Biological activities of *Eremostachys laevigata* Bunge. grown in Iran

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Abstract: Essential oil from flowers, stems, and roots of *Eremostachys laevigata* Bunge. gathered in Iran was analyzed using gas chromatography (GC) and gas chromatography/mass spectroscopy (GC/MS), and 23, 21, and 9 compounds were identified, respectively. The primary components of all three oils were found to be 1,8‒cineole, benzaldehyde, and piperitenone oxide: 18.3%, 18.7%, and 2.5%; 17.9%, 7.7%, and 63.3%; and 15.7%, 21.3%, and 1.2%, respectively. The oils derived from flowers and stems also contained cis-piperitone oxide as a major component (10.1% and 12.2%, respectively). *E. laevigata* oil showed antibacterial activity, particularly towards Gram-positive bacteria; additionally antioxidant activity was induced with IC_{50} of flowers, stems and roots of *E. laevigata* (277.1, 495.0, and 212.6 μg/ml), respectively. Furthermore, under β-carotene-linoleic acid test assay the flower, stem, and root oils of *E. laevigata* had a high antibacterial effect.

Keywords: *Eremostachys laevigata*, antibacterial activity, 1,8-cineole, α-pinene, antioxidant activity.

INTRODUCTION

Although spices have been used for thousands of years and are known for their flavour, taste and colour in the food, they are not always recognized for their medicinal value. In recent years, extensive research has demonstrated the likelihood that the phytochemicals present in spices may have a potential role in the prevention of a number of illnesses, including cancer, cardiovascular disease, diabetes, and neurological and pulmonary diseases (Hirasa 1998). The spice industry is continually on the lookout for novel and little-used spices in hopes of discovering new and marketable properties and uses.

The genus *Eremostachys*, which belongs to Lamiaceae family, consists of about 60 species that are mainly present in South Western Asia (Rechinger 1982). Fifteen species of *Eremostachys* grow wildly throughout many regions of Iran: *E. azerbaijaanica, E. codonocalyx, E. hyoscyamoides,E. pulvinaris,* and *E. adenantha* are endemic (Mozaffarian, 1996). Studies of the phytonutrient properties of a few *Eremostachys* species have revealed the presence of such flavonoids as chrysoeriol glycosides and luteolin (Azizian & Cutler, 1982). Monoterpen glycosides have been identified in *Eremostachys fetissovii* and a furanolabdane diterpene glycoside in *Eremostachys glabra* (Gella & Vavilova, 1981; Delazar *et al*., 2006). Two bioactive ferulic acid derivatives from *E. glabra* (Delazar, Shoeb, *et al*., 2004), and three antioxidant phenylethanoid glycosides from rhizomes of *E. pulvinaris* (Delazar, Sarker, *et al*., 2004) have been identified and studied. *E. laevigata* Bung. which is an everlasting herb is widely distributed in Zagroos mountaines in West of Iran (Ghahreman, 1996). This plant is traditionally used in Lorestan province for relief of the pain caused by insects or reptiles bites and it is also used in industry for dyeing. We have not found any studies of the phytochemical and biological activities of *E. laevigata* Bung., and we believe this paper to be the first such study of this plant.

In traditional folk medicine, extracts of the roots and flowers of *E. laciniata* have been taken orally to treat liver complaints, allergies, and headache (Said *et al*., 2002). A variety of monoterpenes and sesquiterpenes have been isolated in essential oils derived from *E. laciniata* (Navaei & Mirza, 2006). Free-radical scavenging properties were observed in a crude extract of this plant (Erdemoglu *et al*., 2006). As a continuation of previous studies on Iranian flora, we now report on the isolation of three iridoid glucosides—phloyoside I, phlomiol, and pulchelloside—from the rhizomes of *E. laciniata,* and their structure and bioactivity.

MATERIALS AND METHODS

Plant material

Flowers, leaves, and stems of *Eremostachys laevigata* were collected during the flowering stage in July 2004 from 15 km north of Khorramabad, Lorestan Province, Iran, at an altitude of 1700 m. Voucher specimens (No. 63029) were deposited with the Herbarium of the Research Institute of Forests and Rangelands inTehran, Iran.

Isolation of oil

Fresh flowers (70 g), leaves (80 g), and stems (90 g) of *E. laevigata* were hydrodistilled for 3 h using a Cleavenger-type apparatus according to the British Pharmacopoeia (1998). The decanted oil was dried over anhydrous sodium sulfate.

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GC analysis

GC analysis was performed on a Shimadzu 15A gas chromatograph equipped with a flame ionization detector (250ºC) and a split/splitless injector (250°C). The carrier gas employed was nitrogen (1 mL/min). A DB-5 capillary column (50m × 0.2 mm; film thickness 0.32 μm) was used and programmed as follows: temperature kept at 60°C for 3 min, then heated to 220°C at a rate of 5 °C/min, and then kept constant at 220°C for 5 min. A Shimadzu CR4A Chromatopac was used to calculate the relative percentage of all compounds from peak areas without the employment of correction factors.

GC/MS analysis

The samples were assayed with GC/MS using a Hewlett-Packard 5973 with an HP-5MS column (30 m × 0.25 mm; film thickness 0.25 μm). Again, the column temperature was kept at 60°C for 3 min, then heated to 220 °C at a rate of 5°C/min and then kept constant at 220 °C for 5 min. Helium was used as the carrier gas with a flow rate of 1mL/min. MS was taken at 70 eV.

Qualitative and quantitative analyses

The constituent of each oil were identified by using gas chromatography and comparing their retention indices with those reported in the literature (Adams, 2007; Davies, 1990; Jennings & Shibamoto, 1980) or with authentic compounds available in our laboratories. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8–C_{28}) under the same operating conditions. Further identification was made by comparison of mass spectra on both columns with those reported in the literature or stored in the Wiley 275, the NIST 02, and our own libraries. Relative concentrations of components were calculated based on GC peak areas without employing correction factors.

Antimicrobial assay

The broth dilution method (Davies, 1990) was employed to evaluate antibacterial activity by determining the the minimum bactericidal concentration (MBC) and the minimum 118 inhibitory concentration (MIC). Five bacteria species were selected as being representative of the classes of Gram-positive and Gram-negative and were tested: Gram-positive bacteria *Staphylococcus aureus* (PTCC 1113), *S. epidermidis* (PTCC 1349), and *S. saprophyticus* (PTCC 1379), and the Gram-negative bacteria *Escherichia coli* (PTCC 1330) and *Pseudomonas aeruginosa* (PTCC 1310), as identified by the Research Centre of Science and Industry, Tehran, Iran. Microorganisms obtained from enrichment culture in 1 mL of Mueller-Hinton broth incubated for 12 h at 37°C were cultured on Mueller-Hinton agar medium. To measure antibacterial activity, 40 μL of diluted essential oil (40 μL oil in 2 mL DMSO 10%) was added to a 200 μL microbial suspension (1 loop from medium in physiological serum that compared with a 0.5 McFarland standard) in well 1 in a micro plate, and 100 μL from this well was added to a 100 μL microbial suspension in well 2, and this was continued until 8 wells in the micro plate were filled. The micro plates were incubated for 24 h at 37°C.

Antioxidant activity

DPPH assay

Although the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay most commonly involves a hydrogen atom transfer reaction, based on kinetic data, an electron transfer mechanism has also been suggested (Rezazadeh *et al.,* 2006; Mirza *et al.,* 2004). A published DPPH radical-scavenging activity (RSA) assay method (Mirza, 2001) was employed with minor modifications to determine the RSA of the root, flower, and stem oils of *Eremoestachys laevigata*. Stock solutions (10 mg/ml) of the synthetic standard antioxidant BHT and of the oils were dissolved in methanol, creating dilutions with concentrations ranging from 1 to 5×10^{-10} mg/ml. The diluted solutions (2 ml) were mixed with 2 ml of freshly prepared 80 μg/ml DPPH methanol solution and allowed to stand for 30 min in the dark at room temperature. Ultraviolet absorbance of these solutions was recorded on a spectrometer at 517 nm using a blank containing the same concentration of oil or BHT without DPPH. Inhibition of free radical DPPH in percent (I%) was calculated as follows:

$I\%=[(A_{\mathrm{blank}}-A_{\mathrm{sample}})/A_{\mathrm{blank}}]\times 100$

where A_{blank} is the absorbance of the control reaction containing all reagents except the test compound, and A_{sample} is the absorbance of the test compound. Inhibition percentages were plotted against concentrations of the sample to calculate the sample concentration providing 50% inhibition (IC_{50}). All tests were carried out in triplicate, and IC_{50} values were reported as means ± SD of triplicates.

β-Carotene/linoleic acid bleaching assay

The β-carotene/linoleic acid bleaching assay was employed to determine antioxidant activity by measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides arising from linoleic acid oxidation. The method described by Miraliakbari and Shahidi (2008) was used with slight modifications. A stock solution of *β*-carotene and linoleic acid was prepared with 25 μl linoleic acid, 0.5 mg *β*-carotene in 1 ml chloroform, and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 ml of aerated distilled water added to the residue. The 2 g/l samples were dissolved in DMSO and 350 μl of each sample solution was added to 2.5 ml of the above mixture in test tubes, which were then incubated in a hot water bath at 50 °C for 2 h, together with two blanks, one containing the same volume of DMSO instead of the extracts and the other the antioxidant BHT as a positive control. The test tube with BHT maintained its yellow color throughout the period of incubation. The absorbance was measured at

470 nm on an ultraviolet spectrometer. Antioxidant activities (inhibition percentage, I%) of the samples were calculated using the following equation:

$I\% = (A_{\beta\text{-carotene after 2h assay}}/A_{\text{initial }\beta\text{-carotene}}) \times 100$

where $A_{\beta\text{-carotene}}$ after 2 h assay is the absorbance of β-carotene remaining in the samples after 2 h, and $A_{\text{initial }\beta\text{-carotene}}$ is the absorbance of β-carotene at the beginning of the experiment. All tests were carried out in triplicate, and inhibition percentages were reported as means ± SD of triplicates.

RESULTS

Essential oils analysis

Table 1 shows the chemical components identified in the three oils of *E. laevigata* and their percentage composition. There were 23 compounds identified in the flower oil, representing 92.5% of the oil composition. The main compounds were 1,8-cineole (18.3%), benzaldehyde (17.9%), piperitenone oxide (15.7%), and cis-piperitone oxide (10.1%). Another notable constituent was 1-octen-3-ol (5.8%).

In the stem oil, 21 compounds were identified, representing 93.0% of the oil composition. The main compounds were piperitenone oxide (21.3%), 1,8−cineole (18.7%), and cis-piperitone oxide (12.2%), while benzaldehyde (7.7%) was found in large amounts. In the root oil, enzaldehyde (63.3%) and 1-octen-3-ol (11.5%) were found to be the main components among the 9 constituents isolated, representing 90.0% of the total components detected.

Antibacterial activity

The in vitro antibacterial activity of *E. laevigata* oil against five bacteria species known to cause gastrointestinal, respiratory, skin, and urinary disorders and selected as representative of the classes of Gram-positive and Gram-negative was evaluated by determining the MIC and the MBC using the broth dilution method. Table 2 displays the results obtained in the antibacterial assay.

Antioxidant activity

In the present study, root oil was found to possess the weakest radical-scavenging activity (212.0 ± 4.3 μg/ml^{-1}). In fact, the positive control BHT showed stronger activity than any of the samples showed activity (88.4 ± 0.4 μg/ml^{-1}). In the β-carotene/linoleic acid bleaching assay % inhibition rate of the root oil (67.8 ± 1.2) was found to be nearest to that of the positive control BHT (96.2 ± 0.9) (table 3).

DISCUSSION

Three essential oils isolated by hydrodistillation from the aerial parts of *E. laevigata* were obtained in yields of 0.55%, 0.45%, and 0.35% (w/w), respectively. The composition of the essential oils of the flower, stem and root oils were listed in table 1, in which the percentage and retention indices of components are given. Twenty-three constituents, representing 92.5% of the total components in the flower oil of *E. laevigata*, were characterized by 1,8-cineole (18.3%), benzaldehyde (17.9%), piperitenone oxide (15.7%), and cis-piperitone oxide (10.1%). Twenty-one compounds identified in the stem oil, representing 93.0% and piperitenone oxide (21.3%), 1,8−cineole (18.7%), and cis-piperitone oxide (12.2%) were main compounds. So, in the root oil, enzaldehyde (63.3%) and 1-octen-3-ol (11.5%) were found to be the main components, representing 90.0% of the nine components in the oil. Monoterpenes were the most abundant components found in the flower, stem, and root oils (84.6%, 82.9%, and 88.1%, respectively), benzaldehyde proving to be the major constituent (17.9%, 7.7%, and 63.3%, respectively). Another notable constituent was 1,8-cineole (18.3%, 18.7% and 2.5%, respectively). The amount of oil isolated from the three oils of *E. laevigata* Bung. by hydrodistilation, based on dry weight was 0.1% (w/w) Lamiaceae family in Turkey on the basis of essential oil content has been classified as rich (>2%), moderately rich (0.5-2%) and poor (<0.5%) (Baser, 1993). Previous studies have shown that *Eremostachys* are in general poor in oil (Kacabas & Karaman, 2001). The chemical composition of the flower, stem, and root oils from *Eremostachys laevigata* is presented in table 1; and components are listed in order of their retention indices as determind on a DB-5 column. Comparison of components in *E. laevigata* with other species demonstrated that germacrene-D is also found as the major constituent in the essential oils of *Phlomis persica* (32.5%), *Phlomis chorassanica* (51.5%), *Phlomis anisodonta* (65%), *Phlomis lanceolata* (47%), *Nepeta ucrainica* (39.7%), *Stachys schtschegleevii* (25.8%), *Teucrium salviastrum*(21.6%), *Marrabium cunceatum* (24.1%) (Sarkhail *et al.,* 2004; Sarkhail *et al.,* 2005; Rezazadeh *et al.,* 2006). β-Caryophyllene is the major compound of the oil of *Teucrium sativarum* (29.3%), *Phlomis olivieri* (16.1%), *Marrabium vulgare* (11.6%), *Marrabium parviflorum* (15.6%), *Phlomis bruguieri* (11%), *Salvia nemorosa* (41.6%)*, Salvia virgata* (46.6%), *Salvia aethiopis* (27.5%) (Mirza, 2001; Khanavi *et al.,* 2005; Mirza & Baher, 2003; Mirza & Sefidkon, 1999; Sefidkon & Mirza, 1999; Chalchat *et al.,* 2001). Furthermore, dodecanal has been reported to be the main constituent of the oils of *Nepeta saccharata* (25.5), *Stachys laxa* (1.6%) and *Astrodaucus persicus* (15.5%) (Bigdeli *et al.,* 2004; Mos-hafi *et al.,* 2005; Sajjadi & Mehregan, 2003; Farag, 1989).

The *in vitro* antibacterial activity of *E. laevigata* oils against five bacteria species, selected as representatives of the classes of Gram-positive and Gram-negative and known to cause respiratory, evaluated by determining the

Table 1: Percentage composition of the flower, stem and root oils of *E. laegaca*

Compound	RI	Flower oil	Stem oil	Root oil
Benzaldehyde	961	17.9	7.7	63.3
1-Octen-3-ol	978	5.83	2.6	11.5
β-Pinene	980		2.1	
β-Terpinene	1018	1.8	-	-
p-Cymene	1026	-	1.2	1.2
Limonene	1031	0.7	-	-
1,8–Cineole	1033	18.3	18.7	2.5
Benzene acetaldehyde	1043	1.6	3.1	5.2
(*Z*)-β-Ocimene	1050	-	-	-
Tetra methyl pyrazine	1085	1.7	2.6	-
Linalool	1098	0.4	-	-
α-Campholenal	1125	0.4	-	-
trans-Limonene oxide	1139	2.1	-	-
Pinocarvone	1162	1.6	-	-
α-Terpineol	1189	1.5	3.2	-
Myrtenol	1193	2.1	2.2	-
p-Cymen-9-ol	1206	-	0.6	-
cis-Piperitone oxide	1210	10.1	12.2	-
(*Z*)-Ocimenone	1231	-	0.9	-
Geranial	1290	1.1	-	-
(*2E,4E*)-Decadinenal	1291	-	1.3	-
Thymol	1298	1.8	2.8	3.2
Carvacrol	1356	-	-	-
Eugenol	1360	-	0.7	-
Piperitenone oxide	1368	15.7	21.3	1.2
α-Copaene	1376	0.6	-	0.8
β-Bourbonene	1381	0.9	-	-
β-Caryophyllene	1418	0.8	-	-
Germacrene D	1480	1.7	0.5	1.1
β-E-ionone	1485	-	0.6	-
(Z)-α-Bisabolene	1504	-	3.0	-
Caryophyllene oxide	1581	0.8	1.9	-
Valeranone	1672	3.1	3.8	-

[a] Retention indices as determind on a DB-5 column using the homologous series of n-alkane

MIC and the MBC using the broth dilution method. The results obtained in the antibacterial assay are shown in Table 2. Our sample showed particular effectiveness against Gram-positive bacteria, as evidenced by lower MIC values. Gram-negative bacteria did not seem to be vulnerable to the oil. Antioxidant activity of the oils derived from roots, flowers, and stems of *E. laevigata* was assayed independently by DPPH and β-carotene/linoleic acid bleaching assay. With DPPH, the antioxidant reacts with the stable free radical 1,1-diphenyl-2-picrylhydrazyl to convert it to 1,1-diphenyl-2-picrylhydrazine with discoloration, the degree of discoloration indicating the free radical scavenging potential of the antioxidant. Known antioxidants such as ascorbic acid, cysteine, glutathione, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol), and tocopherol reduce and decolorize 1,1-diphenyl-2-picrylhydrazyl because of their hydrogen-donating ability. The synergistic effects of fenolic acids such as polyphenols and rosmarinic acid and chemicals such as flavonoids might account as well for the radical scavenging activity observed in methanol extracts. In the present study, root oil was found to possess the weakest radical-scavenging activity (212.0 ± 4.3 μg/ml^{-1}). In fact, the positive control BHT showed stronger activity than any of the samples showed activity (88.4 ± 0.4 μg/ml^{-1}). In the β-carotene/linoleic acid bleaching assay, the absence of an antioxidant results in rapid discoloration of β-carotene, due to the formation of free radicals by coupled oxidation of linoleic acid and β-carotene. The free radical generated by the abstraction of a hydrogen atom from one of the diallylic methylene group of the linoleic acid attacks the highly unsaturated β-carotene molecules, causing oxidation and partial breaking down of the β-carotene. The system consequently loses its chromophore and the characteristic orange color displayed under spectrophotometric

Table 2: Antibacterial activity of of the flower, stem and root oils of *E. laegaca* based on dilution method and using four reagent*

Bacterial Species	Gram +/-	Flower oil	Stem oil	Root oil	Gentamicin	Penicillin	Sefazolin	Norfloxacin
Staphylococcus Aureus PTCC 1113	+	27.3	13.4	9.0	0.0	0.0	15.7	0.0
Staphylococcus epidermidis PTCC 1349	+	19.3	17.3	11.2	30.3	21.0	30.3	31.0
Staphylococcus Saprophyticus PTCC 1379	+	11.8	14.2	12.5	0.0	25.0	20.0	11.0
Erichia coli PTCC 1330	–	11.0	0.0	0.0	19.0	0.0	17.7	28.3
Pseudomonas aeruginosa PTCC 1310	-	0.0	0.0	0.0	15.6	0.0	15.3	30.3

*Values are the means diameter of inhibitory zones (mm)

monitoring. The % inhibition rate of the root oil (67.8 ± 1.2) was found to be nearest to that of the positive control BHT (96.2 ± 0.9) (table 3). The rapid increase of peroxides accompanies auto-oxidation of linoleic acid without volatiles and methanol extracts. There is a relationship between the presence of some phenolic nuclei in essential oils and extracts and inhibition of hydro peroxide formation (Farag *et al.,* 1989; Esmaeili and Amiri, 2011; Esmaeili *et al.,* 2009, 2010). Others have reported that the antioxidative effectiveness in natural sources is mostly due to phenolic compounds.

CONCLUSION

Essential oils of aromatic plant species have numerous uses: in traditional medicine for treatment of infectious diseases; for food preservation; in production of soaps, perfumes, and toiletries; and as insecticides and anti-parasitics.

The present study indicated that 1,8–cineole, benzaldehyde, and piperitenone constitute the main components of the root, stem, and flower oils of *E. laevigata*. 1,8-Cineole as an abundant component in the oil of *E. laevigata* is one of common ingredient in perfumery and has been used in soap, detergent, beauty care and household products. The substance is partially non-toxic, and is a permitted food additive (GRAS) in both U.S. and the UE (inchem). All three oils contained monoterpene hydrocarbon, a macrocyclic olefin which possesses anti-inflammatory and anticarcinogenic properties and along with its derivatives may also have a potential role in plant defense. The % inhibition capacity of the root oil *E. laegaca* was found to be higher than the sample.

Our sample showed activity particularly against Gram-positive bacteria, as evidenced by the lower MIC values returned in the Gram-positive bacteria studied. Gram-negative bacteria did not appear to be sensitive to the oil.

Table 3: Antioxidative capacities of the flower, stems and roots oils of *E. laegaca* [a]

Plant oils	Test system	
	DPPH IC_{50} (μg/ml)	β-Carotene/ linoleic acid (% inhibition rate)
Flower *E. laegaca*	277.1± 4.3	31.6± 0.8
Stem *E. laegaca*	495.0± 0.6	43.7± 1.1
Root *E. laegaca*	212.6± 1.8	67.8±2.1
BHT	18.2 ± 0.4	96.2 ± 0.9

[a] Results are means of three different experiments.

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