TWO BIOACTIVE FERULIC ACID DERIVATIVES FROM *EREMOSTACHYS GLABRA*

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ABSTRACT

Two ferulic acid derivatives, hexacosyl-(*E*)-ferulate [**1**] and leucosceptoside A [**2**], have been isolated from the rhizomes of *Eremostachys glabra*. The chemical structures of these compounds have been elucidated by UV, ESIMS, ^{1}H NMR and ^{13}C NMR spectroscopic analyses, and also by comparing experimental data with respective literature data. The free radical scavenging activity and general toxicity of these compounds have been assessed. While none of these compounds has shown any significant general toxicity in the brine shrimp lethality assay (LD_{50}>1 mg/mL), compounds **1** and **2** displayed significant antioxidant activity in the DPPH assay (RC_{50} = 0.0976 mg/mL and 0.0148 mg/mL, respectively).

Key words: *Eremostachys glabra*, Lamiaceae, natural antioxidant, DPPH assay, brine shrimp lethality assay, hexacosyl-(*E*)-ferulate, leucosceptoside A, *Eremostachys pulvinaris*.

INTRODUCTION

The genus *Eremostachys* (family: Lamiaceae *alt.* Labiatae) comprises about 60 species that occur mainly in Central Asian countries, *e.g.* Armenia, Turk-menistan and USSR (1). A number of *Eremostachys* species, e.g. *E. glabra*, *E. laciniata*, *E. lanata* and *E. labiosa* are also well distributed in Iran. Previous phytochemical investigations on just a few species of the genus *Eremostachys* revealed the presence of flavonoids, *e.g.* luteolin and chrysoeriol glycosides and monoterpene glycosides (1, 2).

According to the unpublished information gathered from indigenous knowledge and traditional practice, in Iran, the rhizomes of *Eremostachys glabra* Boiss are apparently used as local analgesic and anti-inflammatory. No phyto-chemical investigation has previously been carried out on *E. glabra*. We now report on the isolation, structural determination and biological activity of two ferulic acid derivatives, hexacosyl-(*E*)-ferulate [**1**] and leucosceptoside A [**2**] from the rhizomes of this plant.

MATERIALS AND METHODS

General procedures

NMR spectra were recorded on a Bruker 250 MHz NMR Spectrometer using $CDCl_3$ and CD_3OD for the compounds **1** and **2**, respectively. The residual solvent peaks were used as internal standards. ESIMS analysis was performed on Finnigan MAT95 spectrometer.

Plant material

The rhizomes of *Eremostachys glabra* Boiss. (Syn: *E. pulvinaris*) were collected during September-October 2002 from Tabriz (altitude of 1,400 meters) in Eastern Azarbaijan province (Iran) and the identity was confirmed by anatomical examination in comparison with the herbarium specimen retained in the School of Pharmacy, Tabriz University of Medical Sciences, Iran.

Extraction, isolation and structure elucidation

The dried, ground rhizomes of *E. glabra* (100 g) were Soxhlet extracted, successively, with *n*-hexane, dichloromethane (DCM) and methanol (MeOH). The amounts of dried *n*-hexane, dichloromethane and MeOH extracts were 366.1 mg, 269.5 mg and 15.01 g, respectively. The DCM extract was subject to vacuum liquid chromatography (VLC) on silica gel 60H using a step gradient of *n*-hexane:ethyl acetate (100:0,

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90:10, 80:20, 60:40, 40:60, 20:80, 100:0). Further purification of *n*-hexane/EtOAc (80:20) fraction was carried out by PTLC on silica gel GF_{254} using chloroform ($CHCl_3$) as the mobile phase resulting in the isolation of compound **1** (4.2 mg, R_f=0.55). The MeOH extract (2 g) was subjected to Sep-Pack (ODS) fractionation using a step gradient of MeOH-water mixture (10:90, 20:80, 40:60, 60:40, 80:20 and 100:0). The preparative reversed-phase HPLC analysis [Luna C_{18} column 10 µm, 250 mm × 21.2 mm; mobile phase: 0 to15 min, isocratic 10% acetonitrile (ACN) in water; 15 to 45 min gradient 10 to 27% ACN in water; 45 to 55 min, isocratic ACN 27% in water; flow-rate: 20 mL/min, detection at 220 nm, 300 nm] of the 40% methanolic Sep-Pack fraction (132 mg) resulted in the isolation of compound **2** (2.3 mg, retention time: 37.6 min). The chemical structures of these compounds have been elucidated unambiguously by UV, ESIMS, NMR spectroscopic analyses, and also by comparing experimental data with literature data.

Hexacosyl-(*E*)-ferulate [**1**]. Amorphous solid, UV λ_{max} (EtOH): 235, 296, 327; ESIMS *m/z* 559 $[M+1]^+$; ^{1}HNMR (250 MHz, $CDCl_3$): δ 0.84 (3H, *t*, *J*=6.80 Hz, CH_3), 1.22 [48H, *s*, 24 x CH_2], 3.89 (3H, *s*, OMe), 4.15 (2H, *t*, *J*=6.8 Hz, O-CH_2), 5.78 (1H, *br s*, OH), 6.25 (1H, *d*, *J*=16.0 Hz, H-8), 6.88 (1H, *d*, *J*=8.2 Hz, H-5), 7.00 (1H, *d*, *J*=1.8 Hz, H-2), 7.04 (1H, *dd*, *J*=8.2, 1.8, H-6), 7.57 (1H, *d*, *J*=16 Hz, H-7).

Leucosceptoside A [**2**]. Gum, UV λ_{max} (MeOH): 295 (sh), 331; ESIMS *m/z* 639 $[M+H]^+$; ^{1}H NMR (250 MHz, CD_3OD): Table 1; ^{13}C NMR (62.5 MHz, CD_3OD): Table 1.

Antioxidant assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. The method used by Takao *et al.* (3) was adopted with suitable modifications (4). A solution of DPPH (0.08 mg/mL) in $CHCl_3$ or MeOH was used. The compound **1** was dissolved in $CHCl_3$ to obtain a concentration of 1 x 10^{-1} mg/mL. Dilutions were made to obtained concentrations of 5.00x10^{-2}, 2.5 x10^{-2}, 1.25x10^{-2}, 6.25x10^{-3}, 3.13x10^{-3}, 1.56x10^{-3} mg/mL. Diluted solutions (5 ml each) were mixed with DPPH (5 mL) and allowed half hour for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in duplicate and average absorption was noted for each concentration. Data were processed using EXCEL and the concentration that caused a 50% reduction in absorbance (RC_{50}) was calculated. The same procedure was followed for the standard (Trolox). For compound **2**, the same method was used (as mentioned above), but the solution was prepared in MeOH instead of $CHCl_3$ and standard quercetin was used as a positive standard.

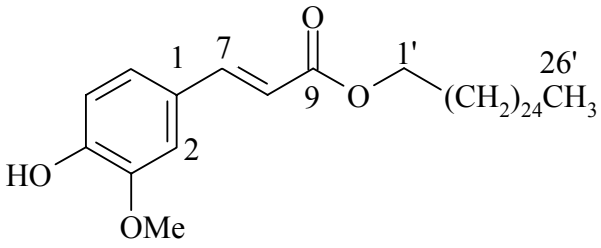
Brine shrimp lethality assay

The method described by Meyer *et al* (5) was adopted to study the general toxicity of the compounds **1** and **2**. Waterlife brand brine shrimp (*Artemia salina*) eggs were purchased from The Pet Shop, Kittybrewster Shopping Complex, Aberdeen, UK. The eggs were hatched in a conical flask containing 300 ml artificial seawater made by dissolving distilled water. The flasks were well aerated with the aid of an air pump, and kept in a water bath at 29-30^o C. A bright light was left on. The nauplii hatched within 48 h. The compounds **1** and **2** were dissolved in DMSO to obtain a concentration of 1 mg/ml separately. These were serially diluted two-times and seven different concentrations were obtained. Solution of each concentration (1 mL) was transferred into clean sterile universal vials with a pipette and aerated seawater (10 mL) was added. About 10 nauplii were transferred into each vial with pipette. A check count was performed. The number alive after 24 h was noted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30 sec of observation. The controls used were DMSO, normal saline and podophyllotoxin.

RESULTS AND DISCUSSION

A combination of VLC and PTLC of the DCM extract of *E. glabra* afforded **1** and Sep-Pak fractionation followed by prep-HPLC of the MeOH extract produced **2**. The ESIMS analysis of **1** showed the $[M+1]^+$ ion at *m/z* 559, suggesting the molecular weight of 558 and solving for $C_{36}H_{62}O_4$ which was consistent with the structure of hexacosyl-(*E*)-ferulate. The ^{1}H NMR spectrum showed signals characteristic for a ferulic acid moiety (δ 6.25, *d*, *J*=16.0 Hz; 6.88, *d*, *J*=8.2 Hz; 7.00, *d*, *J*=1.8 Hz; 7.04, *dd*, *J*=8.2, 1.8; 7.57, *d*, *J*=16 Hz; 3.89, *s*) and a hexacosyl unit (δ 0.84, *t*, *J*=6.80 Hz; 1.22, *s*; 4.15, *t*, *J*=6.8 Hz). The deshielded nature of the oxymethylene signal (δ 4.15) confirmed its link to the carbonyl of the ferulic acid moiety and thus provided evidence for ester formation. The spectroscopic data were in good agreement with those of published data for hexacosyl-(*E*)-ferulate (6).

The UV spectrum of **2** showed absorptions at 295 (sh) and 331 nm. The ESIMS analysis of **2** displayed the $[M+H]^+$ ion at *m/z* 639, suggesting the molecular weight of 638 and solving for $C_{30}H_{38}O_{15}$ which was consistent with the structure of leucosceptoside A. The ^{1}H and ^{13}C NMR spectra (table 1) showed all signals corresponding



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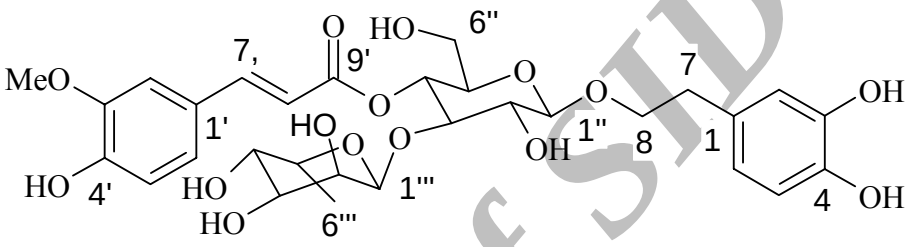

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Table 1. ^{1}H (250 MHz , coupling constant *J* in Hz in parentheses) and ^{13}C NMR (62.5 MHz) data of **2**

Carbon number	Chemical shift δ in ppm		Carbon number	Chemical shift δ in ppm	
	δ_H	δ_C		δ_H	δ_C
1	-	131.2	Glucose moiety		
2	6.70 d (2.0)	116.8	1''	4.36 d (7.5)	103.9
3	-	144.4	2''	3.40*	75.8
4	-	145.9	3''	3.80 t (9.5)	81.2
5	6.68 d (8.0)	116.2	4''	4.94 t (9.5)	70.3
6	6.57 dd (2.0, 8.0)	121.3	5''	3.70*	75.9
7	2.80 m	36.7	6''	3.54*, 3.79*	62.4
8	4.1 m* 3.8 m*	72.0	Rhamnose moiety		
1'	-	127.4	1'''	5.19 d (1.5)	102.7
2'	7.06 d (2.0)	115.2	2'''	3.78*	71.8
3'	-	149.1	3'''	3.58*	72.0
4'	-	150.5	4'''	3.28*	73.5
5'	6.78 d (8.5)	116.2	5'''	3.56*	70.3
6'	6.96 dd (2.0, 8.5)	124.1	6'''	1.12 d (6.5)	18.4
7'	7.59 d (16.0)	147.6			
8'	6.28 d (16.0)	115.3			
9'	-	167.9			
OMe	3.88 s	56.4			

* Overlapped peaks.

to a ferulic acid, a 3,4-dihydroxyphenyl ethanol, a glucose and a rhamnose moieties. The unambiguous identification of this compound was achieved by direct comparison of these spectroscopic data with those published for leucosceptoside A [**2**] (7).

There is no report on the occurrence of ferulic acid derivatives, **1** and **2**, in the genus *Eremostachys*. However, isolation of hexacosyl-(*E*)-ferulate [**1**] was previously reported from *Erythrina excelsa* (6). Within the family Lamiaceae, phenylethanoid glycoside ester of ferulic acid, leucosceptoside A [**2**], has previously been reported from other genera, *Phlomis grandiflora*, *Marrubium velutinum*, *Stachys officinalis* and *Prostanthera melissifolia* (8-14).

DPPH assay measures the free radical scavenging capacity of a compound. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant which can donate an electron to DPPH, the purple color, which is typical to free DPPH radical, decays, and the change in absorbance at 517 nm is followed spectrophotometrically. In cases where the structure of the electron donor is not known (e.g., a plant extract), this method can afford data on the reduction potential of the sample, and hence can be helpful in comparing the reduction potential of unknown materials. The antioxidant activity of **1** and **2** was determined by this method and the RC_{50} values were found to 9.76 x 10^{-2} and 1.48 x 10^{-2} mg/mL, respectively. The RC_{50} value of the positive controls, trolox and quercetin, were 3.07 x 10^{-3} and 2.78 x 10^{-5} mg/mL, respectively. Since reactive oxygen species are important contributors to tissue injury and inflammation, the antioxidant properties of **1** and **2** probably contribute to the antiinflamatory properties of the *E. glabra* (traditional use).

The brine shrimp lethality assay, which has been proven to be an effective and rapid assay method to screen compounds for potential general toxicity and cytotoxic activity (5) was used to determine the general toxicity of **1** and **2**. The LD_{50} of these compounds were > 1.0 mg/mL. LD_{50} value of podophyllotoxin, a well known cytotoxic lignan was 2.79 x 10^{-3} mg/mL

Hexacosyl-(*E*)-ferulate [**1**] has characteristic coconut-like essence. As this compound possesses significant antioxidant property and has no toxicity towards brine shrimps, it has the potential of being developed as a drug or used as a flavouring agent in food industry. The hexacosyl moiety of **1** has previously been reported to have various types of pharmacological activities, *e.g.* promotion of maturation of central neurons in culture and neurotrophic activity (15, 16), attenuated degeneration of cholinergic neurons (17), reduction of cell loss in some neurodegenerative disorders like Alzheimer's disease or stroke (15), inhibition of glucose-stimulated insulin secretion (18) and increasing the phagocytosis activity of cultured macrophages (19).

The phenylethanoid glycosides acteoside, which is structurally very similar to **2**, showed inhibitory effect on histamine and bradykinin induced contractions of guinea-pig ileum, and *in vivo* antiinflammatory activity when administered orally to rats mainly in the fourth hour after the administration of the phlogistic agent. The antiinflammatory properties were thought to be due to the inhibition of bradykinin and histamine (20). Also the five other similar phenylethanoids, isolated from *Plantago lanceolata* showed inhibitory effects on arachidonic acid-induced mouse ear edema (21). It is therefore reasonable to say that compound **2** may also possess similar pharmacological properties.

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REFERENCES

1. Azizian, D., Cutler, D. F. Anatomical, cytological and phytochemical studies on *Phlomis* L *and Eremostachys* Bunge (Labiatae), Botanical Journal of the Linnean Society 1982; 85: 225-248.
2. Gella, E. V., Vavilova, N. K. Monoterpene glycosides of *Eremostachys fetissovii*, Khim Prir Soedin. 1981; 3:390-391.
3. Takao, T., Watanabe, N., Yagi, I., Sakata, K. A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish, Biosci. Biotech. Biochem 1994;58:1780-1783.
4. Kumarasamy, Y., Fergusson, M., Nahar, L., Sarker, S. D. Bioactivity of moschamindole from *Centarea moschata*. Pharmaceutical Biology. 2002; 40:307-310.
5. Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobson, J. B., Nicholas, D. E., McLaughlin, J. .L, Brine shrimp A convenient general bioassay for active plant constituents, Planta Medica. 1982; 45: 31-34.

6. Wandji, J., Nkengfack, A. E., Fomum, Z.T. A new prenylated isoflavone and long chain esters from two *Erythrina species*, Journal of Natural Products 1990; 53: 1425-1429.
7. Ismail, L. D., Et-Azizi, M. M., Khalifa, T. I., Stermitz, F. R. Verbascoside derivatives and iridoid glycoside from *Penstemon crandallii*, Phytochemistry 1995; 39: 1391-1393.
8. Takeda, Y., Kinugawa, M., Masuda, T., Honda, G., Otsuka, H., Sezik, E., Yesilada, E. Phlomisethanoside, a phenylethanoid glycoside from *Phlomis grandiflora var. grandiflora*, Phytochemistry 1999; 51:323-325.
9. Karioti, A., Skaltsa, H., Heilmann, J., Sticher, O. Acylated flavonoid and phenylethanoid glycosides from *Marrubium velutinum*, Phytochemistry 2003; 64: 655-660.
10. Çalis, I., Baŝaran, A. A., Saracoğlu, I., Stcher O. Iridoid and phenylpropanoid glycosides from *Stachys macrantha*, Phytochemistry 1991; 31: 167-169.
11. Çalis, I., Saracoğlu, I**.,** Baŝaran, A. A., Sticher, O. Two phenethyl alcohol glycosides from *Scutellaria orientalis* subsp. *Pinnatifida*, Phytochemistry 1993; 32: 1621-1623.
12. Çalis, I., Hosny, M., Khalifa, T., Rüedi, P. Phenylpropanoid glycosides from *Marrubium alysson*, Phytochemistry 1992; 31: 3624-3626.
13. Kisiel, W., Piozzi, F. Phenylethanoid glycosides from *Prostanthera melissifolia*, Phytochemistry 51:1083-1085.
14. Miyase, T., Yamamoto, R., Ueno, A. Phenylethanoid glycosides from *Stachys officinalis*, Phytochemistry 1996; 43: 475-479.
15. Borg, J., Kesslak, P. J., Cotman, C.W. Peripheral administration of long chain fatty alcohol promotes septal cholinergic neurons survival after fimbria-fornix transection, Brain Research. 1990; 518: 295-298.
16. Borg, J.,Toazara, J., Hietter, H., Neny, M., Schmitt, G., Luu, B. Neurotrophic effect of naturally occurring long chain fatty alcohol on cultured CNS neurons, FEBS Letters. 1987; 213: 406-410.
17. Azzous, M., Kennel, P. F., Warter, J. M., Poindron, P., Borg, J. Hexacosanol has been shown to possess neutrotrophic properties on cultured neurons and to attenuate the degeneration of cholinergic neurons after injury, Experimental Neurology. 1996;138:189-197.
18. Damge, C., Hillaire-Buys, D., Koenig, M., Gross, R., Hoeltzel, A., Chapal, J., Balboni, G., Borg, J., Ribes, G. Effect of n-hexacosanol on insulin secretion in the rat, European Journal of Pharmacology. 1995; 274:133-139.
19. Moosbrugger, L., Bischoff, P., Beck, J.P., Luu, B., Borg, J. Studies on the immunological effects of fatty alcohols- I. Effect of n-hexacosanol on murine macrophages in culture, International Journal of Immunopharmacology. 1992; 14: 293-302.
20. Schapoval, E. E. S., de Vargas, M. R. W., Chaves, C. G., Bridi, R., Zuanazzi, J. A., Henriques, A. T. Antiinflammatory and antinociceptive activities of extracts and isolated compounds from *Stachytarpheta cayennensis*, J. Ethnopharmacology 1998; 60: 53-59.
21. Murai, M., Tamayama, Y., Nishibe, S. Phenylethanoids in the herb of *Plantago lanceolata* and inhibitory effect on arachidonic acid-induced mouse ear edema, Planta Medica 1995;61: 479-480.