

CYTOTOXIC TERPENOIDS FROM *SATUREJA MACRANTHA* C. A. MEY.¹AHMAD REZA GOHARI, ²ABBAS HADJIAKHOONDI, ³SEYED ESMAEIL SADAT-EBRAHIMI, ¹SOODABEH SAEIDNIA, ³ABBAS SHAFIEE¹Medicinal Plant Research Center, ²Department of Pharmacognosy, Medicinal Plant Research Center and Pharmaceutical Sciences Research Center, ³Department of Medicinal Chemistry, Faculty of Pharmacy, Medicinal Plant Research Center and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT

From the aerial parts of *Satureja macrantha* C. A. Mey, one monoterpene (**1**), two triterpenoids (**2,3**) and one sesquiterpene (**4**) were isolated. Their structures were determined to be thymol (**1**), oleanolic acid (**2**), ursolic acid (**3**) and caryophyllene oxide (**4**), by using ¹H and ¹³C-NMR, FTIR and EIMS spectra. Brine shrimp cytotoxicity effects of the crude extracts and isolated compounds were examined. Among them compounds **1** (612 μM), **2** (17 μM) and **3** (29 μM) were effective against *Artemia salina* larva.

Keywords: *Satureja macrantha*, *Artemia salina*, Thymol, Caryophyllene oxide, Ursolic acid

INTRODUCTION

The genus *Satureja* (Labiatae), which is generally called Marzeh in Persian language, has 13 species in Iran. One of these species is *S. macrantha* that grows in north-west of Iran as well as east parts of Iraq (1). Previous phytochemical investigations on some species of this genus revealed the presence of phenolic acids (2, 3), flavones (4), anthocyanins (5), sterols (6), diterpenes (7, 8) and triterpenes (9). Ethanolic extract of *S. parvifolia* showed antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *E. Coli* (10). The essential oil of *S. hortensis* has been reported to be an antispasmodic agent (11). Anti-HIV activity from water extract of *S. obovata* (12) and antioxidant effect of the hexan extract of *S. thymbrā* have been reported (13). In addition, cytotoxic effects of the crude extracts of *S. odora* and *S. parvifolia* (14) have been shown. Thymol is one of the most important monoterpenes which has been identified in the volatile oil of *S. mutica* and *S. atropatana* (15). We have previously reported the presence of caryophyllene oxide in the essential oil of *S. mutica* (15). Literature survey shows that the chemical composition and biological activity of *S. macrantha* has not been investigated. In this report the isolation, structural elucidation and cytotoxic activity of terpenoid constituents of *S. macrantha* is described.

MATERIALS AND METHODS

General procedures

The FT-IR spectra were recorded on a Nicolet 550 instrument. ¹H and ¹³C-NMR spectra were

measured on a Varian FT-400 unity plus (400 MHz for ¹H and 100 MHz for ¹³C) spectrometer and also a JEOL JNM-LA500 spectrometer with tetramethyl silane as an internal standard. Chemical shifts are given in δ. EIMS spectra were recorded on a Finnigan MAT EI TSQ-70 eV. Silica gel 60 F₂₅₄ precoated plates (Merck) were used for TLC. Silica gel for column chromatography (Mesh 200) was purchased from Merck Company. The spots were detected by spraying anisaldehyde- H₂SO₄ reagent followed by heating.

Plant material

Aerial parts of *S. macrantha* C. A. Mey at flowering stage were collected from Uroomieh, in the north-west of Iran, during flowering stage in September 2001. Voucher specimen (78409 TARI) was deposited at the Herbarium of the Institute of Forests and Rangelands Researches. Plant specimen was identified by Dr. Vali-allah Mozaffarian from the same institute.

Extraction, isolation and structural elucidation

Dried aerial parts of *S. macrantha* (800 g) were cut into small pieces and successively extracted with diethyl ether, methanol and water at room temperature (for 72 hours) using percolation method. The solvent was evaporated by a rotary evaporator to give 35.5 g, 48.9 g and 54.4 g residues from diethyl ether, methanol and aqueous extracts respectively.

The residue of the diethyl ether extract (15 g) was subjected to silica gel column chromatography (CC) with Hexane: CHCl₃ (8:2, 0:1) and MeOH as eluent, to give eight fractions (A-H). Fraction F (795 mg) was subjected to Lobar (Si-60) CC with

Correspondence: A. Shafiee, Department of Medicinal Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran E-mail: ashafiee@ams.ac.ir

Hexane: EtOAc (19:1, 1:1 and 0:1) to obtain compound **1** (143 mg, $R_f = 0.5$ in Toluene:CHCl₃: EtOH, 4:4:1). Fraction G (1.376 g) was separated by silica gel CC with Hexane: EtOAc (7:3) to obtain four fractions (G1-G4). Fraction G3 was subjected to silica gel CC with Hexane: EtOAc (3:1) and then CHCl₃: EtOAc (19:1) to give compound **2** (64 mg, $R_f = 0.22$ in CHCl₃: Hexane, 4:1). Compound **3** (37 mg, $R_f = 0.2$ in CHCl₃: Hexane, 4:1) was obtained from fraction G4 after CC with Hexane: EtOAc (3:2). Three grams of diethyl ether extract was subjected to silica gel CC using CHCl₃: MeOH (95: 5) and then further purification was carried out by PTLC (Preparative Thin Layer Chromatography) on silica gel GF₂₅₄ using Hexane: CHCl₃: EtOH (1: 7: 1) as the mobile phase resulting in the isolation of compound **4** (18 mg, $R_f = 0.4$).

Thymol [**1**]. Colorless crystal. mix m.p. with an authentic sample: 50-51°C. IR ν_{max} (KBr) cm⁻¹ 3434, 2955, 2868, 1615, 1456, 1427, 1296, 1156, 938, 812, 740. EIMS m/z (rel. int.): 150(M⁺, 47), 135(100), 132(53), 115(24), 105(15), 91(38). ¹H-NMR (400 MHz, CDCl₃): 1.23 (3H, *s*, H-9), 1.25 (3H, *s*, H-10), 2.26(3H, *s*, H-7), 3.16 (1H, *m*, H-8), 4.70 (1H, *s*, OH-3), 6.56 (1H, *s*, H-2), 6.73 (1H, *d*, $J=7.6$ Hz, H-6), 7.08 (1H, *d*, $J=7.6$ Hz, H-5).

Oleanolic acid [**2**]. White amorphous powder. m.p.: 271-273° C. IR ν_{max} (KBr) cm⁻¹ 3442, 3022, 2930, 1711, 1610, 1465, 1368, 1214, 789. EIMS m/z (rel. int.): 456(M⁺, 5), 248(100), 207(16), 203(41), 191(7), 189(7), 133(8). ¹H-NMR (400 MHz, CDCl₃): 0.75, 0.77, 0.90, 0.91, 0.93, 0.98 (each 3H, *s*, CH₃ ×6), 1.13 (3H, *s*, H-27), 2.82 (1H, *dd*, $J=3.6, 13.2$ Hz, H-18), 3.23 (1H, *dd*, $J=11.2, 4.4$ Hz, H-3), 5.27 (1H, *t*, $J=3.5$ Hz, H-12).

Ursolic acid [**3**]. White amorphous powder. m.p.: 279-281° C. IR ν_{max} (KBr) cm⁻¹ 3437, 3022, 2924, 1711, 1455, 1363, 1214, 743. EIMS m/z (rel. int.): 456(M⁺, 7), 248(100), 207(15), 203(52), 191(8), 189(12), 133(20). ¹H-NMR (500 MHz, pyridine-*d*₅): 0.88 (3H, *s*, H-25), 0.95 (3H, *d*, $J=6.4$ Hz, H-30), 1.00 (3H, *d*, $J=6.4$ Hz, H-29), 1.02 (3H, *s*, H-24), 1.05 (3H, *s*, H-26), 1.22 (3H, *s*, H-27), 1.24 (3H, *s*, H-23), 2.12 (1H, *dt*, $J=12.3, 4.2$ Hz, H-16a), 2.33 (1H, *dt*, $J=12.2, 4.2$ Hz, H-16b), 2.63 (1H, *d*, $J=11.3$ Hz, H-18), 3.45 (1H, *dd*, $J=10.1, 5.8$ Hz, H-3), 5.48 (1H, *br s*, H-12).

Caryophyllene oxide [**4**]. White amorphous powder, m.p.: 60-61° C. IR ν_{max} (KBr) cm⁻¹ 3058, 2925, 2863, 1624, 1460, 1383, 1260, 1070, 866. EIMS m/z (rel. int.): 220 (M⁺, 3), 187(5), 138(12), 121(28), 109(37), 91(75), 79(100), 69(49). ¹H-NMR (400 MHz, CDCl₃): 0.99 (3H, *s*, H-12), 1.01 (3H, *s*, H-13), 1.20 (3H, *s*, H-14), 1.76 (2H, *m*, H-3), 2.10 (2H, *m*, H-6), 2.25 (1H, *m*, H-7a), 2.34

(1H, *m*, H-7b), 2.61 (1H, *m*, H-9), 2.87 (1H, *dd*, $J=10.4, 4$ Hz, H-5), 4.86 (1H, *brs*, H-15a), 4.97 (1H, *brs*, H-15b).

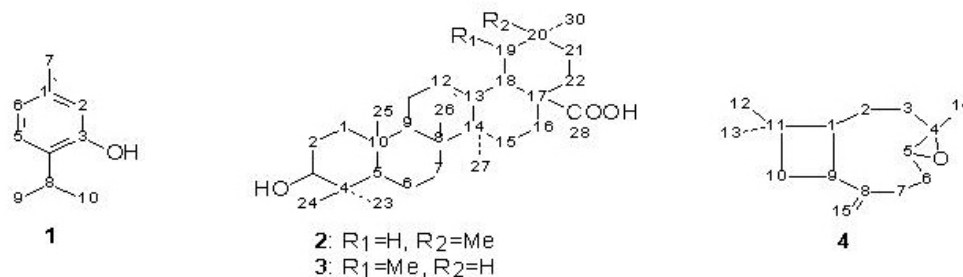
Brine Shrimp Lethality Assay (BSA)

The cytotoxicity of the compounds were determined by adaptation of a previously described method (14). Water life brand brine shrimp (*Artemia salina*) eggs were purchased from the Shilat Center (Tehran). The eggs were hatched in a flask containing 300ml artificial seawater made from distilled water. The flask was well aerated with the aid of an air pump, and kept in a water bath at 29-30 °C and illuminated by a bright light. The nauplii hatched within 48h. The extracts and pure compounds were dissolved in normal saline. Different concentrations were obtained by serial dilution. Solution of each concentration (500 µl) was transferred into clean 24 wells plates via a pipette, and to them was added aerated seawater having 10-20 nauplii (500 µl). A check count was performed, and the number of alives were noted after 24h. The mortality end point of the bioassay was determined as the absence of controlled forward motion during 30 sec of observation. The controls used were seawater and a well-known cytotoxic alkaloid, berberine hydrochloride (LC₅₀= 26 µM). Lethality percentage was determined and LC₅₀ calculated based on Probit Analysis with 95% of confidence interval.

RESULTS AND DISCUSSION

Dried aerial parts of *S. macrantha* were successively extracted with diethyl ether, methanol and water. These extracts were concentrated and examined with BSA. Minimum lethal concentrations of MeOH and aqueous extracts were more than 1000 µg/ml, therefore they were inactive. Diethyl ether extract showed cytotoxic activity against *Artemia salina* (LC₅₀= 186 µg/ml). This extract was used for further isolation on silica gel column chromatography, Lobar and PTLC to obtain compounds **1** (143 mg), **2** (64 mg), **3** (37 mg) and **4** (18 mg).

Compound **1** showed to be thymol based on its spectral data, m.p. and mix m.p. in comparison with an authentic sample. Compound **2** gave a positive Liebermann-Burchardt test and its mass spectrum showed a molecular ion at m/z 456 corresponding to C₃₀H₄₈O₃. Fragment ion at m/z 248 which had a formula C₁₆H₂₄O₂ is formed by a retro-Diels-Alder fragmentation between C-9 and C-11, and between C-8 and C-14. Such cleavage is characteristic of pentacyclic triterpenoids having a double bond at C-12 like a olea-12-ene skeleton (16). Fragment ions at m/z 438 [M-H₂O]⁺, 410 [M-HCOOH]⁺, 203 [248-COOH]⁺ were also observed in the mass spectrum (16).

Figure 1. Isolated compounds from *S. macrantha*Table 1. ¹³C-NMR of terpenoids isolated from *Satureja macrantha*.

No.	1 ^a	2 ^a	3 ^b	4 ^a	No.	1 ^a	2 ^a	3 ^b	4 ^a
1	136.6	38.4	39.4	48.7	16		22.9	24.9	
2	116.0	27.2	28.1	29.7	17		46.6	48.0	
3	152.5	79.1	78.1	30.2	18		41.0	53.6	
4	131.3	38.8	39.1	59.8	19		45.9	39.5	
5	126.2	55.2	55.8	63.7	20		30.7	39.4	
6	121.6	18.3	18.8	39.1	21		33.8	31.1	
7	20.8	32.6	33.6	39.7	22		32.4	37.5	
8	26.7	39.3	40.0	151.8	23		28.1	28.8	
9	22.6	47.6	48.0	50.7	24		15.6	16.6	
10	22.6	37.1	37.3	27.2	25		15.3	15.7	
11		23.4	23.6	34.0	26		17.0	17.5	
12		122.6	125.7	17.0	27		25.9	23.9	
13		143.6	139.3	21.6	28		183.8	179.9	
14		41.6	45.5	29.9	29		33.1	17.5	
15		27.7	28.7	112.7	30		23.6	21.4	

a) In CDCl₃; b) in pyridine-*d*₅.

Table 2. Attached proton test of compound 2 and 4.

Compound	-CH ₃	-CH ₂	-CH	-C	-C=O
2	15.30 (C-25)	18.29 (C-6)	40.95 (C-18)	30.65 (C-20)	183.79 (C-28)
	15.54 (C-24)	22.88 (C-16)	47.61 (C-9)	37.04 (C-10)	
	15.98 (C-26)	23.38 (C-11)	55.18 (C-5)	38.72 (C-4)	
	23.55 (C-30)	27.11 (C-2)	79.10 (C-3)	39.26 (C-8)	
	25.92 (C-27)	27.65 (C-15)	122.62 (C-12)	41.58 (C-14)	
	28.07 (C-23)	32.40 (C-22)		46.54 (C-17)	
	33.04 (C-29)	32.60 (C-7)		143.56 (C-13)	
		33.77 (C-21)			
		38.39 (C-1)			
		45.84 (C-19)			
4	16.95 (C-12)	27.16 (C-10)	48.68 (C-1)	33.98 (C-11)	-
	21.58 (C-13)	29.73 (C-2)	50.68 (C-9)	59.77 (C-4)	
	29.85 (C-14)	30.15 (C-3)	63.70 (C-5)	151.78 (C-8)	
		39.10 (C-6)			
		39.70 (C-7)			
	112.73 (C-15)				

The $^1\text{H-NMR}$ spectrum of compound **2** showed seven tertiary methyl groups at δ 0.75, 0.77, 0.90, 0.91, 0.93, 0.98 and 1.13 on an oleanane skeleton. A doublet doublet of one proton at δ 2.82 and a triplet of one vinyl proton at δ 5.27 were assigned to H-18 and H-12, respectively, suggesting an olea-12-ene skeleton. One methine proton at δ 3.23 (*dd*, $J=$ 11.2 and 4.4 Hz) showed that compound **2** has at least one hydroxyl group. In $^{13}\text{C-NMR}$ spectrum, the signal corresponding to the carboxyl C-28 appeared at δ 183.8 (see Table 1). The spectral data were similar to those reported for oleanolic acid (17, 18).

The mass spectrum of **3** showed a molecular ion at m/z 456 corresponding to a molecular formula $\text{C}_{30}\text{H}_{48}\text{O}_3$. The fragmentation of **3** is similar to **2**. The $^1\text{H-NMR}$ spectrum of compound **3** displayed five tertiary methyl groups at δ 0.88 (H-25), 1.02 (H-24), 1.05 (H-26), 1.22 (H-27), 1.24 (H-23) and two secondary methyl group at δ 0.95 (H-30) and 1.00 (H-29) on an ursane skeleton. A doublet of one proton at δ 2.63 and a triplet of one proton at δ 5.48 were assigned to H-18 and H-12, respectively, suggesting an urs-12-ene skeleton. One methine proton at δ 3.45 (*dd*, $J=$ 10.1 and 5.8 Hz) demonstrated that **3** has one hydroxyl group. In $^{13}\text{C-NMR}$ spectrum, the signal corresponding to the carboxyl C-28 appeared at δ 179.9 (see Table 1) which was consistent with the structure of ursolic acid. The spectral data were also similar to those reported for ursolic acid (17, 18).

The mass spectrum of **4** showed a molecular ion at m/z 220 corresponding to a molecular formula of

$\text{C}_{15}\text{H}_{24}\text{O}$ which was consistent with the structure of caryophyllene oxide. The $^1\text{H-NMR}$ spectrum of compound **4** showed three methyl groups at δ 0.99 (H-12), 1.01 (H-13), 1.20 (H-14). Two olefinic protons (H-15) showed two broad singlet signals in δ 4.86 and 4.97. A doublet doublet signal of one proton was shifted downfield at δ 2.87 (H-5) because of the presence of epoxi group. Table 2 shows Attached Proton Test (APT) of compound **2** and **4** to confirm the type of each carbon signals (CH_3 -, CH_2 -, CH -, C -, $-\text{CO}$). These results are in good agreements with identified structures.

Oleanolic acid and Ursolic acid are the main triterpenoid components that previously have been reported from *Dracocephalum kotschyi* and *D. subcapitatum* belonging to Labiatae family (17, 19). Thymol and caryophyllene oxide have been found in the essential oil of some other species of *Satureja* (15).

The results obtained from BSA showed that only compounds **1** ($\text{LC}_{50}=$ 612 μM), **2** (17 μM) and **3** (29 μM) were effective against *Artemia salina*. Among them compound **2** (oleanolic acid) is more effective than positive control (berberine hydrochloride).

ACKNOWLEDGEMENT

This work was supported by grants from Research council of Tehran University of Medical Sciences and Iran TWAS Chapter Based at ISMO. The authors wish to thank Dr. V. Mozaffarian and Mr. I. Mehregan for their help in collection and identification of the plant material.

REFERENCES

1. Rechinger KH. Flora Iranica, Labiatae. Hedge IC (ed). Akademische Druck Verlagsantalt, Graz, Austria, 1986; 150: 495-504.
2. Zgorka G, Glowinski K. Variation of free phenolic acids in medicinal plants belonging to the lamiaceae family. J Pharm Biomed Anal 2001; 26: 79-87.
3. Pedersen JA. Distribution and taxonomic implications of some phenolic in the family lamiaceae determined by ESR spectroscopy. Biochem Syst Ecol 2000; 28: 229-253.
4. Ferreres F, Barberan FA, Tomas F. Free flavone aglycones and flavonoid glycosides from *Satureja obovata*. Int J Crude Drug Res 1987; 25: 246-250.
5. Saito N, Harborne JB. Correlations between anthocyanin type, pollinator and flower colour in the labiatae. Phytochemistry 1992; 31: 3009-3015.
6. Escudero J, Lopez JC, Rabanal RM, Valverde S. Secondary metabolites from *Satureja* species. New triterpenoid from *Satureja acinos*. J Nat Prod 1985; 48: 128-131.
7. Labbe C, Castillo M, Fainia F, Coll J, Connolly JD. Rearranged isopimarenes and other diterpenoids from *Satureja gilliesii*. Phytochemistry 1994; 36: 735-738.
8. Alvarenga SA, Gastmans JP, Rodrigues GV. A computer-assisted approach for chemotaxonomic studies of diterpenes in Lamiaceae. Phytochemistry 2001; 56: 583-595.
9. Connolly JD, Hill RA. Dictionary of Terpenoids. London: Chapman and Hall; 1991
10. Feresin GE, Tapia AA, Bustos DA. Antibacterial activity of some medicinal plants from San Juan Argentina. Fitoterapia 2000; 71: 429-432.
11. Hajhashemi V, Sadraei H, Ghannadi AR, Mohseni M. Antispasmodic and anti-diarrheal effect of *Satureja hortensis* L. essential oil. J Ethnopharmacol 2000; 71: 187-192.

12. Bedoya LM, Sanchez S, Abad MJ, Bermejo P, Alcami J. Anti-HIV activity of medicinal plant extracts. *J Ethnopharmacol* 2001; 77: 113-116.
13. Madsen HL, Sorensen B, Skibsted LH, Bertelsen G. The antioxidative activity of summer savory (*Satureja hortensis* L.) and rosmary (*Rosmarinus officinalis* L.) in dressing stored exposed to light or in darkness. *Food Chemistry* 1998; 63: 173-180.
14. Mongelli E, Martino V, Coussio J, Ciccio G. Screening of Argentine medicinal plants using the Brine Shrimp Microwell Cytotoxicity assay. *Int J Pharmacognosy* 1996; 34: 249-254.
15. Gohari AR, Hadijakhoondi A, Shafiee A, Sadat-Ebrahimi SE, Mozaffarian V. Chemical composition of the essential oils of *Satureja atropatana* and *Satureja mutica* growing widely in Iran. *J Essent Oil Res* 2005; 17: 17-18.
16. Lahlou HE, Hirai N, Tsuda M, Ohigashi H. Triterpene phytoalexins from nectarine fruits. *Phytochemistry* 1999; 52: 623-629.
17. Saeidnia S, Gohari AR, Uchiyama N, Ito M, Honda G, Kiuchi F. Two new monoterpene glycosides and trypanocidal terpenoids from *Dracocephalum kotschyi*. *Chem Pharm Bull* 2004; 52: 1249-1250.
18. Hung CY, Yen GC. Extraction and identification of antioxidative components of Hsian-tiao (*Mesona procumbens* Hemsl.). *Lebensm-Wiss U-Technol* 2001; 34: 306-311.
19. Saeidnia S, Gohari AR, Ito M, Kiuchi F, Honda G. Bioactive constituents from *Dracocephalum subcapitatum* (O. Kunntze) Lipsky. *Z Naturforsch* 2005; 60 c: 22-24.

Archive of SID