the retention times and mass spectral data with those for standard alkanes (C8-C20), and computer matching with the NIST21, NIST107 and WILEY229 library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature.[19]

Statistical Analysis

All experiments were done in triplicate measurements and presented as the Mean ± SD. Data were analyzed by Excel 2010 Microsoft.

Results and Discussion

In the present study, general toxicity, anti-proliferative and free radical scavenging activities of n-hexane, DCM and MeOH extracts from rhizomes of *E. macrophylla* were determined and the results are shown in Table 1.

The antioxidant activity of the extracts was determined by DPPH method, based on the ability of compounds act as a free radical scavenger or hydrogen donor. Also BSLT a general screening assay for determination of compounds toxicity towards brine shrimp. Both techniques are simple, inexpensive and utilize a small amount of the test material.[24]

Table 1. General toxicity, antioxidant and anti-proliferative activities of n-hexane, DCM and MeOH extracts of *E. macrophylla* rhizomes.

	General toxicity*	**Antioxidant effect****	**Antiproliferative activities(IC_{50}: µg/mL)**		
EMR extracts	LC_{50} (µg/mL)	RC_{50} (µg/mL)	HT29	A549	HUVEC
n-hexane	69 ± 7.5	595 ± 17	>1000	253.16 ± 28.47	>1000
DCM	119 ± 13	463 ± 2	194.96 ±47.28	228.98 ± 70.24	>1000
MeOH	>1000	751 ± 11	>1000	>1000	>1000

Experiment was performed in triplicate and expressed as Mean ± SD.

* The LC_{50} value of podophyllotoxin as positive control was 2.8 ± 0.1 µg/mL.

** The RC_{50} value for quercetin as positive control was 3.9 ± 0.1 µg/mL.

Table 2. Antibacterial activity results of n.hexane, DCM and MeOH extracts of *E. macrophylla* rhizomes.

	Inhibition zone diameter (mm)			
Bacterial Species	n-hexane extract	DCM extract	MeOH extract	Amikacin (positive control)
Staphylococcus aureus	-	16 ± 1.41	-	22± 0.43
Staphylococcus epidermidis	-	24 ± 1.42	-	21± 0.21

*The disc diameter was 6 mm.

Table 3. GC-MS analysis of *E. macrophylla* rhizomes n.hexane extract.

Retention time (min)	Kovats index	Compound name	Yield (%)
8.273	-	Butane	1.21
8.437	-	Pentane, 2-bromo-	0.96
13.364	913	Ether, 3-butenyl propyl	1.49
14.186	933	3-Hexyl hydroperoxide	0.89
15.053	955	5-Hexen-2-one	7.91
49.168	-	Decanoic acid, 1-methylethyl ester	7.43
54.846	-	2-Cyclopentene-1-undecanoic acid	74.76

According to Table 1 DCM fraction was the most efficient extract in DPPH assay and n-hexane extract showed the most potent effect in BSLT in comparing with podophyllotoxin as a well-known standard cytotoxic lignan. Furthermore, MeOH extract indicated weak antioxidant effect and toxicity against *A. salina* in comparison to other extracts.

In the next step, the cytotoxic activities (IC_{50}) of *E. macrophylla* rhizome extracts against two cancer cell lines and one normal cell line were evaluated and the results were shown in Table 1.

DCM extract showed the potent anti-proliferative effects against the HT29 cell line, but n-hexane and MeOH extracts didn't have any significant effect. Also in the assessment of the anti-proliferative assay on A549 cell line, cytotoxic effects were seen by n-hexane and DCM fractions. In addition, these three extracts of *E. macrophylla* didn't show any significant effect against HUVEC as a normal cell line that was used in this study. Regarding to *in vitro* cytotoxic activities of n-hexane and DCM extracts of *E. macrophylla* on HT29 and A549 Cells as cancer cells and absence of any significant side effects on normal cells, the mentioned extracts were suggested as a natural resource of potential antitumor agents in the future.

In the antimicrobial assessment, among the 5 different species of examining microorganisims, including two strains of gram negative species (*Pseudomonas aeroghinosa* and *Escherichia coli*) , two gram positive species namely *Staphylococcus epidermidis* and *Staphylococcus aureus* and a fungi (*C. albicans*), only DCM extract showed antibacterial effects on two gram positive strains that the results were shown in Table 2.

DCM extract as the most active part, displayed antibacterial activity against two gram positive microorganisms and the most noteworthy activity of this extract was against *S. aureus* with the Minimum Inhibitory Concentration (MIC) value of 3 mg/ml.

Considering of obtained results, DCM extract was the most potent fraction in antioxidant, anti-proliferative and antibacterial effects. The potent activities of this fraction in comparison to others might be due to the existence of high amounts of compounds in this extract with antioxidant, cytotoxic and antibacterial effects. Pursuant to our previously published paper, phytochemical analyses of DCM extract of *E. macrophylla* rhizomes by GC-MS showed the presence of linear alkanes, fatty acids, steroids, polycyclic aromatic hydrocarbons and terpenoids as major active constituents.[19]

Antioxidant, cytotoxic and antibacterial effects of fatty acids, steroids were confirmed previously.[25-31] Likewise, some polycyclic aromatic hydrocarbons and terpenoids have been reported for these bioactive properties.[32-35] Therefore, the potential biological efficacy of DCM extract may be related to the combination of these compounds and their synergies correlation with each other.

In the case of n-hexane extract as the other effective fraction with potent cytotoxic response against *A. salina* and A549 cell line, fatty acid derivatives (82.19%) were identified by GC-MS analysis as volatile part of this fraction (Table 3). As mentioned above anti-proliferative activity could be related to fatty acid content.[30] Previous phytochemical investigations on the non-volatile part of the n-hexane extract of *E. laciniata* as other species of this genus indicated the presence of stigmasterol and β-sitosterol with steroid structure, which had good anti-proliferative effects on A549 cells.[8,36]

Conclusion

This is the first report on the antioxidant, general toxicity, cytotoxic and antibacterial effects of the rhizomes of *E. macrophylla*. The findings demonstrated the *in vitro* antioxidant, anti-proliferative and antibacterial effects of DCM extracts of *E. macrophylla* rhizomes with any deleterious effects on normal cells. Additionally, n-

hexane extract of these rhizomes had a cytotoxic effect on A549 cell line, which may have high clinical importance in future. The obtained results showed that more studies should be focused on the isolation of active and pure ingredients and clarification of the anti-neoplastic mechanism of them.

Conflict of interests

The authors claim that there is no conflict of interest.

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