# CYTOTOXIC TERPENOIDS FROM SATUREJA MACRANTHA C. A. MEY.

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# 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 <sup>1</sup>H and <sup>13</sup>C-NMR, FTIR and EIMS spectra. Brine shrimp cytotoxicity effects of the crude extracts and isolated compounds were examined. Among them compounds 1 (612  $\mu$ M), 2 (17  $\mu$ M) and 3 (29  $\mu$ M) 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. thymbra 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 monoterpens 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. <sup>1</sup>H and <sup>13</sup>C-NMR spectra were

measured on a Varian FT-400 unity plus (400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C) spectrometer and also a JEOL JNM-LA500 spectrometer with tetramethyl silane as an internal standard. Chemical shifts are given in  $\delta$ . EIMS spectra were recorded on a Finnigan MAT EI TSQ-70 eV. Silica gel 60 F<sub>254</sub> precoated plates (Merck) were used for TLC. Silica gel for column chromatography (Mesh 200) was purchased from Merch Company. The spots were detected by spraying anisaldehyde- H<sub>2</sub>SO<sub>4</sub> 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:  $CHCl_3$  (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

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Hexane: EtOAc (19:1, 1:1 and 0:1) to obtain compound 1 (143 mg,  $R_f = 0.5$  in Toluene:CHCl<sub>3</sub>: 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 CHCl<sub>3</sub>: EtOAc (19:1) to give compound 2 (64 mg,  $R_f = 0.22$  in CHCl<sub>3</sub>: Hexane, 4:1). Compound **3** (37 mg,  $R_f = 0.2$  in CHCl<sub>3</sub>: 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 CHCl<sub>3</sub>: MeOH (95: 5) and then further purification was carried out by PTLC (Preparative Thin Layer Chromatography) on silica gel GF<sub>254</sub> using Hexane: CHCl<sub>3</sub>: 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  $v_{max}$  (KBr) cm<sup>-1</sup> 3434, 2955, 2868, 1615, 1456, 1427, 1296, 1156, 938, 812, 740. EIMS *m*/*z* (rel. int.): 150(M<sup>+</sup>, 47), 135(100), 132(53), 115(24), 105(15), 91(38). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): 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  $v_{max}$  (KBr) cm<sup>-1</sup> 3442, 3022, 2930, 1711, 1610, 1465, 1368, 1214, 789. EIMS *m*/*z* (rel. int.): 456(M<sup>+</sup>, 5), 248(100), 207(16), 203(41), 191(7), 189(7), 133(8). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): 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  $v_{max}$  (KBr) cm<sup>-1</sup> 3437, 3022, 2924, 1711, 1455, 1363, 1214, 743. EIMS *m/z* (rel. int.): 456(M<sup>+</sup>, 7), 248(100), 207(15), 203(52), 191(8), 189(12), 133(20). <sup>1</sup>H-NMR (500 MHz, pyridine*d*<sub>5</sub>): 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.:  $60-61^{\circ}$  C. IR  $v_{max}$  (KBr) cm<sup>-1</sup> 3058, 2925, 2863, 1624, 1460, 1383, 1260, 1070, 866. EIMS *m*/*z* (rel. int.): 220 (M<sup>+</sup>,3), 187(5), 138(12), 121(28), 109(37), 91(75), 79(100), 69(49). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): 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 (LC<sub>50</sub>= 26  $\mu$ M). 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  $\mu$ g/ml, therefore they were inactive. Diethyl ether extract showed cytotoxic activity against *Artemia salina* (LC<sub>50</sub>= 186  $\mu$ g/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  $C_{30}H_{48}O_3$ . Fragment ion at m/z 428 which had a formula  $C_{16}H_{24}O_2$  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-H<sub>2</sub>O]<sup>+</sup>, 410 [M-HCOOH]<sup>+</sup>, 203 [248-COOH]<sup>+</sup> were also observed in the mass spectrum (16).



Figure 1. Isolated compounds from S. macrantha

<b>Table 1.</b> C-INMIK of terpenolas isolated from Satureja macrantha.										
No.	1 <sup>a</sup>	$2^{a}$	3 <sup>b</sup>	<b>4</b> <sup>a</sup>		No.	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	<b>4</b> <sup>a</sup>
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	

**Table 1.** <sup>13</sup>C-NMR of terpenoids isolated from Satureja macrantha.

a) In CDCl<sub>3</sub>; b) in pyridine-*d*<sub>5</sub>.

Compound	-СНЗ	-CH2	-СН	-C	-C=O
2	15.30 (C-25) 15.54 (C-24) 15.98 (C-26) 23.55 (C-30) 25.92 (C-27) 28.07 (C-23) 33.04 (C-29)	18.29 (C-6) 22.88 (C-16) 23.38(C-11) 27.11(C-2) 27.65(C-15) 32.40 (C-22) 32.60 (C-7) 33.77 (C-21) 38.39 (C-1) 45.84 (C-19)	40.95 (C-18) 47.61 (C-9) 55.18 (C-5) 79.10 (C-3) 122.62 (C-12)	30.65 (C-20) 37.04 (C-10) 38.72 (C-4) 39.26(C-8) 41.58 (C-14) 46.54 (C-17) 143.56 (C-13)	183.79 (C-28)
4	16.95 (C-12) 21.58 (C-13) 29.85 (C-14)	27.16 (C-10) 29.73 (C-2) 30.15 (C-3) 39.10 (C-6) 39.70 (C-7) 112.73 (C-15)	48.68 (C-1) 50.68 (C-9) 63.70 (C-5)	33.98 (C-11) 59.77 (C-4) 151.78 (C-8)	_

The <sup>1</sup>H-NMR spectrum of compound **2** showed seven tertiary methyl groups at  $\delta$  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  $\delta$  2.82 and a triplet of one vinyl proton at  $\delta$  5.27 were assigned to H-18 and H-12, respectively, suggesting an olea-12-ene skeleton. One methine proton at  $\delta$  3.23 (*dd*, *J*= 11.2 and 4.4 Hz) showed that compound **2** has at least one hydroxyl group. In <sup>13</sup>C-NMR spectrum, the signal corresponding to the carboxyl C-28 appeared at  $\delta$  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  $C_{30}H_{48}O_3$ . The fragmentation of **3** is similar to **2**. The <sup>1</sup>H-NMR spectrum of compound **3** displayed five tertiary methyl groups at  $\delta$  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  $\delta$  0.95 (H-30) and 1.00 (H-29) on an ursane skeleton. A doublet of one proton at  $\delta$  2.63 and a triplet of one proton at  $\delta$ 5.48 were assigned to H-18 and H-12, respectively, suggesting an urs-12-ene skeleton. One methine proton at  $\delta$  3.45 (*dd*, *J*= 10.1 and 5.8 Hz) demonstrated that **3** has one hydroxyl group. In <sup>13</sup>C-NMR spectrum, the signal corresponding to the carboxyl C-28 appeared at  $\delta$  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

 $C_{15}H_{24}O$  which was consistent with the structure of caryophyllene oxide. The <sup>1</sup>H-NMR spectrum of compound **4** showed three methyl groups at  $\delta$  0.99 (H-12), 1.01 (H-13), 1.20 (H-14). Two olefinic protons (H-15) showed two broad singlet signals in  $\delta$  4.86 and 4.97. A doublet doublet signal of one proton was shifted downfield at  $\delta$  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<sub>3</sub>-,CH<sub>2-</sub>, CH,-C, -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** (LC<sub>50</sub>= 612  $\mu$ M), **2** (17  $\mu$ M) and **3** (29  $\mu$ M) were effective against *Artemia salina*. Among them compound **2** (oleanolic acid) is more effective than positive control (berberine hydrochloride).

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