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Di-, and Triterpenoids Isolation and LC-MS Analysis of *Salvia marashica* Extracts with Bioactivity Studies

Sibel Kıran Aydın [1], Abdülselam Ertaş [2], Mehmet Boğa [3], Ebru Erol [4], Gülbahar Ö. Alim Toraman [5], Tuba Kuşman Sayğı [6], Belkıs Halfon [7] and Gülaçtı Topçu [5*]

[1]*Health Sciences Institute, Bezmialem Vakif University, İstanbul, Türkiye*
[2]*Dept. of Pharmacognosy, Faculty of Pharmacy, Dicle University, Diyarbakır, Türkiye*
[3]*Dept. of Analytical Chemistry, Faculty of Pharmacy, Dicle University, Diyarbakır, Türkiye*
[4]*Dept. of Analytical Chemistry, Faculty of Pharmacy, Bezmialem Vakif University, İstanbul, Türkiye*
[5]*Dept. of Pharmacognosy, Faculty of Pharmacy, Bezmialem Vakif University, İstanbul, Türkiye*
[6]*Dept. of Pharmacognosy, Faculty of Pharmacy, Van Yüzüncü Yil University, Van, Türkiye*
[7]*Dept. of Chemistry, Faculty of Arts and Science, Bogazici University, İstanbul, Türkiye*

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Abstract: In this study, dichloromethane, acetone, and methanol extracts of the aerial parts of the *Salvia marashica* plant which is an endemic species to Anatolia, were investigated. The total phenolic amounts of these extracts were determined as pyrocatechol equivalent and total flavonoids as quercetin equivalent. Antioxidant activity was determined by four complementary methods including inhibition of lipid peroxidation (by *β*-carotene color expression), DPPH free radical scavenging activity, ABTS cation radical scavenging activity and CUPRAC methods. Anticholinesterase activity of the extracts was investigated by the Ellman method against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes. Viability and cytotoxic activity tests were carried out on the fibroblast L929 cells and cytotoxic A549 lung cancer cells, respectively. The triterpenoids and diterpenoids constitute the major secondary metabolites of the *S. marashica* acetone and methanol extracts isolated by chromatographic methods. Their structures were determined based on spectroscopic methods, namely NMR and mass analyses. Ten terpenoids were obtained from either acetone or methanol extracts of the *S. marashica*. Seven of them were triterpenoids, elucidated as lupeol, lupeol-3-acetate, lup-12, 20(29)-diene, lup-20(29)-ene, *α*-amyrin-tetracosanoate, oleanolic acid and ursolic acid besides a steroid *β*-sitosterol. Two abietane diterpenes, abieta-8,11,13-triene **(1)** and 18-acetoxymethylene-abieta-8,11,13-triene **(2)**, were obtained from the acetone extract which were isolated from a *Salvia* species for the first time in the present study. The methanol extract was found to be very rich in rosmarinic acid determined by LC-MS/MS analysis.

Keywords: *Salvia marashica*; terpenoids; flavonoids; NMR; LC-MS/MS; bioactivity.

1. Introduction

Salvia species belongs to Lamiaceae (Labiatae) family plants which are rich in terpenoids. Over 900-1000 *Salvia* species grown throughout the world while there are over 100 *Salvia* species in Turkey, 53 of them being endemic to Turkey [1-3]. Secondary metabolites of *Salvia* species consist of

* Corresponding author: E-Mail: gtopcu@bezmialem.edu.tr

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terpenoids and steroids, flavonoids and other phenolic compounds. *Salvia* species can exhibit anti-inflammatory [4], anticholinesterase [5], antiviral [6], hepatotoxic [7], cytotoxic-antitumor activities [8, 9] due to their constituents, particularly diterpenoids [5, 10] and triterpenoids [11] while their flavonoids showed antioxidant [12] and antimicrobial effects [13-15].

Salvia marashica A. İlçim, F. Celep & Doğan is an endemic species that grows in rocky mountain slopes of Kahramanmaraş in south-eastern part of Turkey at an altitude of 850-1700 m. This species was first collected by Dr. Ahmet İlçim from the rocky slopes of Ahır Mountain, where the species is rare and local, and then introduced to the science world in 2009 [16]. The epithet name of the species is derived from the city Kahramanmaraş where the type sample was collected.

This plant is a perennial suffruticose herb usually flowers in April and May having lilac color and pleasant fragrance. Stems are many, ascending to erect with a 30-70 cm tall. *S. marashica* resembles three other endemic species of Turkey, *S. rosifolia, S. huberi,* and *S. pisidica*. However, it differs by its larger, serrate, and densely pilose terminal leaf segment, multicellular and black-headed glandular pilose hairs. There is a detailed morphological comparison of *S. marashica* with those three endemic species [16] in the literature. *S. marashica* flowers in April, the other two species (*S. rosifolia* and S. *huberi*) grow in eastern and north-eastern Anatolia, respectively. They flower from June to August while *S. pisidica* grows in western Anatolia.

2. Materials and Methods

2.1. General Experimental Procedure

All chemicals were used in analytical purity and were supplied from Sigma Aldrich and Merck companies. 1D and 2D NMR spectral analyses were obtained on a Varian ID-6508 600 MHz instrument using TMS as an internal standard for chemical shifts. The LCQ-Deca Ion Trap Mass Spectrometer was used for mass analyses of the pure compounds. Rotary Evaporator (Buchi L-100), and Microplate Elisa Reader (Eon Biotek-960) were also used as other equipment. The acetone and methanol extracts were analyzed by LC-MS/MS using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument to detect polar compounds (flavonoids and phenolics). The liquid chromatography instrument was equipped with LC-30AD binary pumps, DGU-20A3R degasser, CTO-10ASvp column oven, and SIL-30AC autosampler. The chromatographic separation was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm×4.6 mm, 3µm) analytical column. The column temperature was fixed at 40ºC. The elution gradient consisted of mobile phase A (water, 5mM ammonium formate, and 0.1% formic acid) and mobile phase B (methanol, 5 mM ammonium formate, and 0.1% formic acid). The gradient program with the following proportions of solvent B was applied t (min), B%: (0, 40), (20, 90), (23.99, 90), (24, 40), (29, 40). The solvent flow rate was maintained at 0.5 mL/min, and the injection volume was settled as 4 µL.

2.2. Plant Material

The aerial parts of *S. marashica* was collected from South Anatolia (Kahramanmaraş, upper Ceyhan Valley, Ahır Mountain, Maksutlu village, at 1450 – 1600 m, in May 2014 by Dr. Serpil Demirci Kayıran and Dr. Tuba Kuşman Sayğı. It was identified by Dr. S. Demirci Kayıran, and a voucher specimen (ISTE No: 98045) is deposited in the Herbarium of Istanbul University.

2.3. Extraction and Isolation

The aerial parts of the collected plant were air-dried (2065 g). Powdered aerial parts of the plant were extracted with dichloromethane, acetone, and methanol, respectively. The dichloromethane extract is 19.0 g, acetone extract is 9.7, g and methanol extract (SMM: *S. marashica* methanol extract) is 13.05 g. Similarities of the contents of dichloromethane and acetone extracts were observed by TLC spots checking under UV lights at 254 and 366 nm, and then occurring spots colors detection, followed by spraying cerium (IV) sulfate reagent on the TLC plate, then dried in an oven at 105^0C for 5 minutes and the observation of the occurring spots colors. Due to the occurrence of the same spots

on the TLC plate, the dichloromethane and the acetone extracts were combined, and was named as SMA. The mixed extract (SMA=28.7 g) is named the acetone extract, which was fractionated on a silica gel column, and seven compounds were isolated from the acetone extract and then purified mainly by preparative thin-layer chromatographic technique. The column was eluted with petroleum ether, followed by a gradient of dichloromethane and acetone up to 100%, respectively, and then 5% gradient of methanol up to 100%. From the main column of the acetone extract, during the elution of petroleum ether (PE) and dichloromethane (DCM) (9:1), compound **1** was isolated and then purified and applied on Si-gel preparative TLC plates developing on a PE-DCM (85: 15) solvent system which afforded pure compound **1** (15.9 mg). From the fraction 29 of the acetone extract, compound **2** was obtained during elution of DCM-PE (6:4) as (89 mg). Compound **3** (Amyryltetracosanoate (Urs-12-en-tetracosanoate)) was obtained from the methanol extract during the elution of the DCM-PE (1:1) solvent system as a white powder (9,8 mg).

3-Acetyllupeol (Lup-20(29)-ene-3-acetate) **(4)** was also obtained from the main acetone extract by the elution of DCM-PE (6:4) from the fraction 27 (25.6 mg). The other two lupane triterpenoids, both without any substituents, having two double bonds; lup-12,20(29)-diene **(5)** (72 mg), and the other one having only a double bond between C-20 and C-29 which is called lup-20(29)-ene **(6)** (197.3 mg) were obtained from the acetone extract by DCM- PE (8:2) elution, and they were then purified in very small quantities by using Sephadex LH-20 column.

Lupeol **(7)** was obtained from both acetone and methanol extracts during the elution with 100 % chloroform (totally 600 mg) as white powder which was found to be the most abundant compound in the plant [17, 18]. From the methanol extract, oleanolic acid **(8)** (1.4 g) and ursolic acid **(9)** (1.6 g) were also isolated, as well as *β*-sitosterol **(10)** (38,2 mg). The yield % of each compound was calculated based on dried plant amount. According to the calculations, the yield % of the compounds are: **1** (0,00076%), **2** (0.0041%), **3** (0,00047%), **4** (0.0012%), **5** (0.0035%), **6** (0.0095%), **7** (1.4%), **8** (0.06779%), **9** (0,07748%), **10** (0.00184%).

2.4. Determination of Cnolic Content and Flavonoid contents

The total phenolic and flavonoid contents of the studied samples were calculated as equivalent to pyrocatechol and quercetin, respectively [19, 20].

2.5. Antioxidant Activity

β-Carotene-linoleic acid test system [21], ABTS cation radical [22], DPPH free radical [23], and CUPRAC (Cupper (II) ion reducing antioxidant capacity) [24] test methods were used to determine the antioxidant properties of the samples. In these four antioxidant test methods, BHT (Butylated Hydroxytoluene) was used as a reference compound.

2.5.1. β-Carotene/Linoleic Acid Bleaching Assay

β-Carotene (0.5 mg) in 1 mL of chloroform was added into linoleic acid (25 μL) and then tween 40 emulsifiers (200 mg) mixture. After evaporating chloroform, 100 mL of distilled water saturated with oxygen was added and shaken; 160 μL of this mixture was then transferred into different test tubes containing 40 μL of the sample solutions at different concentrations. The emulsion was added to each tube, the zero time absorbance of the values was read at 470 nm. The mixture was incubated for 2 h at 50 °C [21].

2.5.2. ABTS Cation Radical Decolorization Assay

ABTS (7.0 mMol) in H_2O was added to 2.45 mM potassium persulfate to produce $ABTS^{\bullet+}$ and the solution was stored in the dark at 25 ºC for 12 h. The prepared solution was diluted with ethanol to get an absorbance of 0.700 ± 0.025 at 734 nm. $ABTS^{\bullet+}$ solution (160 μL) was added to each sample solution at different concentrations. After 30 min, the percentage inhibition at 734 nm was read for

each concentration relative to a blank absorbance (methanol) [22]. The following equation was used to calculate the scavenging capability of $ABTS^{\bullet+}$:

$$\text{ABTS}^{\bullet+}\text{ scavenging effect (Inhibition \%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

2.5.3. DPPH Free Radical Scavenging Assay

DPPH (0.1 mM 160 μL) solution in methanol was added to 40 μL of sample solutions in methanol at different concentrations. After 30 minutes, the absorbance values were read at 517 nm. The DPPH free radical scavenging potential was calculated using the following equation:

$$\text{DPPH scavenging effect (Inhibition \%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

A_{Control} is the initial concentration of the $DPPH^{\bullet}$
A_{Sample} is the absorbance of the remaining concentration of $DPPH^{\bullet}$ in the presence of the extracts or positive controls [23].

2.5.4. Cupric Reducing Antioxidant Capacity (CUPRAC)

Samples were dissolved in distilled water to prepare their stock solution at 1000 μg/mL concentration. Aliquots of 61 mL of 1.0×10^{-2} M copper (II) chloride, 61 μL of NH_4OAc buffer (1 M, pH 7.0), and 61 μL of 7.5×10^{-3} M neocuproine solution were mixed, x µL sample solution (2.5, 6.25, 12.5, and 25 μL) and $(67 - x)$ μL distilled water were added to make the final volume 250 μL. The tubes were stopped, and after 1 h, the absorbance at 450 nm was measured against a reagent blank [24].

2.6. Anticholinesterase Activity

A spectrophotometric method developed by Ellman et al. (1961) was used to indicate the acetyl- and butyryl-cholinesterase inhibitory activities. Aliquots of 150 μL of 100 mM sodium phosphate buffer (pH 8.0), 10 μL of sample solution, and 20 μL BChE (or AChE) solution were stirred and incubated for 15 min at 25 ºC, then DTNB (10 μL) is added to the mixture. In the next step, by the addition of butyrylthiocholine iodide (or acetylthiocholine iodide) (10 μL), the reaction was started. At the end, the final concentration of the tested solutions was 200 μg/mL. BioTek Power Wave XS at 412 nm was used to monitor the hydrolysis of these substrates [25].

2.7. Cytotoxic Activity (MTT Assay)

MTT test was used to determine cytotoxicity. Cytotoxic activities were carried out to KÜBTAL (Kırıkkale University) with service procurement. This test is a sensitive method that uses tetrazolium salt of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to measure cell proliferation. If viability is calculated below 70%, it means the sample has a cytotoxic potential. L929 Fibroblast cells and A549 cytotoxic cells were inoculated into 96 well plates at 1.0 x 10 4 cells per well. Cells were left to incubate for 24 hours. Samples were prepared at 1mg/mL. It was sterilized under UV and passed through a 0.20 µm filter. 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5µg/mL concentrations determined from the extracts were applied to the cells and incubated for 24 hours. As a control, only the nutrient medium was applied to the cells. At the end of 24 hours, the media in the wells were discarded, and 100 µL of medium and 10 µL of MTT solution were added to each well. After 3.5 hours of incubation at 37°C, 150 µL of DMSO was added to the wells and incubated for 15

minutes. The absorbance values of the 96-well plate were read at 570 nm in an ELISA plate reader to determine cell viability [26].

2.8. Identification and Quantitation of Ohenolic Compounds by LC-MS/MS

A previously established and validated LC-MS/MS method was applied to determine the phenolic contents in the acetone and methanol extracts of *Salvia marashica*. The validation parameters of this study were linearity, recovery, repeatability, limits of the detection (LOD), and limits of the quantification (LOQ). Detailed procedures of uncertainty evaluation have been previously reported in the literature [27]. Validation parameters are given in Table 7.

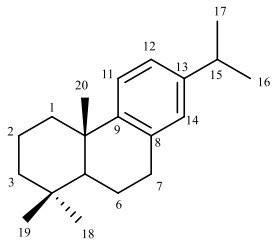
3. Results and Discussion

3.1. Structure Elucidation

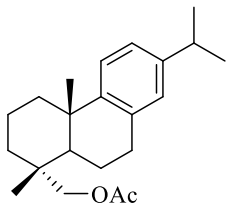
In the present study, totally of 10 terpenoids were obtained from both acetone and methanol extracts of the *S. marashica* plant. Four of them were lupane triterpenoids (lupeol, 3-acetyl-lupeol, lup-12,20(29)-diene, lup-20(29)-ene) isolated from the acetone extract. The six compounds including two diterpenoids **1** and **2** and the other three triterpenoids; oleanolic acid, ursolic acid, and α-amyrin-tetracosanoate, and a steroid *β*-sitosterol were obtained from the methanol extract of *S. marashica* [28-30].

From *Salvia pomifera*, a series of new abietane diterpenoids; pomiferins A-G, and two known abietane diterpenoids; ferruginyl-12-methyl ether and 18-hydroxy-8,11,13-trien-7-one, have been isolated [31, 32], some of them have similar structures to the compounds **1** and **2**, but none of them was exactly showed the same structure [33]. In fact, compound **1** has been previously obtained by the acetylation of pomiferin A, isolated from *S. pomifera* [31]. The ^{1}H NMR and ^{13}C NMR data of compound **1** were not given in detail before. Therefore, its spectral data were now given based on 1D and 2 D NMR studies [Table 1], and HRMS. The main ion peak was observed as $[M+1]^{+}$ at *m/z* 271.24097, corresponding to a molecular formula $C_{20}H_{31}$ which was verified structure of compound **1** as abieta-8,11,13-triene. Compound **2** was previously isolated from *Nepeta teydea* [34] which also belongs to the Lamiaceae family, its ^{13}C NMR data were also presented in Table 1.

Among lupane triterpenoids, lup-12,20(29)-diene, lup-20(29)-ene were isolated together. They could not be separated from each other on the Silica-gel column, and then by using Sephadex LH-20 on a small column, they were isolated from each other in a small quantity (2 and 3 mg, respectively). Based on our previous studies, it should be noted that lupane triterpenoids have been found in some *Salvia* species which are distributed in eastern and south-eastern Anatolia, rather than in western Anatolia, such as *S. macrochlamys* [17], *S. kronenburgii* [35, 36], *S. trichoclada* [18, 37]. All the compounds have been previously isolated from some *Salvia* species, except diterpenes **(1)** and **(2)**.



Abieta-8,11,13-triene **(1)**

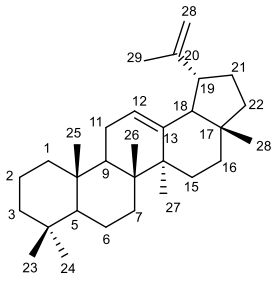


18-Acetoxymethylene-abieta-8,11,13-triene **(2)**

Figure 1. Structures of two abietane diterpenoids isolated from *S. marashica*

Table 1. NMR data for compounds **1** and **2** (500 MHz in $CDCl_3$, δ in ppm, and *J* in Hz

Position	Compound 1		Compound 2	
	^{1}H	^{13}C	^{1}H	^{13}C
1α 1β	1.41 m 2.34 dt (1.5 and 12.7)	38.86	1.55 m 2.28 dt (1.6 and 12.8)	38.30
2α 2β	1.73 m 1.87 m	19.12	1.74 m 1.89 m	18.90
3α 3β	1.19 m 1.44 m	41.74	1.21 m 1.41 m	36.50
4	-	30.51	-	31.70
5	1.35 bd (11.1)	50.43	1.34 br d (11.8)	44.20
6α 6β	1.60 m 1.77 m	19.34	1.83 m 1.88 m	18.80
7α 7β	2.82 m 2.92 dd (4.2 and 10.5)	30.51	2.83 m 2.85 dd (6.3 and 10.8)	30.10
8	-	134.95	-	134.30
9	-	147.63	-	146.90
10	-	37.53	-	37.23
11	7.18 d (8.2)	124.28	7.17 d (8.3)	124.10
12	6.98 dd (1.2 and 8.2)	123.78	6.99 dd (1.75 and 8.3)	123.70
13	-	145.40	-	145.65
14	6.88 br s	126.81	6.89 br s	126.85
15	2.84 sept (6.4)	33.34	2.82 sept (7.0)	33.40
16	1.24 d (6.4)	24.01	1.22 d (7.0)	23.80
17	1.23 d (6.4)	23.99	1.22 d (7.0)	23.80
18	0,94 s	33.44	3.68 (11.0) 3.97 (10.8)	72.30
19	0,92 s	21.63	0.93 s	17.60
20	1.22 s	24.90	1.21 s	25.20
$OCO\underline{CH_3}$	-	-	2.02 s	21.50
$O\underline{C}OCH_3$	-	-	-	171.00



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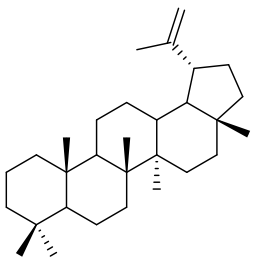

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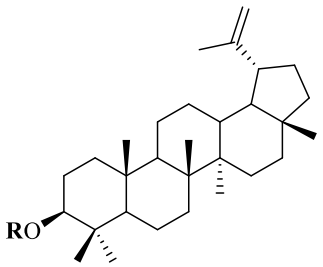
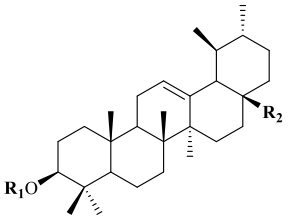


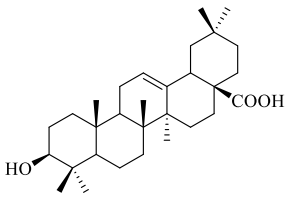
R= H **7**

R= Ac **4**



R_1= H, R_2= COOH **9**

R_1= $CO(CH_2)_{22}CH_3$, R_2= CH_3 **3**



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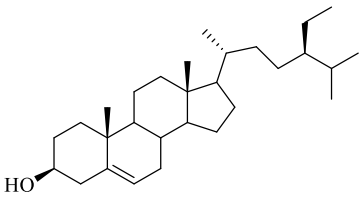

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Figure 2. Structures of the triterpenoids and a steroid isolated from *S. marashica*

3.2. Determination of Total Phenolic and Total Flavonoid Contents

The total phenolic and total flavonoid contents of the two extracts were determined as equivalent to pyrocatechol and quercetin, respectively. The methanol (SMM) extract of the plant was fairly rich in total phenolic content compared to the acetone (SMA) extract. However, both extracts exhibited to have less amount flavonoids about 25-30 µg QEs/mg extract (Table 2).

Table 2. Results of total phenolic and total flavonoid contents of the *S. marashica* acetone (SMA) and the methanol (SMM) extracts

Extracts	Total phenolic content (µg PEs/mg extract)[b]	Total flavonoid content (µg QEs/mg extract)[c]
SMA	46.19±2.14	25.13±1.15
SMM	172.38±3.37	30.46±0.18

[b]Phenolic content equivalent to pyrocatechol (y=0.021x+0.0396 R^2 = 0.9993)
[c]Flavonoid content equivalent to quercetin (y=0.1543x-0.0497 R^2 = 0.9931)

3.3. Antioxidant Activity

3.3.1. β-Carotene/Linoleic Acid Bleaching Assay, DPPH Free Radical Scavenging Assay, ABTS Cation Radical Decolorization Assay

Table 3. Results of antioxidant activity (inhibition %) of the two extracts and standard BHT

Extracts	β-Carotene-Linoleic acid	DPPH free radical	ABTS cation radical
SMA	69.98±1.70	208.95±3.65	93.72±1.39
SMM	67.83±1.15	33.59±0.72	15.04±0.08
BHT	11.30±0.03	58.86±0.50	22.29±0.67

Values are given as the mean and standard deviation of three parallel measurements.

3.3.2. Cupric Reducing Antioxidant Capacity (CUPRAC)

The CUPRAC antioxidant determination method was studied at four different concentrations (10, 25, 50, 100 µg/mL) (Table 4). In this method, the methanol extract was found to be more active than the acetone extract. However, it was determined that the methanol extract showed lower activity than the standard BHT.

Table 4. CUPRAC test assay of the two extracts and standard BHT

Extracts	10 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
SMA	0.157±0.003	0.318±0.020	0.496±0.020	0.745±0.050
SMM	0.362±0.060	0.751±0.020	1.377±0.050	2.378±0.140
BHT	0.491±0.065	1.117±0.098	1.887±0.045	3.337±0.184

Values are given as mean and standard deviation of three parallel measurements.

3.3.3 Anticholinesterase Activity

According to the anticholinesterase activity results (Table 5), the extracts were tested at a concentration of 200 µg/mL which were not shown acetylcholinesterase enzyme inhibition. However, in the butyrylcholinesterase enzyme inhibition test, the methanol extract exhibited better activity rather than acetone extract with 74.59 % inhibition which was close to the standard compound galantamine (76.52 %).

Table 5. Results of anticholinesterase activity of the two extracts

Extracts	AChE (Inhibition%)	BChE (Inhibition%)
SMA	N.A.	50.63±1.93
SMM	N.A.	74.59±3.27
Galantamine[b]	76.08±0,39	76.52±0.41

Values are given as mean and standard deviation of three parallel measurements.
b: Reference, N.A.: Not Active

3.3.4. Viability/Cytotoxic Activity

Viability/Cytotoxicity test results are given in Figures **3** and **4**. The cell viability on the fibroblast L929 cell lines of both extracts was found to be very similar and fairly high (90-80 %) at four concentrations (12.5, 25.0, 50.0, and 100 µg/mL).

In contrast, their cytotoxic activity was found to be fairly low on A549 lung cancer cell lines, and both extracts exhibited fairly close results to each other, given as µg/mL at the four concentrations. The two extracts showed almost the same cell viability on the fibroblasts L929 cells. However, the acetone extract showed better cytotoxic activity than that of methanol extract, but none of the results showed a dose-dependent relationship.

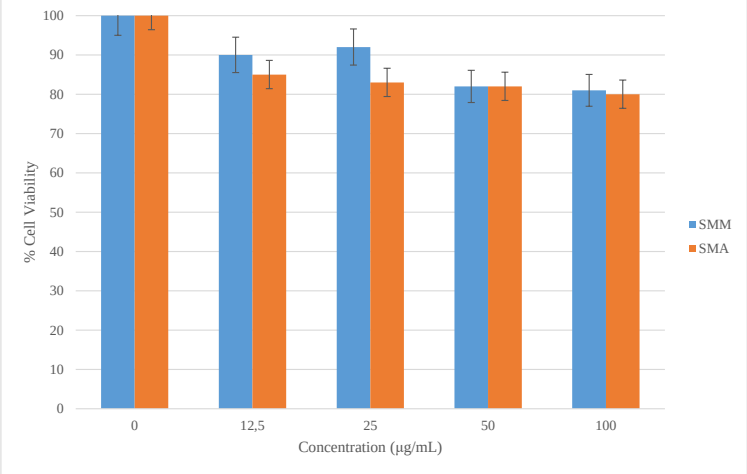

Figure 3. Cell Viability of the L929 cells after treatment with the extracts

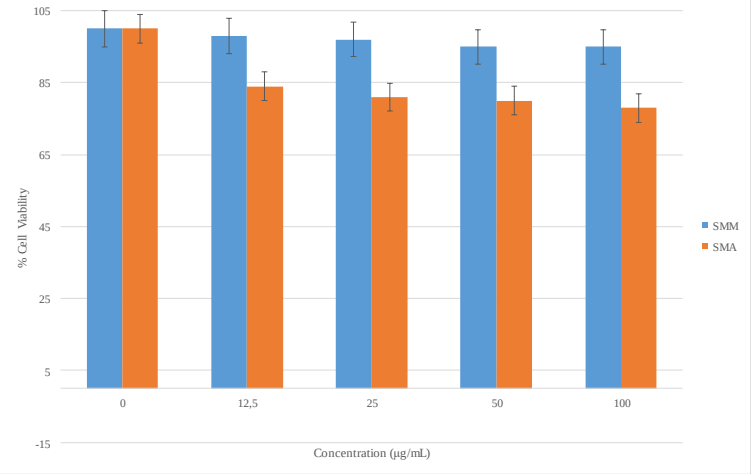

Figure 4. Cell Viability of the A549 cells after treatment with the extracts

3.3.5. LC-MS/MS

In fact, the methanol extract was found to have fairly rich in total phenolic amounts as (172,38 µg PEs/mg extract) compared to the acetone extract (Table 2). LC-MS/MS qualitative and quantitative analyses results allowed us detection of 27 compounds; out of 15 are flavonoids, the rest of them being phenolics, particularly phenolic acids. Rosmarinic acid was found to be the highest phenolic compound (33330,62±1534,89 µg analyte/g) in the LC-MS/MS analysis of the methanol extract, which has strong antioxidant properties [38, 39, 40]. It is followed by trans caffeic acid (752,65±35,39), tannic acid (346,55±12,83), quinic acid (333,45±12,31), and protocatechuic acid (234,62±8,98) as µg analyte/g in the extracts. Total flavonoids amount in both extracts was found to be very small compared to the phenolics. Even, some flavonoids amounts were could not be measured, they have detected as the only trace. Another point, the presence of a high amount of rosmarinic acid in the methanol extract, created a problem for the detection of the compounds in small quantities (Table 6).

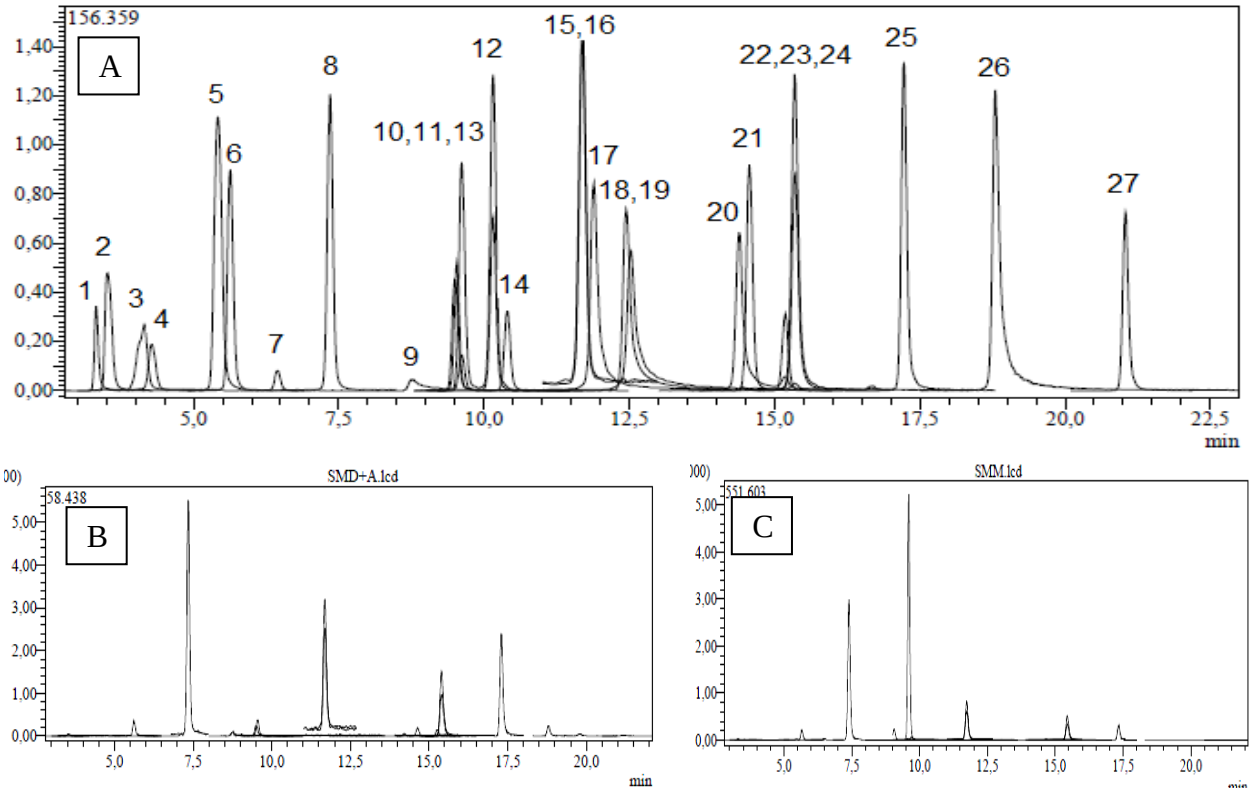

Figure 3. LC-MS/MS chromatograms of **A**: 250 ppb standard mix, **B**: *S. marashica* acetone extract, **C:** *S. marashica* methanol extract

1: Quinic acid, 2: Malic acid, 3: tr-Aconitic acid, 4: Gallic acid, 5: Chlorogenic acid, 6: Protocatechuic acid, 7: Tannic acid, 8: tr- caffeic acid, 9: Vanillin, 10: p-Coumaric acid, 11: Rosmarinic acid, 12: Rutin, 13: Hesperidin, 14: Hyperoside, 15: 4-OH Benzoic acid, 16: Salicylic acid, 17: Myricetin, 18: Fisetin,19: Coumarin, 20: Quercetin, 21: Naringenin, 22: Hesperetin, 23: Luteolin, 24: Kaempferol, 25: Apigenin, 26: Rhamnetin, 27: Chrysin.

Table 6. Secondary metabolites of SMA and SMM Extracts by LC-MS/MS

Compounds	Main Ion(m/z)[a]	MS^2(Collision Energy)[b]	Amount (µg analyte/g extract)[c]	
			SMA	SMM
Quinic acid	190.95	85 (22),93 (22)	9.45±0.36	333.45±12.31
Malic acid	133.05	115 (14),71 (17)	72.67±3.52	223.04±11.35
(E)-Aconitic acid	172.85	85 (12).129 (9)	<LOD	<LOD
Gallic acid	169.05	125 (14),79 (25)	1.31±0.02	6.06±0.03
Chlorogenic acid	353.0	191 (17)	<LOD	54.57±2.51
Protocatechuic acid	152.95	109 (16),108 (26)	40.29±2.01	234.62±8.98
Tannic acid	182.95	124 (22),78 (34)	<LOD	346.55±12.83
(E)-Caffeic acid	178.95	135 (15),134 (24),89 (31)	139.53±8.57	**752.65±35.39**
Vanilin	151.05	136 (17),92 (21)	100.76±4.98	11.98±0.45
p-Coumaric acid	162.95	119 (15),93 (31)	42.79±1.62	52.97±2.09
Rosmarinic acid	358.90	161 (17),133 (42)	288.07±13.76	**33330.62±1534.89**
Rutin	609.10	300 (37),271 (51),301(38)	<LOD	32.10±2.50
Hesperidin	611.10	303,465	5.97±0.09	284.28±11.23
Hyperoside	463.10	300,301	<LOD	51.74±2.46
4-Hydroxybenzoic acid	136.95	93,65	49.66±2.34	138.78±7.03
Sinapinic acid	136.95	93,65,75	48.96±2.29	125.72±5.56
Myricetin	317.00	179,151,137	<LOD	<LOD
Fisetin	284.95	135,121	<LOD	<LOD
Coumarin	146.95	103,91,77	<LOQ	<LOQ
Quercetin	300.90	179,151,121	<LOQ	<LOQ
Naringenin	270.95	151,119,107	<LOQ	<LOQ
Hesperetin	300.95	164,136,108	9.27±0.45	7.61±0.36
Luteolin	284.95	175,151,133	33.27±1.59	125.31±6.76
Kaempferol	284.95	217,133,151	35.07±1.63	135.55±7.21
Apigenin	268.95	151,117	37.91±1.87	54.78±2.59
Rhamnetin	314.95	165,121,300	<LOD	<LOD
Chrysin	253.00	143,119,107	<LOD	<LOD

As a result, both extracts were found to be rich in terpenic compounds, particularly triterpenoids. Among triterpenoids, mainly lupeol, ursolic acid, and oleanolic acid were found to be high amounts in the plant. Thus, *S. maraschica* should be considered as a rich source for these triterpenoids with their cytotoxic and anti-inflammatory properties. Most of those triterpenoids have been previously obtained from several *Salvia* species by our group and investigated for their antioxidant and anticholinesterase activities. Particularly ursolic acid and oleanolic acid showed high and selective anticholinesterase activity [18]. Furthermore, this endemic plant was also found to be fairly rich in rosmarinic acid which is a well-known phenolic compound with high antioxidant and anticholinesterase activities [18, 37, 40] as well as anti-inflammatory, neuroprotective, antiviral, antimutagenic, anti-epileptic, antinociceptive, antidepressant, and anti-anxiolytic properties [41].

Table 7. Analytical parameters of LC-MS/MS method

No	Analyte	RT[a]	Linear Regression Equation	r^2[b]	RSD %[c]	Linearity Range (mg/L)	LOD/LOQ[d] (µg/L)	Rec. (%)	U[e]
1	Quinic acid	3.36	y=33.6626x+25132.9	0.9927	0.0388	250-10000	22.3 / 74.5	103.3	4.8
2	Malic acid	3.60	y=93.6102x-5673.77	0.9975	0.1214	250-10000	19.2 / 64.1	101.4	5.3
3	*(E)*-Aconitic acid	4.13	y=79.2908x-28416.2	0.9933	0.3908	250-10000	15.6 / 51.9	102.8	4.9
4	Gallic acid	4.25	y=358.069x+26417.5	0.9901	0.4734	25-1000	4.8 / 15.9	102.3	5.1
5	Chlorogenic acid	5.29	y=48.9828x+26779.7	0.9932	0.1882	250-10000	7.3 / 24.3	99.7	4.9
6	Protocatechuic acid	5.51	y=36.8568x+6197.38	0.9991	0.5958	100-4000	25.8 / 85.9	100.2	5.1
7	Tannic acid	6.30	y=90.2704x+30233.2	0.9955	0.9075	100-4000	10.2 / 34.2	97.8	5.1
8	*(E)*-Caffeic acid	7.11	y=1585.16x+83957.5	0.9942	1.0080	25-1000	4.4 / 14.7	98.6	5.2
9	Vanillin	8.57	y=44.5478x-574.867	0.9995	0.4094	250-10000	10.1 / 33.7	99.2	4.9
10	*p*-Coumaric acid	9.17	y=73.5303x+27064.3	0.9909	1.1358	100-4000	15.2 / 50.8	98.4	5.1
11	Rosmarinic acid	9.19	y=18.0298x-1149.86	0.9992	0.5220	250-10000	10.4 / 34.8	101.7	4.9
12	Rutin	9.67	y=51.8835x+3841.66	0.9971	0.8146	250-10000	17.0 / 56.6	102.2	5.0
13	Hesperidin	9.69	y=195.773x+105641	0.9973	0.1363	250-10000	21.6 / 71.9	100.2	4.9
14	Hyperoside	9.96	y=0.978146x+827.221	0.9549	0.2135	100-4000	12.4 / 41.4	98.5	4.9
15	4-Hydroxy benzoic acid	11.38	y=635.003x+54284.6	0.9925	1.4013	25-1000	3.0 / 10.0	106.2	5.2
16	Salicylic acid	11.39	y=915.178x+72571.4	0.9904	0.6619	25-1000	4 / 13.3	106.2	5.0
17	Myricetin	11.42	y=54.2823x+5414.67	0.9991	2.8247	100-4000	9.9 / 32.9	106.0	5.9
18	Fisetin	12.10	y=331.870x+34409.0	0.9988	2.4262	100-4000	10.7 / 35.6	96.9	5.5
19	Coumarin	12.18	y=236.639x+34370.3	0.9924	0.4203	100-4000	9.1 / 30.4	104.4	4.9
20	Quercetin	13.93	y=206.102x+1693.14	0.9995	4.3149	25-1000	2.0 / 6.8	98.9	7.1
21	Naringenin	14.15	y=1100.55x+39055.7	0.9956	2.0200	25-1000	2.6 / 8.8	97.0	5.5
22	Hesperetin	14.80	y=160.323x+6545.07	0.9961	1.0164	25-1000	3.3/ 11.0	102.4	5.3
23	Luteolin	14.84	y=111.474x+3057.10	0.9992	3.9487	25-1000	5.8 / 19.4	105.4	6.9
24	Kaempferol	14.85	y=20.9677x+571.241	0.9917	0.5885	25-1000	2.0 / 6.6	99.1	5.2
25	Apigenin	16.73	y=543.793x+18525.6	0.9954	0.6782	25-1000	0.1 / 0.3	98.9	5.3
26	Rhamnetin	18.41	y=110.091x+632.444	0.9994	2.5678	25-1000	0.2 / 0.7	100.8	6.1
27	Chrysin	20.60	y=698.787x+23531.7	0.9965	1.5530	25-1000	0.05 / 0.17	102.2	5.3

[a]RT: Retention time, [b]r^2: coefficient of determination, [c]RSD: relative standard deviation, [d]LOD/LOQ (µg/L): Limit of detection/Limit of quantification, [e]U (%): Percent relative uncertainty at 95% confidence level (k=2).

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Supporting Information

Supporting information accompanies with this paper on http://www.acgpubs.org/journal/records-of-natural-products

ORCID

Sibel Kıran Aydın: 0000-0003-1810-5188
Abdülselam Ertaş: 0000-0002-2193-8386
Mehmet Boğa: 0000-0003-1810-5188
Ebru Erol: 0000-0001-6342-4298
Gülbahar Ö. Alim Toraman: 0000-0002-7816-7551
Tuba Kuşman Sayğı: 0000-0002-1771-1453
Belkıs Haflon: 0000-0001-5845-5081
Gülaçtı Topçu: 0000-0002-7946-6545

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