Antioxidant activity tests on novel triterpenoids from *Salvia macrochlamys*

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Dedicated to Prof. Dr. Atta-ur-Rahman to mark his 65th birthday and to acknowledge his hundreds of articles and many books in the field of phytochemistry

Abstract
The methanol extract of *Salvia macrochlamys* Boiss. and Kotschy was fractionated on a silica gel column to yield a group of terpenic compounds. After separation and cleaning, seven known and three new terpenoid compounds were isolated, and their structures were elucidated by spectroscopic methods, including intensive NMR and MS studies. The crude extract was tested in five different systems for antioxidant activity. The extract and monogynol A (**1**) and its three derivatives (**2-4**) were found to be highly active in a metal chelating test system on ferrous ions.

Keywords: *Salvia macrochlamys*, Labiatae, terpenoids, antioxidant activity

Introduction

Salvia species have been used in folk medicine since ancient times to cure tuberculosis,[1,2] cancer,[3] diabetes,[4] coronary heart diseases, angina pectoris and myocardial infarction.[5,6] Skin diseases such as psoriasis and eczama[7] could be treated by *Salvia* species and they also exhibit oestrogenic activities.[8] In Turkey there are about 90 *Salvia* species, half of them being endemic, and some species are consumed as tea, especially in rural areas.[9] Our group has studied over 50 *Salvia* species for their chemical contents and their biological activities.

From the whole plant, after extraction and chromatographic separation seven known and three new terpenoids were isolated. The known compounds were germanicol, germanicol acetate,[10] lupeol, lupeol acetate,[11] monogynol A,[12,13] ursolic acid[14] and caryophyllene oxide.[15]

The three new compounds were monogynol A (**1**) derivatives 3β-acetylmonogynol A (**2**), 3β-acetyl,22β-hydroxymonogynol A (**3**), 3β-acetyl,21β,22β-dihydroxymonogynol A (**4**) (Fig.1). Their structures were determined by using spectral data including intensive 1D and 2D NMR techniques (DEPT, COSY, HMQC, HMBC and NOESY).

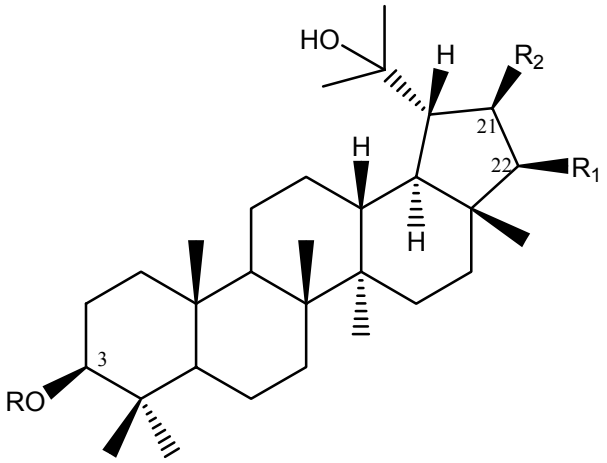
Results and Discussion

The structure of the first new compound was established as 3β-acetylmonogynol A (**2**) on the basis of spectral data. Although it was prepared from monogynol A previously,[16] this is the first time that it was isolated from nature.

The HRMS spectrum of compound **2** exhibited a molecular ion peak at *m/z* 486.4100 corresponding to the molecular formula $C_{32}H_{54}O_3$. This indicated the presence of six degrees of unsaturation, of which five were accounted for by a pentacylic ring system and one by an acetoxy group carbonyl. The ^{13}C NMR spectrum having 9 methyl, 10 methylene, 6 methine and 7 quaternary carbons correlated with the given structure for **2**. In the ^{1}H NMR spectrum of the compound **2,** eight methyl signals were observed at δ 0.83, 0.84, 0.85, 0.87, 1.00, 1.06, 1.24, 1.35 as singlets. The main difference between monogynol A (**1**) and compound **2** was in the chemical shift of H-3 which was observed more downfield resonating at δ 4.49 (1H, dd, *J*= 5.8 and 10.5 Hz) versus to δ 3.20 in lit.[16] The acetyl group was observed in ^{1}H NMR spectrum at δ 2.04 and δ 21.59 for methyl and δ 171.30 for carbonyl in the ^{13}C NMR spectrum. Its IR spectrum verified the presence of the acetyl group with the bands at 1725 and 1260 cm^{-1}. Finally monogynol A (**1**) obtained in this study was acetylated in the usual way and compared with compound **2** on a TLC plate, and both were found to be identical,[16] their ^{1}H NMR and IR data were also found to be exactly the same.

The structure of the second new compound was deduced as 3β-acetyl,22β-hydroxymonogynol A (**3**). The HRMS of compound **3** exhibited a molecular ion at *m/z* 502.4075 corresponding to the molecular formula $C_{32}H_{54}O_4$ with six double-bond equivalents, of which five were accounted for by a pentacyclic ring system skeleton and one by carbonyl of the acetoxy group. Spectral properties of ^{1}H and ^{13}C NMR were quite similar to those of compound **2**, with the exception of additional methine proton at δ 3.53 as a double doublet. Its location was ascertained through a HMBC experiment and ^{13}C NMR shift values of the methyl signals. In the APT and HSQC experiments no meaningful chemical shift difference was observed for the A, B, C and D ring protons and carbons compared to compound **2**; therefore, the second hydroxyl group should probably be at ring E, also C-18 (δ 45.68) and C-19 (δ 47.51) on ring E were resonated almost at the same frequencies with those of compound **2**. Both C-18 and C-19 atoms showed HMBC correlation (Fig. 2) with methyl protons at δ 0.82 which was assigned to Me-28. The most indicative information was obtained by the observation of a three-bond away correlations between protons of Me-28 and C-22 (δ 79.55), and protons of Me-28 and C-16 (δ 33.47) which unambiguosly indicated the location of the second hydroxyl group to be at C-22.

The HSQC and HMBC experiments also allowed us to determine the methyl groups at δ 0.94 and 1.07 must belong to Me-27 and Me-26, respectively, particularly by the observation of two- and three-bond away correlations between both methyl protons with C-8 (δ 41.60) and C-14 (δ 43.74) in the latter experiment. In addition, methyl protons at δ 0.85 and 0.87 showed three-bond away correlations with the carbon at δ 55.5 (C-5) which led to their assignments as C-23 and C-25 methyls, respectively. The stereochemistry of the hydroxyl group at C-22 was deduced as β, by studying on a Dreiding model, and from the *J* value of the peak at δ 3.53 (dd, *J*= 7.80 and 9.75 Hz) which was verified by a NOESY experiment following from NOESY correlations between H-22 and H-18. Thus, the structure of compound **3** was determined as 3β-acetyl,22β-hydroxymonogynol A based on mass and intensive NMR spectral data including ^{1}H, APT, HSQC, HMBC and NOESY experiments.



(**1**) Monogynol A R = H R_1 = H R_2 = H
(**2**) 3β-Acetylmonogynol A R = Ac R_1 = H R_2 = H
(**3**) 3β-Acetyl,22β-hydroxymonogynol A R = Ac R_1 = OH R_2 = H
(**4**) 3β-Acetyl,21β,22β-dihydroxymonogynol A R = Ac R_1 = OH R_2 = OH

Figure 1. Chemical formulae of the compounds **1**–**4**.

Table 1. ^{1}H and ^{13}C NMR data of compounds **2**, **3** and **4** (in $CDCl_3$, *J* values in parantheses as Hz)

C	^{1}H **(2)**	^{1}H (**3**)	^{1}H (**4**)	^{13}C **(2)**	^{13}C (**3**)	^{13}C (**4**)
1	0.97 m; 1.74m	0.99 m; 1.70m	1.00 m; 1.71 m	38.03	38.61	38.68
2	1.66 dddd (3, 4, 12, 13)	1.61 m	1.60 m; 1.90 m	23.91	23.91	23.89
3	4.49 dd (5.8 ,10.5)	4.48 dd (6.0 , 10.6)	4.48 dd (5.9 , 10.5)	81.15	81.17	81.17
4	-	-	-	38.27	38.01	38.20
5	0.80 brd	0.80 brd	0.78 brd	55.74	55.50	55.52
6			1.39 m; 1.52 m	18.40	18.43	18.36
7	1.40 m	1.41 m	1.28 m; 1.60 m	35.10	34.70	34.66
8	-	-	-	41.26	41.60	42.06
9	1.27 m	1.28 m	1.28 m	50.90	50.45	50.75
10	-	-	-	37.32	37.23	37.86
11			1.25 m; 1.51 m	21.41	21.56	21.87
12		1.84 m		26.76	27.36	27. 29
13		1.67 m	1.89 m	37.23	37.16	36.95
14	-	-	-	42.83	43.74	44.81
15		1.27 m	1.26 m; 1.38 m	29.61	29.56	29.22
16				33.96	33.47	33.69
17	-	-	-	42.21	46.63	41.69
18	1.43 m	1.42 m	1.59 dd (9.6, 11.5)	45.26	45.68	45.02
19	1.83 br dd (8.2, 10.5)	1.81 m	1.93 t (9.6)	45.26	47.51	46.22
20	-	-	-	71.40	73.17	74.79
21			4.20 dd (9.36, 9.60)	29.79	32.13	79.18
22		3.53 dd (7.8, 9.75)	3.53 d (9.36)	41.04	79.55	84.20
23	0.85	0.85	0.83	28.17	28.16	28.17
24	0.84	0.84	0.82	16.74	16.72	16.72
25	0.87	0.87	0.84	16.63	16.42	16.56
26	1.06	1.07	1.06	16.73	16.71	16.59
27	1.00	0.94	0.97	15.13	15.01	15.22
28	0.83	0.82	0.80	14.38	12.39	13.97
29	1.24	1.15	1.24	26.32	25.46	29.56
30	1.35	1.23	1.34	28.18	31.74	32.34
OAc	2.04	2.05	2.03	21.59 171.30	21.50 171.74	21.50 171.76

The third new compound was also a derivative of monogynol A and its structure was deduced as 3β-acetyl,21β,22β-dihydroxymonogynol A (**4**) on the basis of spectral data. The HRMS of **4** exhibited a molecular ion peak at *m/z* 518.3996 corresponding to the molecular formula $C_{32}H_{54}O_5$ which correlated with the ^{13}C NMR findings consisting of 9 methyl, 8 methylene, 8 methine and 7 quaternary carbons. The ^{1}H NMR spectrum of compound **4** showed similar skeleton profile to those of **2** and **3** with eight methyl signals at δ 0.80, 0.82, 0.83, 0.84, 0.97, 1.06, 1.24 and 1.34 as singlets. There were three methine signals which should be adjacent to oxygenated substituents, followed from ^{1}H and ^{13}C NMR spectra at δ 4.48 (1H, dd, *J*=5.9, 10.5 Hz), 4.20 (1H, dd, *J*=9.36, 9.60 Hz) and 3.53 (1H, d, *J*=9.36 Hz) and corresponding carbons at δ 81.17 (C-3), 79.18 (C-21) and 84.20 (C-22) respectively, determined by HSQC spectrum. Their locations were identified through COSY and HMBC experiments (Fig. 2). The COSY experiment allowed us to observe a COSY correlation between the signals at δ 3.53 and 4.20 indicating that they must be vicinal protons. However, there was no COSY relation between these signals and the signal at δ 4.48 which attached to C-3 proton. This signal gave a COSY correlation with the signal at δ 1.60 (m) indicating that the latter signal should belong to one of the C-2 protons. The signal at δ 1.60 further gave a COSY correlation with its geminal proton at δ 1.90 as well as the signal at δ 1.00 which is assigned to one of the C-1 protons (H-1α). Observation of direct correlations between C-1 signal at δ 38.68 and proton signals at δ 1.00 and 1.71 (H-1β) verified that both signals belong to C-1 protons. Since C-5, C-6 and C-7 are resonated at exactly the same ppm with those observed in monogynol A, and there was no interaction in the COSY experiment between the signal at δ 4.48 (H-3) and the methine protons at δ 3.53 and 4.20, the latter two protons should take place on other than A and B rings by attaching to the oxygenated carbons. On the other hand, these two protons, therefore their adjacent oxygenated substituents could not be located at ring C or D because of their multiplicities, the only possible place for them was found to be on ring E. The observation of COSY correlation between the signal at δ 4.20 dd (*J*= 9.36 and 9.60 Hz) and a triplet signal at δ 1.93 (H-19) is attributed to their vicinity, and therefore the location of the former signal at C-21 on ring E. A three-bond away correlation between C-19 (δ 46.22) with Me-29 and Me-30 (δ 1.24 and 1.34) were clearly observed, as well as correlation between C-18 with Me-28 (δ 0.80) by HMBC experiment (Fig. 2). In fact, the location of two secondary hydroxyl groups at C-21 and C-22 were also determined by HMBC experiments following two-, three- and four-bond away correlations. A two-bond away correlation was observed between H-19 with C-21, and a three-bond away correlation between proton signal of Me-28 (δ 0.80) with C-22 (δ 84.20), and four-bond away correlation Me-28 with C-21 (δ 79.18) which indicating the location of both secondary hydroxyl groups should be on ring E. Since their vicinity, both H-21 and H-22 showed a two-bond away HMBC correlation with each other carbons. Two-bond correlations were further observed between C-20 signal at δ 74.79 with two methyl singlets at δ 1.24 and 1.34. Assignments of the all methyl groups were made based on their HSQC and HMBC correlations (Fig.2). Three-bond away correlations were observed between C-3 (δ 81.17) with the methyl signals at δ 0.82 and 0.83, which allowed the assignments of the latter signals to Me-24 and Me-

23, respectively. The stereochemistry of the hydroxyl groups located at C-21 and C-22 were deduced from their *J* values and by studying on a Dreiding model as well as by NOESY experiments. Thus, both methine protons, attached to the secondary hydroxyl groups on ring E, showed a NOESY correlation with each other, as well as with H-18, while no correlation was observed between them with either H-19 or Me-28 protons.

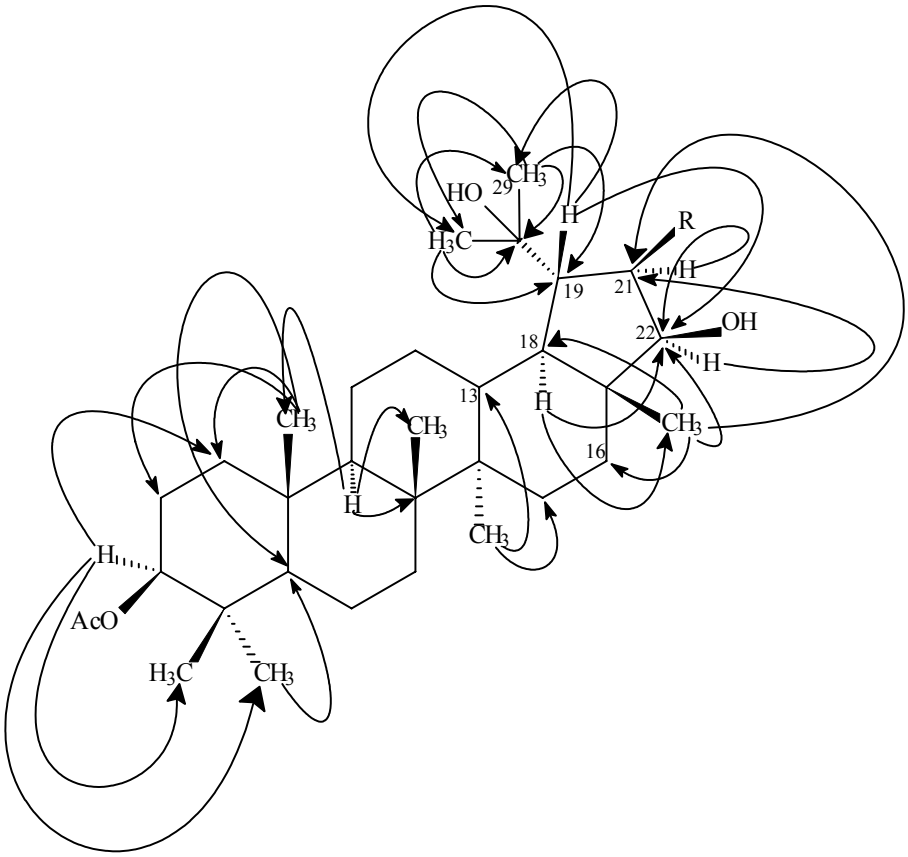


Figure 2. HMBC correlation of compounds **3** and **4**.

The mass fragmentation patterns of all three new compounds (**2-4**) followed by APCI (atomic pressure-chemical ionization) technique and they are shown in Figure 3 giving the main fragments (*a*), (*b*), (*c*) and (*d*) which are characteristic for lupane ring by the cleavage of ring C at the ring junctions.[17]

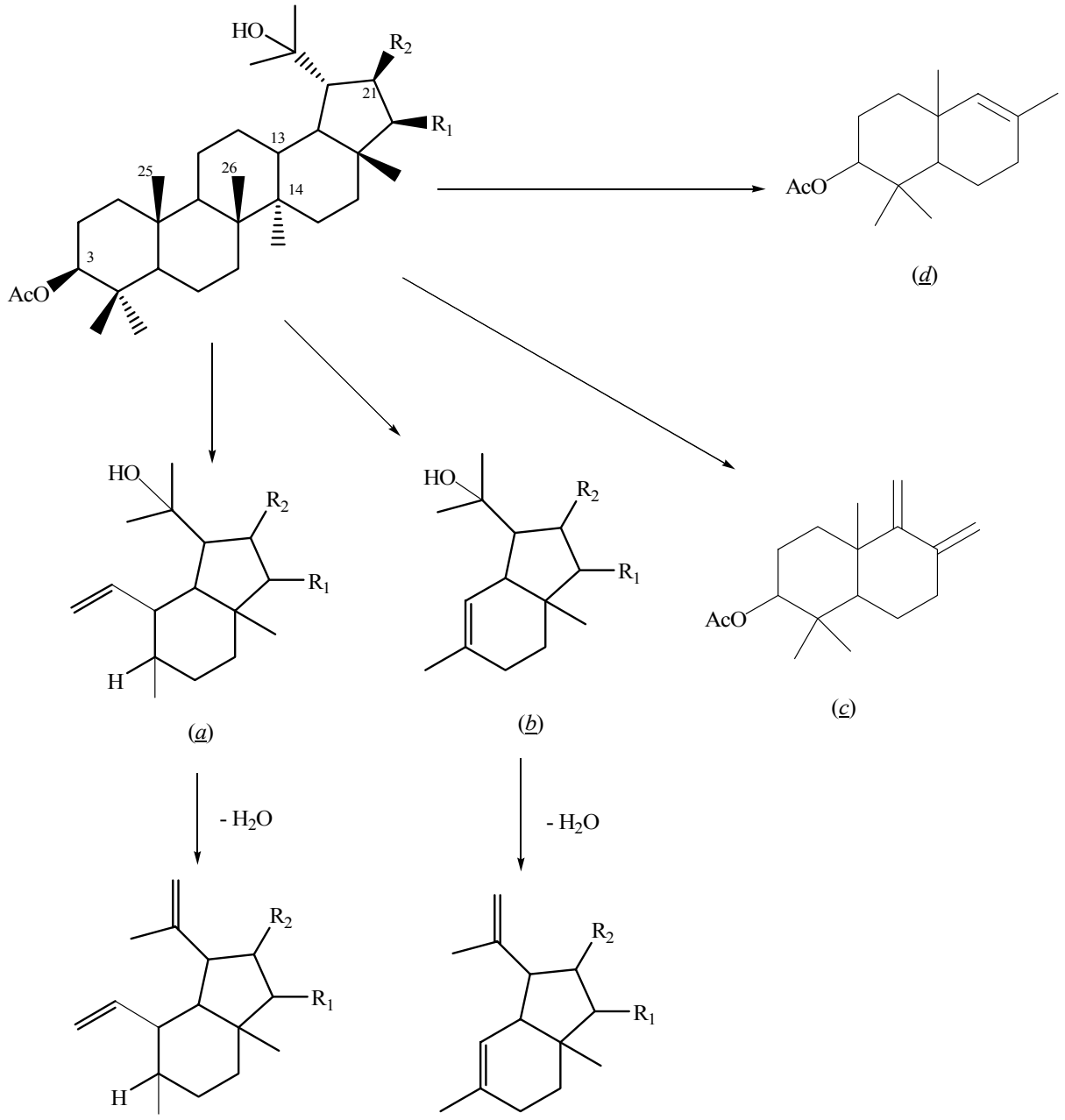


Figure 3. The mass fragment patterns of the three new compounds **2-4**.

The extract of *Salvia macrochlamys* was tested in five different methods for potential antioxidant activity consisting of free radical scavenging activity by DPPH (Table 2), antioxidant activity by β-caroten–linoleic acid (Fig. 4), superoxide anion radical scavenging activity (Fig.5) in PMS-NADH system, CUPRAC test for Cu (II) reducing capacity (Fig. 6), and metal chelating activity with ferrozine (Fig. 7). While the extract was not found to be active in the DPPH test system it showed moderate activity, in general (Table 2). Particularly, in inhibition of superoxide anion radical generation, the extract showed better activity than standard BHT and a similar

activity to that of ascorbic acid at 12.5-25 μg/mL concentrations, while in inhibition of lipid peroxidation it exhibited less activity, however, there was a linearity with increasing concentration, and inhibition was observed to be the same with that of BHT at 50 μg/mL concentration.

Table 2. DPPH radical scavenging activity (inhibition %) of *S. macrochlamys* extract and triterpenes (**1**-**4**)[a]

Sample	25 μg	50 μg	100 μg
Extract	5.33±0.55	12.67±0.26	19.81±0.76
1	0.24±0.05	0.61±0.01	1.06±0.00
2	0.36±0.07	0.93±0.03	1.50±0.00
3	0.88±0.00	1.50±0.03	2.59±0.03
4	0.41±0.00	0.88±0.04	1.37±0.03
α-Tocopherol[b]	92.66±0.07	93.28±0.07	93.40±0.99
BHT[b]	38.98±0.34	57.48±0.01	78.27±0.63

[a] Values expressed are means±S.D. of three parallel measurements. ($P<0.01$) [b] Reference compounds.

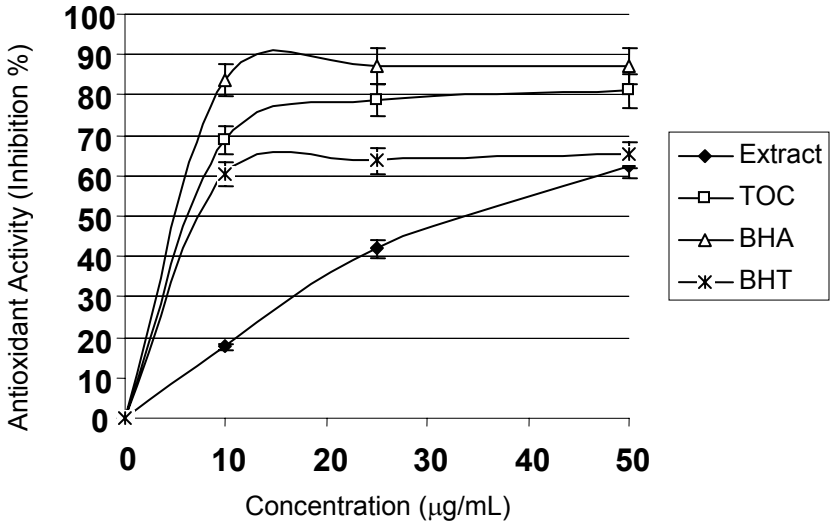


Figure 4. Inhibition (%) of lipid peroxidation of *S. macrochlamys* extract, BHT, BHA and TOC, by the β-carotene bleaching method (BHT: butylated hydroxytoluene; BHA: butylated hydroxyanisole; TOC: α-tocopherol).

It is noteworthy that the plant extract showed high activity in metal chelating system giving better results than those of tested standards TOC, BHT and quercetin (Fig. 7). Therefore,

isolated monogynol A (**1**) and its new derivatives (**2-4**) were investigated in the same test system for their metal chelating activity, and they were found not as active as the crude extract, however, they showed comparible activity results with those of the standards (Fig. 7).

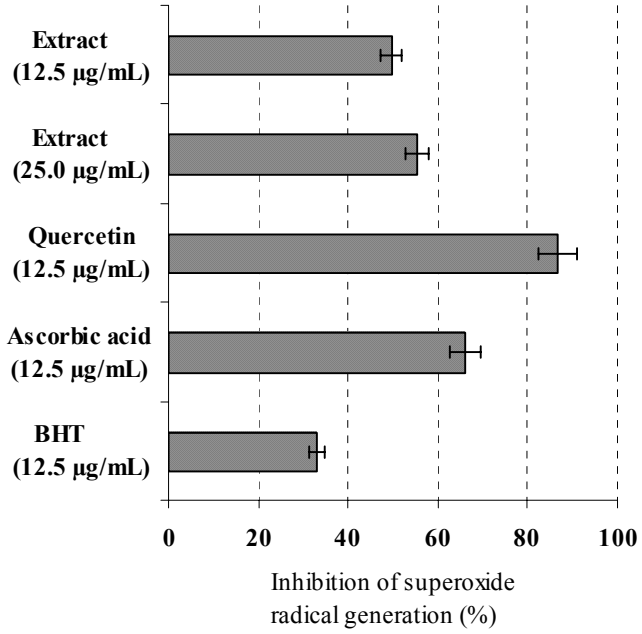


Figure 5. Superoxide anion radical scavenging activity of *S. macrochlamys* extract at 12.5 and 25.0 µg/mL concentrations.

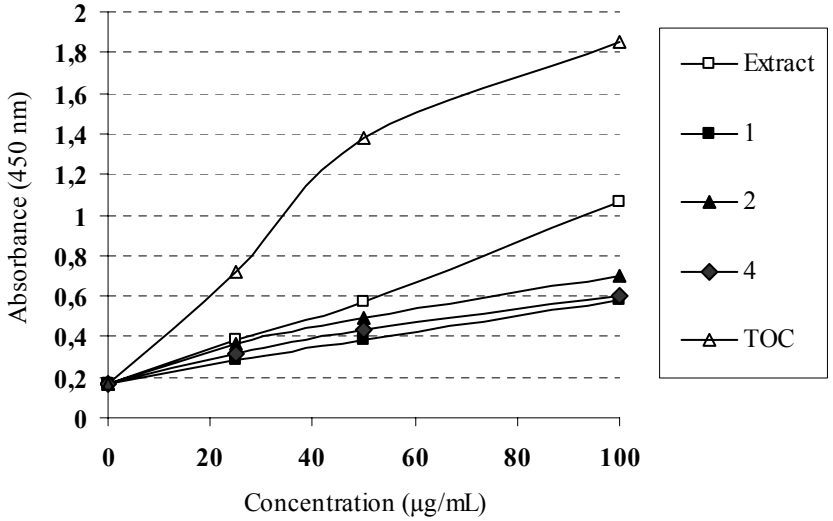


Figure 6. Cupric reducing antioxidant capacity (CUPRAC) of different concentrations of the extract and triterpenes (**1-2**, **4**) comparing with α-tocopherol using spectrophotometric detection of the Cu^{+2}-Cu^{+1} transformation.

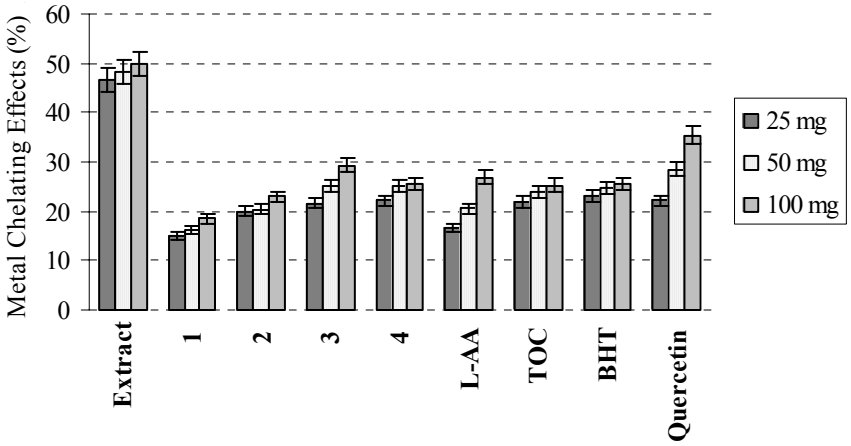


Figure 7. Metal chelating effect of different concentrations of the extract and triterpenes (**1**-**4**) on ferrous ions.

Experimental Section

General Procedures. IR spectra were recorded on Perkin-Elmer in $CHCl_3$; ^1H and ^{13}C NMR on a Varian Mercury-Vx 400 NMR spectrometer operating at 400 MHz for ^1H- and at 100 MHz for ^{13}C NMR in $CDCl_3$ (TMS as an internal standard) (at Boğaziçi University), HRMS were recorded on VG-ZabSpec (Micromass) and APCI on Thermo Deca XP-MAX instrument. All UV-vis measurements were recorded on a Shimadzu UV–1601 (Kyoto, Japan). Ferric chloride, chloroform, methanol, L-ascorbic acid (L-AA), quercetin and copper (II) chloride were obtained from E. Merck (Darmstadt, Germany). β-carotene, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40), 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and α-tocopherol (TOC), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonicacid)-1,2,4-triazine (Ferrozine), nicotinamide adenine dinucleotide (NADH), neocuproine and ammonium acetate were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). Nitrotetrazolium blue chloride (NBT) and N-methylphenazonium methyl sulphate (PMS) were obtained from Fluka Chemie (Fluka Chemie GmbH, Sigma-Aldrich, Sternheim, Germany). Kieselgel 60F 254 (E. Merck) TLC plates were used for preparative separation of the compounds, and silica gel (E. Merck) for column chromatography.
Plant Material. The whole plant *Salvia macrochlamys* Boiss. and Kotschy (Labiatae = Lamiaceae family) was collected from eastern Turkey, Bitlis (Alacabük Dağı) in an altitude 2100 m. The plant was collected and identified by Dr. Fevzi Özgökçe (Van, 100. Yıl University), a voucher specimen is deposited in the Herbarium of the Faculty of Sciences and Literature, Department of Botany, Van, F12939.

Extraction and Isolation. Dried and powdered plant material (275 g) was extracted with MeOH in a Soxhlet apparatus and dried under a vacuum (25.6 g), a crude residue was obtained. The residue was re-extracted with $CHCl_3$ and dried (15 g), which was fractioned in a silica gel column (2.5x100 cm) eluting with petroleum ether (40-60^o) followed by a gradient of CH_2Cl_2 up to 100 % a gradient of acetone and MeOH both up to 100 %. The fractions obtained from this column were controlled on TLC plates, and similar ones were combined. By preparative TLC plates the following compounds were isolated from Frac. 32-36 germanicol acetate (9 mg), lupeol acetate (11 mg), from Frac.37-39 lupeol (13 mg), germanicol (8 mg), caryophyllene oxide (12 mg), from Frac. 48-51 monogynol A (**1**) (4.5 mg), from Frac. 62-65 ursolic acid (23 mg), 3β-acetylmonogynol A (**2**) (13.5 mg) and 3β-acetyl,22β-hydroxymonogynol A (**3**) (15mg), and from Frac.76-77.4, 3β-acetyl,21β,22β-dihydroxymonogynol A (**4**) (20 mg).

Compound characterization

3β-Acetylmonogynol A (2). Amorphous compound, $[\alpha]_D$ +20.85 ($CHCl_3$; c 0.1), IR $\nu^{CHCl}{}_3$ $cm^{-1:}$ 3350, 2960, 2900, 2885, 1722, 1360, 1265, 1170, 1135, 1050, 925, 740. ^1H and ^{13}C NMR given in Table 1. APCI-MS: *m/z* 485.13 $[M-1]^+$ (27), 467.13 $[M-H_2O]^+$ (18), 425.07 $[M-HOAc-1]^+$ (100) (base peak), 407.27 (15), 248.93 $[(\underline{d})$ -H $]^+$ (17), 236.13 $(\underline{a})^+$ (4), 234.93 $[(\underline{a})$ - H$]^+$ (13), 219.07 $[(\underline{c})$- Ac-1$]^+$ (20), 217.07 $[(\underline{a})$ - H_2O-1$]^+$ (20), 191.07 $[(\underline{b})$ –OH$]^+$ (20) (See Figure 3). HRMS Calcd. $C_{32}H_{54}O_2$: 486.4075. Found: 486.4100.

3β-Acetyl,22β-hydroxymonogynol A (3). Amorphous compound, IR $\nu^{CHCl}{}_3$ $cm^{-1:}$ 3360, 2950, 2900, 2880, 1725, 1360, 1265, 1170, 1135, 1050, 925, 740. ^1H and ^{13}C NMR are given in Table 1. APCI-MS: *m/z* 502.10 $[M]^+$ (49), 483.14 $[M-H_2O-1]^+$ (20), 465.18 $[M-2xH_2O-1]^+$ (25), 441.16 $[M-HOAc-1]^+$ (100) (base peak), 427.39 $[M-HOAc-Me]^+$ (73), 423.33 $[M-HOAc-H_2O]^+$ (20), 367.38 $[M-HOAc-Me-C_3H_7O-1]^+$ (12), 251.22 $[(\underline{a})$ -1$]^+$ (8), 234.33 $[(\underline{a})$ -$H_2O]^+$ (8), 224.38 $[\underline{b}]^+$ (5), 220.15 $[(\underline{c})$ – Ac$]^+$ (2), 216.01 $[234-H_2O]^+$ (6), 206.13 $[(\underline{b})$ - $H_2O]^+$ (5), 191.15 $[(\underline{d})$ - Ac + H$]^+$ (13) (See Figure 3). HRMS: Calcd. $C_{32}H_{54}O_3$: 502.4022. Found: 502.4075.

3β-Acetyl,21β,22β-dihydroxymonogynol A (4). Amorphous compound, IR $\nu^{CHCl}{}_3$ $cm^{-1:}$ 3353, 2960, 2910, 2880, 1725, 1720, 1360, 1265, 1260,1170, 1130, 1050, 922, 740. ^1H and ^{13}C NMR are given in Table 1. APCI-MS: *m/z* 518.12 $[M]^+$ (27), 500.15 $[M-H_2O]^+$ (35), 482.45 $[M-2xH_2O]^+$ (17), 458.17 $[M-HOAc]^+$ (100)(base peak), 443.12 $[M-HOAc-Me]^+$ (65), 425.17 $[443.12-H_2O]^+$ (17), 268.25 $[\underline{a}]^+$ (21), 262.35 $[\underline{c}]^+$ (11), 251.22 $[(\underline{d})$ + H$]^+$ (7), 240.21 $[\underline{b}]^+$ (16), 233.39 (27), 222.39 $[(\underline{b})$ - $H_2O]^+$ (15), 208.28 $[(\underline{d})$ - Ac$]^+$ (17), 202.41 $[(\underline{c})$ - HOAc$]^+$ (See Figure 3). HRMS: Calcd. $C_{32}H_{54}O_4$: 518.3971. Found: 518.3996.

Acetylation of Monogynol A

5 mg of monogynol A (**1**) was dissolved in pyridine, then 1 mL $(CH_3COO)_2O$ was added and left at room temperature over night. Subsequently diluted with EtOH and evaporated under *vacuum* and compared with compound **2** on TLC.

Antioxidant Activity Tests

Determination of the antioxidant activity with the β-carotene bleaching method

The antioxidant activity of *S. macrochlamys* extract was evaluated by β-carotene-linoleic acid model system.[18,19] β-carotene (0.5 mg) in 1 mL of chloroform was added to 25 μL of linoleic

acid, and 200 mg of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water saturated with oxygen, were added by vigorous shaking. Four thousand microliters of this mixture were transferred into different test tubes containing different concentrations of the sample. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The emulsion system was incubated for 2 h at 50^0C. A blank, devoid of β-carotene, was prepared for background subtraction. BHT and α-tocopherol were used as standards (Fig. 4).

Free radical scavenging activity

The free radical scavenging activity of *S. macrochlamys* extract and triterpenes (**1**-**4**) was determined by the DPPH assay described by Blois.[20] In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.1 mM solution of DPPH in methanol was prepared, and 4 mL of this solution was added to 1 mL of sample solution in methanol at different concentrations. 30 minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity (Table 2). The capability to scavenge the DPPH radical was calculated using the following equation.[20,21]

$$\text{DPPH Scavenging Effect (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \text{ x } 100$$

Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of *S. macrochlamys* extract was based on the method described by Liu et al. with slight modification.[22] Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide radicals were generated in 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 μM) solution, 1 mL NADH (78 μM) solution and sample solutions. The reaction started by adding 1 mL of PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25^oC for 5 min, and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. Results were given as percentage inhibition [21] (Fig. 5).

Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity of the extract and triterpenes (**1**-**2**, **4**) was determined according to the method of Apak et al.[23] To a test tube, 1 mL each of 10 mM Cu (II), 7.5 mM neocuprine, and NH_4Ac buffer (1 M, pH 7.0) solutions were added. Extract and triterpenes (**1**-**2**, **4**) at different concentrations were added to the initial mixture so as to make the final volume 4.1 mL. The tubes were stoppered, and after 1 h, the absorbance at 450 nm was recorded against a reagent blank (Fig. 6).

Metal chelating activity

The chelating activity of *S. macrochlamys* extract and triterpenes (**1**-**4**) on Fe^{2+} was measured as reported by Decker, & Welch.[24] The extract was added to a solution of 2 mM $FeCl_2$ (0.1 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture reached

equilibrium, the absorbance was determined at 562 nm, results were given as percentage inhibition[21] (Fig. 7).

Statistical analysis

Experimental results concerning this study were mean ± S.D. of two parallel measurements. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by student's-*t* test, *p* values <0.05 were regarded as significant, *p* values <0.01 were regarded as very significant.

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