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Terpenoids, essential oil composition, fatty acid profile, and biological activities of Anatolian *Salvia fruticosa* Mill.

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Abstract: The hexane and dichloromethane extracts, obtained by re-extraction of the methanol extract of *Salvia fruticosa* Mill., afforded 7 diterpenoids (carnosol, carnosic acid, carnosic acid 12-methyl ether, rosmadial, isorosmanol, ferruginol, and manool), 4 triterpenoids (α-amyryltetracosanoate, oleanolic acid, ursolic acid, and erythrodiol), a steroid (3-acetylsitosterol), and a flavone (salvigenin). The galls (apples) of the collected plant were separately extracted with hexane to afford fatty acids composed mainly of oleic acid (29%), palmitoleic acid (29%), and stearic acid (23.20%), which exhibited high anticholinesterase activity, particularly against BChE. The essential oil, obtained from the aerial parts of the plant, exhibited high AChE inhibitory activity, consisting of mainly 1,8-cineol (58.89%). The antioxidant activity according to 6 complementary tests and anticholinesterase potential of the methanol extracts and triterpenoids α-amyryltetracosanoate, oleanolic acid, ursolic acid, and sitosterol acetate were also investigated, and methanol extract exhibited the highest antioxidant and anticholinesterase activity, surpassing the other tested extracts and pure compounds.

Key words: Lamiaceae, *Salvia fruticosa*, secondary metabolites, abietane diterpenoids, triterpenoids, essential oil, fatty oil, antioxidant, anticholinesterase

1. Introduction

The plant name *Salvia* (sage) comes from the Latin word salvare, which means healer.[1] *Salvia* species have been used for their beneficial healing properties and some species have been consumed as tea and food since ancient times. The genus *Salvia* L. (Lamiaceae) is one of the most diverse genera, with more than 900 species distributed all around the world. In Turkey, it is represented by 94 taxa, half of which are endemic. The aerial parts of *Salvia* species usually contain flavonoids and triterpenoids, while the roots are rich in diterpenoids. Both sesquiterpenoids and sesterterpenes are rather rare in *Salvia* species, but several sesquiterpenes have been isolated from Turkish, Iranian, and other Middle-East and Mediterranean countries,[2–6] and some sesterterpenes from Turkish and Iranian *Salvia* species.[2,5,6] Since antiquity, *Salvia* species have been used due to their diuretic, antipyretic, antiseptic, antibacterial, and wound-healing properties as well as in the treatment of some

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Dedicated to the memory of Prof Dr Ayhan Sıtkı Demir for his outstanding contributions in the field of asymmetric synthesis

menopausal problems and as a sedative and memory enhancer. They exhibited antituberculosis, cytotoxic, and liver protective activities and they have been used in the treatment of some heart diseases.[7–9] Cardiovascular properties of several *Salvia* extracts (*S. amplexicaulis*, *S. eriophora*, and *S. syriaca*) and their constituents have been investigated in vivo in Wistar Albino rats.[7,8,10] Their antioxidant and anticholinesterase activities have also been investigated over the last 10–15 years by various researchers.[11,12] In Chinese traditional medicine, particularly *S. milthiorrhiza* has been used for many purposes, such as in the treatment of some heart diseases, and as a liver protective and memory enhancer agent.[8,13] In European folk medicine, particularly *S. officinalis* and *S. lavandulaefolia* have been used to treat memory disorders, depression, and cerebral ischemia.[13] Our group has also recently published several publications on the antioxidant and anticholinesterase activity results of the extracts and pure isolates of *Salvia* species.[14–16]

All pharmacopeias accept that *S. officinalis* is a medicinal *Salvia* species. However, *S. fruticosa* Mill. (syn. *S. triloba* L.fil.) has also been accepted as a medicinal *Salvia* species by the European Pharmacopeia and British Pharmacopeia.

S. fruticosa Mill. (syn. *S. triloba* L.fil.) (Labiatae = Lamiaceae) is native to the Eastern and Western Mediterranean including Israel, Palestine, Turkey, Italy, the Canary Islands, and North Africa. There are 19 synonyms of *S. fruticosa* according to the Kew checklist.[17] The well-known names are *S. triloba*, *S. libanotica*, *S. lobryana*, and *S. cypria*. It has medicinal value and culinary use with its sweet nectar and pollen. The plant has been widely used as a Turkish folk medicine. Its fruits are known as "elma çalbası", "dağ elması", and "elma otu" in Turkey.[18] The essential oil, obtained from *S. fruticosa* leaves and branches without galls and flowers, is known as apple oil, and it contains about 55%–62% 1,8-cineol (eucalyptol).[19] Due to this high percentage of 1,8-cineol, *S. fruticosa* has been considered a highly valued medicinal *Salvia* species, even more valuable than *S. officinalis* in the Mediterranean region.

In the first study on *S. fruticosa* to obtain pure secondary metabolites from the air-dried leaves and stems of the plant, by Ulubelen et al.,[20] a new flavone salvigenin was isolated along with an abietane diterpene carnosol, and triterpenoids oleanolic acid, ursolic acid, and β-sitosterol. In a later study, carried out by Ulubelen, 6 abietane diterpenoids were isolated.[21] Among them, trilobinol and trilobinone were elucidated as new abietane diterpenoids, while ferruginol, horminone, 7α-acetylroyleanone, 6β-hydroxyroyleanone, virgatol, and cryptanol were known abietanes.

In the present study on *S. fruticosa*, we isolated 6 abietane diterpenoids, namely carnosol (**1**), carnosic acid (**2**), carnosic acid 12-methyl ether (**3**), rosmadial (**4**), isorosmanol (**5**), and ferruginol (**6**); a labdane diterpenoid, namely manool (**7**); 4 triterpenoids, namely α-amyryltetracosanoate (**8**), ursolic acid, oleanolic acid, and erythrodiol; and a steroid, namely 3-acetylsitosterol, as well as a flavonoid, namely salvigenin, from the hexane and dichloromethane extracts, which were obtained by re-extraction from the methanol extract of the whole plant (Figure 1). The essential oil of the dried aerial parts was also investigated by GC and GC-MS analyses and a monoterpene 1,8-cineol (eucalyptol) was found to be a major constituent of the oil. The monoterpenes α-pinene, β-pinene, β-myrcene, camphor, α-terpineol, and sesquiterpene *trans*-β-caryophyllene were the other main constituents of the oil. These results are in agreement with Koşar et al.,[22] who reported that 1,8-cineole is the dominant compound of *S. fruticosa* essential oil grown in Turkey and the essential oil of *S. fruticosa* grown in Greece[23–25] as well as the essential oil grown in Amman, Jordan.[26] In contrast, in some studies, α-thujone has been reported as the main constituent of the essential oil of *S. fruticosa*.[27,28]

Table 1. The essential oil composition of *S. fruticosa.*

Peak no.	Compounds	RI[a]	Content (%)	Identification method
1	$\alpha-$Pinene	931	5.62	MS, Co-GC, RI
2	Camphene	945	1.59	MS, Co-GC, RI
3	$\beta-$Pinene	975	5.22	MS, Co-GC, RI
4	$\beta-$Myrcene	989	5.24	MS, Co-GC, RI
5	$\alpha-$Terpinene	1012	0.57	MS, Co-GC, RI
6	$p-$Cymene	1018	1.27	MS, Co-GC, RI
7	1,8-Cineol	1023	58.89	MS, Co-GC, RI
8	*cis*-β-Ocimene	1031	0.55	MS, RI
9	γ-Terpinene	1050	0.93	MS, Co-GC, RI
10	Terpinolen	1081	0.01	MS, RI
11	α-Thujone	1098	0.95	MS, Co-GC, RI
12	p-Cymenene	1101	0.40	MS, RI
13	β-Thujone	1108	0.84	MS, RI
14	Camphor	1140	4.48	MS, Co-GC, RI
15	Isoborneol	1148	1.59	MS, Co-GC, RI
16	Terpinen-4-ol	1167	0.63	MS, Co-GC, RI
17	α-Terpineol	1178	3.02	MS, Co-GC, RI
18	*trans*-$\beta-$Caryophyllene	1410	4.17	MS, Co-GC, RI
19	*allo*-Aromadendrene	1428	0.65	MS, Co-GC, RI
20	α-Humulene	1438	1.37	MS, RI
21	Caryophyllene oxide	1570	0.01	MS, Co-GC, RI
22	Viridiflorol	1580	2.00	MS, RI
Total identified 100.00				
Monoterpene hydrocarbons 21.40				
Oxygenated monoterpenoids 70.40				
Sesquiterpene hydrocarbons 6.19				
Oxygenated sesquiterpenoids 2.01				

[a]Kovats index on DB-1 fused silica column; **Co-GC:** Co-injection with authentic compounds; **RI:** Retention Index literature comparison, **MS:** Mass spectrum

2. Results and discussion

The antioxidant potential of extracts of *S. fruticosa* was investigated by 6 complementary test assays including determination of total phenolic and flavonoid content, DPPH free radical scavenging, β-carotene bleaching, superoxide anion radical scavenging, ferric reducing power, and cupric reducing antioxidant capacity (CUPRAC).

The total phenolic and flavonoid contents were determined as pyrocatechol and quercetin equivalents, respectively. The crude methanol extract of *S. fruticosa* is fairly rich in phenolic compounds (80.96%), probably due to its phenolic abietane diterpenes, because it was not found to be rich in flavonoids (17.57%) or other phenolics.

At 100 μg/mL concentration, the crude methanol extract exhibited high DPPH free radical scavenging activity (93.46%) (Table 2) and high lipid peroxidation inhibitory activity (90.44%) in the β-carotene-linoleic acid assay (Table 3). The methanol extract also exhibited a higher metal chelating property (40.36%) than the standard compound quercetin (30.35%) at 100 μg/mL. The methanol extract exhibited very strong superoxide anion radical scavenging activity with $>$80% inhibition at the 4 concentrations tested (12.5, 25, 50, and 100 μg/mL). At 100 μM/mL concentration, it exhibited an inhibition of 96.13 $\pm$ 1.22%, surpassing the standards

quercetin (94.16 ± 1.42) and ascorbic acid (71.67 ± 2.13). The ferric reducing power of the methanol extract and CUPRAC were found to be moderate, when determined at 4 concentrations (5, 10, 25, and 50 μg/mL). The results at 50 μM/mL were 0.65 ± 0.01 (standard BHT = 1.22 ± 0.00) and 2.46 ± 0.01 (standard BHT = 3.91 ± 0.01), respectively.

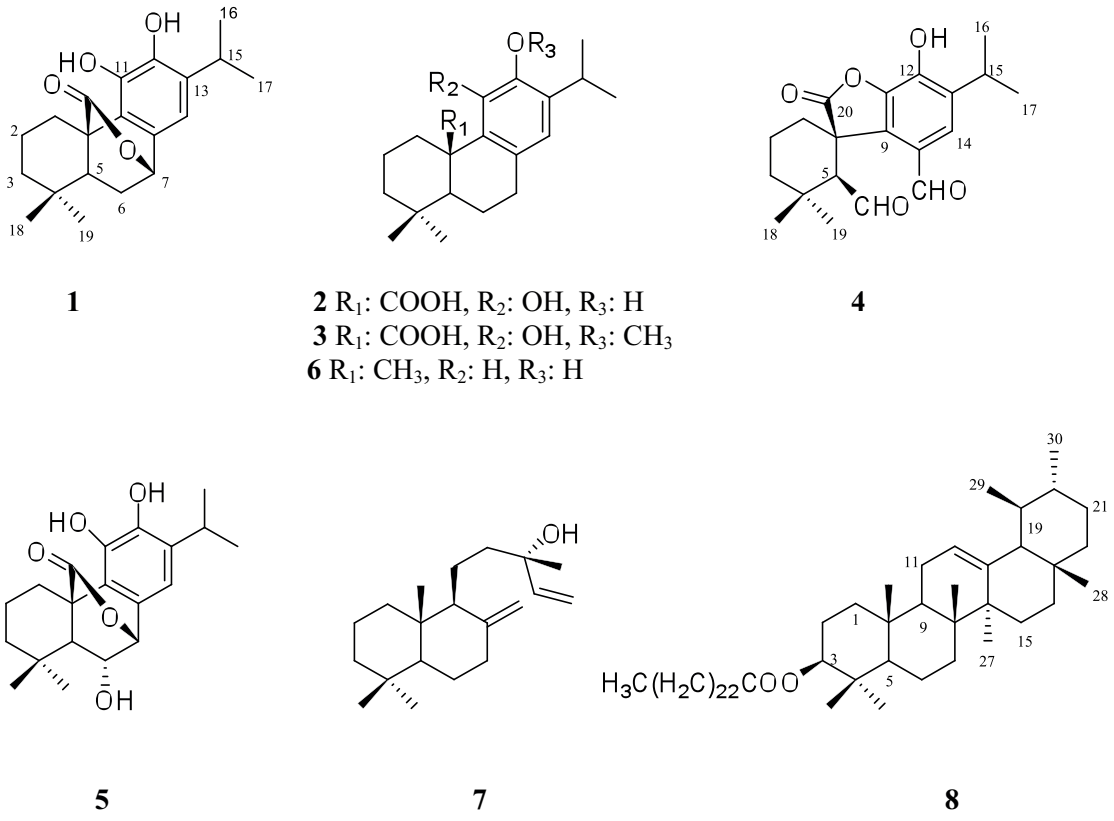

Figure 1. Structures of the compounds isolated from *S. fruticosa*.

Table 2. DPPH free radical scavenging activity of *S. fruticosa* crude methanol extract and triterpenoids.[a]

DPPH (Inhibition %)				
Samples	12.5 μM/mL	25 μM/mL	50 μM/mL	100 μM/mL
S. fruticosa MeOH ext.[¥]	20.30 ± 0.55	45.16 ± 0.66	84.26 ± 0.84	93.46 ± 0.39
α-Amyryltetracosanoate	2.86 ± 1.05	5.56 ± 0.56	6.06 ± 0.87	13.88 ± 0.26
Ursolic acid	–1.38 ± 1.21	–0.22 ± 0.46	–0.71 ± 0.34	0.13 ± 0.31
Oleanolic acid	0.19 ± 0.25	0.41 ± 0.31	3.02 ± 0.34	8.19 ± 0.53
α-Tocopherol*	61.20 ± 0.56	92.70 ± 0.07	95.23 ± 0.07	96.33 ± 0.99
BHT*	37.81 ± 0.68	55.38 ± 0.09	71.79 ± 0.04	92.31 ± 1.00
BHA*	9.89 ± 0.64	21.98 ± 0.34	38.25 ± 0.01	57.31 ± 0.63

[a]Values expressed are means ± SEM of 3 parallel measurements ($P < 0.05$)
*Standard
[¥]Values are given as μg

Table 3. Lipid peroxidation inhibitory activity of *S. fruticosa* crude methanol extract and triterpenoids.[a]

β-Carotene–linoleic acid (Inhibition %)				
Samples	12.5 μM/mL	25 μM/mL	50 μM/mL	100 μM/mL
S. fruticosa MeOH ext.¥	67.72 ± 0.53	71.17 ± 0.94	79.94 ± 0.86	90.44 ± 1.04
α-Amyryltetracosanoate	48.34 ± 0.89	50.55 ± 1.11	56.42 ± 1.08	57.48 ± 1.54
Ursolic acid	22.60 ± 0.65	26.01 ± 0.72	27.36 ± 0.91	31.14 ± 0.92
Oleanolic acid	34.61 ± 1.24	34.31 ± 1.36	34.23 ± 1.45	37.35 ± 1.23
α-Tocopherol*	91.23 ± 0.37	93.89 ± 0.45	95.37 ± 0.24	96.59 ± 0.01
BHT*	89.66 ± 0.39	93.44 ± 0.42	94.77 ± 0.24	95.91 ± 0.02
BHA*	93.34 ± 0.23	94.24 ± 0.02	96.33 ± 0.48	97.00 ± 0.01

[a]Values expressed are means ± SEM of 3 parallel measurements ($P < 0.05$)
*Standard
¥Values are given as μg

Since *S. officinalis* has been used as memory enhancer against cerebral ischemia, depression, and related memory disorders in Europe, in the present study, *S. fruticosa* extracts were further investigated for their anticholinesterase potential against both enzymes and exhibited high inhibition values against AChE (90.70 ± 1.00) and BChE (99.09 ± 3.00) at 100 μg/mL (Tables 4 and 5). The hexane extract of galls showed fairly strong inhibition, particularly against BChE (82.89 ± 2.29) at 100 μg/mL. In contrast, the essential oil exhibited better inhibition against AChE (73.52 ± 0.65) at 100 μg/mL.

Table 4. Acetylcholinesterase activity of the essential oil, crude extract, and apples of *S. fruticosa* and compounds.[a]

	AChE (Inhibition %)			
Sample	25 μM/mL	50 μM/mL	100 μM/mL	200 μM/mL
Essential oil¥	53.67 ± 0.59	67.73 ± 1.70	73.52 ± 0.65	88.56 ± 0.73
Methanol extract of the plant¥	39.61 ± 0.46	58.96 ± 0.90	90.70 ± 1.00	97.83 ± 1.08
Hexane extract of apple¥	23.32 ± 0.56	37.04 ± 2.18	84.27 ± 1.11	91.93 ± 0.96
α-Amyryltetracosanoate	41.80 ± 0.62	44.81 ± 0.71	45.19 ± 0.75	46.39 ± 0.80
Ursolic acid	55.43 ± 0.50	72.80 ± 0.94	75.87 ± 0.92	74.22 ± 0.78
Oleanolic acid	45.32 ± 0.35	50.86 ± 0.41	77.26 ± 0.59	80.81 ± 0.61
3-Acetylsitosterol	42.65 ± 0.38	44.50 ± 0.81	54.70 ± 0.75	69.09 ± 0.82
Galanthamine*	68.36 ± 1.10	74.38 ± 0.65	78.59 ± 0.47	81.41 ± 0.03

[a]Values expressed are means ± SEM of 3 parallel measurements ($P < 0.05$)
*Standard
¥Values are given as μg

In the present study, the abietane diterpenoids carnosol (**1**),[29] carnosic acid (**2**),[30] carnosic acid 12-methyl ether (**3**),[31] rosmadial (**4**),[32] isorosmanol (**5**),[33] and ferruginol (**6**);[34] the labdane diterpenoid manool (**7**);[2] the triterpenoids α-amyryltetracosanoate (**8**),[35] ursolic acid, oleanolic acid, and erythrodiol; the steroid 3-acetylsitosterol; and the flavonoid salvigenin were obtained from the whole plant, collected from Gökova Bay, Muğla. Their structures were elucidated based on spectral analyses, particularly by 1D- and 2D NMR experiments and mass spectroscopic techniques. Although the 7 diterpenoids isolated were known compounds, they were obtained from *S. fruticosa* for the first time, except for carnosol. The essential oil of the dried aerial parts was also investigated by GC and GC-MS analyses and a monoterpene, 1,8-cineol (eucalyptol), was found to be an abundant constituent of the oil (58.89%). The monoterpenes α-pinene (5.62%), β-pinene (5.22%), β-myrcene (5.24%), camphor (4.48%), and α-terpineol (3.02%) and the sesquiterpene *trans*-β-caryophyllene (4.17%) were other major constituents of the oil (Table 1).

Table 5. Butyrylcholinesterase activity of the essential oil, crude extract, and apples of *S. fruticosa and* compounds.[a]

	BChE (Inhibition %)			
Sample	25 μM/mL	50 μM/mL	100 μM/mL	200 μM/mL
Essential Oil[¥]	24.16 ± 1.49	36.83 ± 0.80	51.24 ± 2.99	64.23 ± 1.03
Methanol extract of the plant[¥]	66.84 ± 0.72	86.68 ± 2.55	99.09 ± 3.00	99.88 ± 2.22
Hexane extract of apple[¥]	72.49 ± 0.35	80.11 ± 0.76	82.89 ± 2.29	92.15 ± 2.40
α-Amyryltetracosanoate	17.04 ± 0.67	38.18 ± 0.75	38.89 ± 0.78	41.85 ± 0.82
Ursolic acid	13.29 ± 1.02	27.42 ± 0.92	32.21 ± 0.88	37.35 ± 0.94
Oleanolic acid	11.02 ± 0.12	18.80 ± 0.23	20.16 ± 0.31	25.11 ± 0.43
3-Acetylsitosterol	45.40 ± 0.28	49.82 ± 0.31	54.45 ± 0.37	65.06 ± 0.43
Galanthamine*	40.59 ± 0.88	48.73 ± 0.90	65.02 ± 0.44	75.54 ± 1.05

[a]Values expressed are means ± SEM of 3 parallel measurements ($P < 0.05$)
*Standard
[¥]Values are given as μg

A biogenetic pathway was postulated by Luis et al.[36] for the formation of highly oxidized abietane diterpenoids in the species of *Salvia.* It should be considered that enzymatic dehydrogenation and singlet oxygen play key roles (Figure 2). The co-occurrence of these diterpenoids in *S. columbariae* supports this biogenetic pathway, which seems to create a defense mechanism in the genus against the potential damage caused by highly reactive species of oxygen.[36] According to this postulation, plant NAD^+ enzyme will first lead to the formation of 6,7-didehydro carnosic acid, and then subsequent ether formation with a 4-membered ring in carnosic acid to afford rosmadial, and the oxidation of the latter compound will finally give isorosmanol. In the present study, isolation of 4 abietanes (carnosic acid, 6,7-didehydrocarnosic acid, rosmadial, and isorosmanol) from *S. fruticosa* verifies their hypothetical formation, as seen in Figure 2.

The main triterpenoids of *Salvia* species, oleanolic acid and ursolic acid, were isolated in a previous study[20] as well as in the present study along with 3-acetylsitosterol and α-amyryltetracosanoate. The latter was found in a *Salvia* species for the first time. Both compounds exhibited weak–moderate anticholinesterase activity against both enzymes (Tables 4 and 5). In fact, oleanolic and ursolic acids showed better anticholinesterase activity, especially against AChE, with inhibition % values 77.26 ± 0.59 and 75.87 ± 0.92, respectively, at 100 μM (Table 4).

As a result, the diterpenoids isolated from Anatolian *S. fruticosa* were found to be very similar to or the same as those previously obtained from *S. officinalis*,[37] possibly due to their morphological similarity, belonging to the same subsection of the genus *Salvia.* However, this chemical similarity was not observed for the constituents of *S. tomentosa*,[38] even though it also belongs to the same subsection.

Thus, the anticholinesterase activity results verified the folkloric use of *S. fruticosa* in the Mediterranean region as a neuroprotective and memory enhancing plant, and the higher anticholinesterase activity of methanol extract of *S. fruticosa* rather than its constituents indicated the synergistic effect of the whole plant. Furthermore, the apples' hexane extract should be considered a potential anticholinesterase agent, particularly against BChE, with the essential oil active particularly against AChE, and it can be concluded that whole plant of *S. fruticosa* is a potential anticholinesterase agent in the treatment of Alzheimer disease.

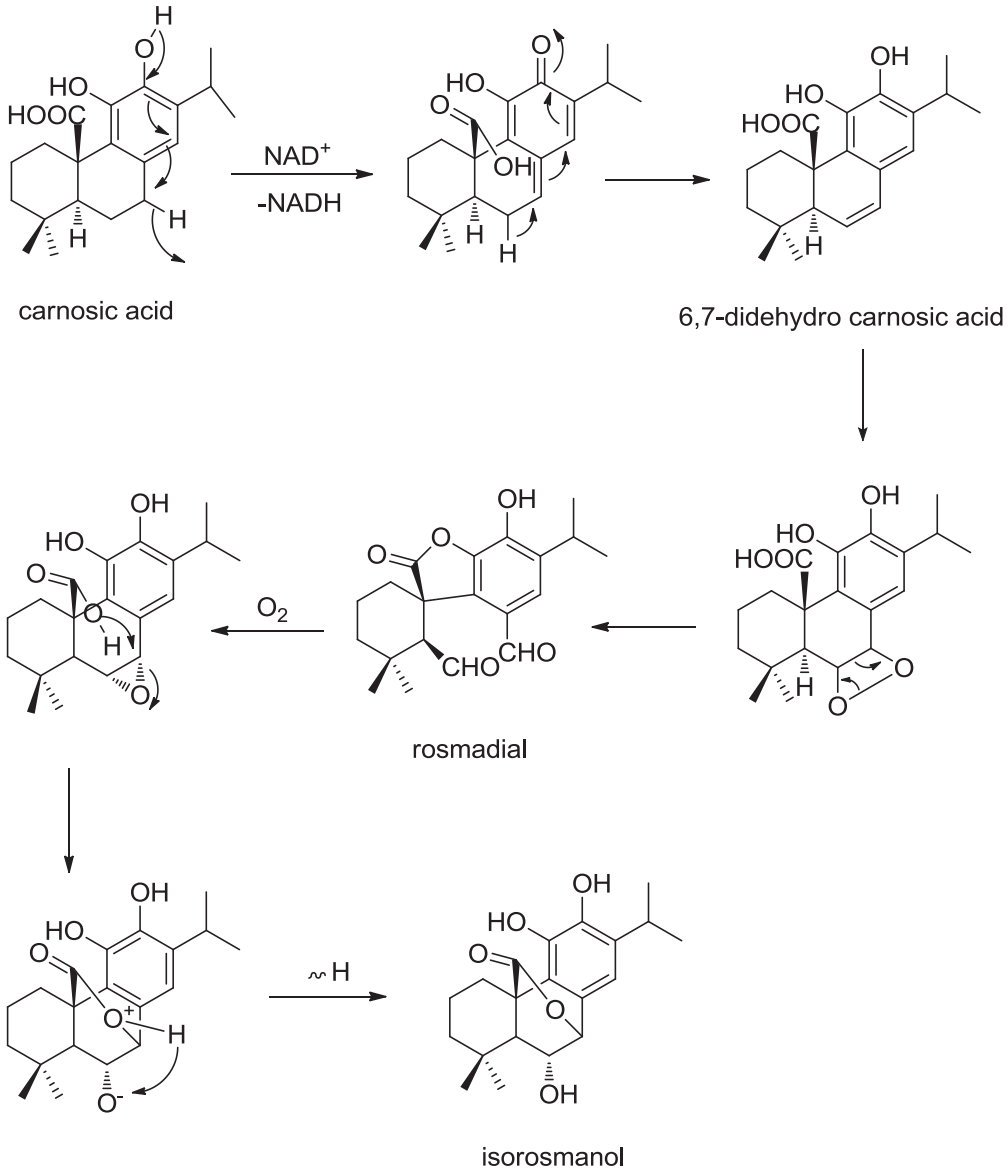

Figure 2. Biogenetic pathway to highly oxidized abietane diterpenes.

3. Experimental

3.1. Materials and methods

3.1.1. General experimental procedure

^{1}H and ^{13}C NMR, COSY, APT, HMQC, and HMBC spectra were recorded on a Varian Mercury-VX at 400 MHz for proton and 100 MHz for carbon and on a Varian ID-6508 at 600 MHz for proton and 150 MHz for carbon with tetramethylsilane (TMS) as an internal standard. Spots and bands were detected by UV Camag spectrometer (254 and 366 nm). Mass spectra ESI-QTOF Bruker Daltonics MicroTOFQ LC-MS/MS and APCI-ION TRAP Thermo Deca XP Max, GC/MS was run on an Agilent 5975 GC-MSD system. β-Carotene, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40), butylated hydroxytoluene (BHT), (+)-catechin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nicotinamideadeninedinucleotide (NADH), electric eel

acetylcholinesterase (AChE, Type-VI-S, EC 3.1.1.7, 425.84 U/mg, Sigma), horse serum butyrylcholinesterase (BChE, EC 3.1.1.8, 11.4 U/mg, Sigma), 5,50-dithiobis (2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide, butyrylthiocholine chloride, and galantamine were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). 2,20-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), Tris-HCl, nitrotetrazoliumbluechloride (NBT), and N-methyl-phenazoniummethylsulphate (PMS) were obtained from Fluka Chemie (Fluka Chemie GmbH, Sternheim, Germany). All other chemicals and solvents were of analytical grade. Column chromatography was conducted with silica gel 60 (0.063–0.200 mm) (Merck No: 1.07734). Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merck No: 1.05554) and preparative TLC was carried out on Merck silica gel 60 GF_{254} plates (Merck No: 1.07730).

3.2. Plant material

Salvia fruticosa Mill. was collected from the Akyaka side of Gökova Bay (Muğla, southwestern Turkey) in 2009 and identified by Assoc Prof Dr Tuncay Dirmenci. A voucher specimen was deposited in the special collection of T. Dirmenci.

3.3. Extraction, fractionation, and isolation

The whole plant of *S. fruticosa* (400 g) was macerated with methanol. The methanol extract (2 g) was mixed with water ($MeOH/H_2O$) (60:40) and was partitioned with hexane and subsequently with dichloromethane (CH_2Cl_2). The hexane extract (490 mg) was fractionated on a silica gel column, and 60 fractions were collected. Similar fractions were combined to obtain in total 11 fractions. Carnosol (**1**), carnosic acid (**2**), carnosic acid 12-methyl ether (**3**), rosmadial (**4**), and ferruginol (**6**); the labdane diterpenoid manool (**7**); the triterpenoids α-amyryltetracosanoate (**8**), ursolic acid, oleanolic acid, and erythrodiol; the steroid 3-acetylsitosterol; and the flavonoid salvigenin were obtained from the hexane extract while isorosmanol (**5**) was not. Salvigenin was obtained from fraction 3 and α-amyryltetracosanoate from fraction 6 (**8**) and purified from a solvent system (CH_2Cl_2/acetone) (90:10). From the main fraction 4, ferruginol (**6**) and manool (**7**) were obtained during elution by hexane/CH_2Cl_2 (50:50). Fraction 5 was purified on prep. silica gel TLC plates using $MeOH/H_2O$ (85/15) to afford 4 subfractions, and then erythrodiol was obtained from subfraction 4. One of the main fractions (fraction 8) was fractionated into 9 subfractions on a Si-gel flash column eluting with hexane and increasing amount of CH_2Cl_2. Following 100% CH_2Cl_2, acetone was added, and then ferruginol (**6**) was obtained during elution with CH_2Cl_2:acetone (95:5). The known triterpenoids oleanolic and ursolic acids were obtained from the same column, and subsequently ferruginol.

The dichloromethane part (1350 mg) was fractionated on a silica gel column and 15 fractions were obtained. From fraction 2, isorosmanol (**5**) was obtained during elution with CH_2Cl_2/acetone (97.5:2.5), and was purified by prep. TLC from the same system ($\times 3$).

3.4. Isolation of the essential oil

The essential oil of the dried aerial parts (excluding galls) of *S. fruticosa* (250 g) was obtained via hydrodistillation by using a Clevenger-type apparatus for 4 h. The oil obtained (1.6 g) was dried over anhydrous sodium sulfate and stored under nitrogen at –20 °C until required.

3.5. Preparation of the hexane extract of the galls (apples) and methyl derivatization

The galls (apples) of *S. fruticosa* (10 g) were dried, powdered, and extracted with 10 mL of hexane (4 × 24 h) at room temperature. After filtration, the solvent was evaporated to dryness in vacuum to give 0.5 g of crude extract. The hexane extract (100 mg) was dissolved in 0.5 M NaOH (2 mL) in a 25-mL flask, and the flask was heated at 50 °C in a water bath. After the addition of 2 mL of BF_3:MeOH reactive, the mixture was boiled for 2 min, and then left until it cooled down. Then flask was filled to 25 mL with saturated NaCl solution. The esters were extracted with *n*-hexane; thus, the organic layer was separated. The hexane layer was washed with a potassium bicarbonate solution (4 mL, 2%) and dried over anhydrous Na_2SO_4 and filtered. The organic solvent was removed under reduced pressure on a rotary evaporator to give methyl esters. Then the derivative was analyzed by GC and GC-MS. The galls afforded fatty acids, composed mainly of oleic acid (29%), palmitoleic acid (29%), stearic acid (23.20%), and linoleic acid (17.40%), while myristic acid (0.12%), palmitic acid (0.12%), and arachidonic acid (1.16%) were present in smaller amounts. According to these results, the total saturated oils' percentage was 24.59% and the total unsaturated oils' percentage was 75.41%.

3.6. Gas chromatography (GC) analysis

GC analyses of the essential oil and fatty acid profile were performed using a Shimadzu GC-17 AAF, V3, 230V series gas chromatograph equipped with a FID and a DB-1 fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The injector temperature and detector temperature were adjusted to 250 and 270 °C, respectively. The carrier gas was He at a flow rate of 1.4 mL/min. Sample size was 1.0 μL with a split ratio of 50:1. Different oven temperature programs were used for the essential oil and methylated hexane extract. In order to analyze the essential oil, the oven temperature was held at 60 °C for 5 min, then increased to 240 °C with 4 °C/min increments, and held at this temperature for 15 min. To analyze the fatty acids, the initial oven temperature was held at 100 °C for 5 min, then increased to 238 °C with 3 °C/min increments, and held at this temperature for 14 min. The percentage compositions of the essential oil were determined with GC solutions software.

3.7. Gas chromatography–mass spectrometry (GC-MS) analysis

GC-MS analyses of the essential oil and fatty acid profile were performed using a Varian Saturn 2100T (E.I. ion trap) equipped with a DB-1 MS fused silica nonpolar capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The carrier gas was helium at a flow rate of 1.4 mL/min. The oven temperatures used for the both tests were the same as those used in the GC analysis mentioned above. Injector and MS transfer line temperatures were set at 220 °C and 290 °C, respectively. Ion source temperature, however, was 200 °C. The injection volume was 0.2 μL with a split ratio of 1:50. EI-MS were obtained at 70 eV ionization energy. Mass range was from m/z 28 to 650 amu. Scan time was 0.5 s with 0.1 interscan delays. Identification of components of the essential oil was based on GC retention indices and computer matching with the Wiley, NIST-2005, and our library as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature.[39] Whenever possible, co-injection was performed with authentic compounds.

3.8. Determination of total phenolic content

The concentrations of the phenolic content in the methanol extract were expressed as micrograms of pyrocatechol equivalents (PEs).[40] The concentration of phenolic compounds was calculated according to the following

equation, obtained from the standard pyrocatechol graphic:

Absorbance = 0.1166 pyrocatechol (μg) – 0.0735 (R^2 = 0.9996)

3.9. Determination of total flavonoid content

The measurement of the flavonoid concentration was based on the standard method with a slight modification and the results were expressed as quercetin equivalents.[40] The concentration of flavonoid compounds was calculated according to the following equation: Absorbance = 0.0665 quercetin (μg) + 0.0157 (R^2 = 0.9972).

3.10. DPPH free radical scavenging activity

The free radical scavenging activity of the samples was determined by the DPPH assay.[40]

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

The antioxidant activity was established by using β-carotene-linoleic acid test system.[40]

3.12. Superoxide anion radical scavenging activity

Measurement of superoxide anion radical scavenging activity of the samples was based on the standard method with slight modification.[40]

3.13. Ferric reducing power

The ferric reducing power of the samples was determined according to the iron(III) reductive assay.[40]

3.14. Cupric reducing antioxidant capacity (CUPRAC)

The CUPRAC of the samples was determined according to the standard method.[40]

3.15. Metal chelating

The chelating activity on Fe^{2+} was measured as reported.[40] The extracts were 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. The results were given as percentage inhibition.[40]

3.16. Anticholinesterase activity

AChE and BChE inhibitory activities were established by slightly modifying the spectrophotometric Ellman method.[41]

3.17. Isolated compounds

Carnosol (1): Crystal, mp: 210–220 °C. ^{1}H and ^{13}C NMR data in Tables 6 and 7. ESI-MS (-) m/z (%) = 330 $[M]^+$ (17), 287 (21), 286 (100), 271 (17), 215 (50), 204 (22), 202 (20).

Table 6. ^{1}H NMR data for compounds **1–5** (in $CDCl_3$)*; J in parentheses (Hz).

	δ				
Position	**1**	**2**	**3**	**4**	**5**
1 α **1** β	2.80dd(14/3) 2.56 ddd (14.3/4.5/4.5)	1.15 m 3.58ddd (14.0/3.4/3.4)	1.23m 3.56d (13)	1.70m 2.20m	1.38dt
2 α **2** β	1.91 m 1.59 m	2.11d (13.5) 1.8m	2.27m 1.56m	1.51m 2.03m	1.92 m 1.61 m
3 α **3** β	1.52 m 1.30m	1.7m 1.38ddd (13.4/13.8/4.5)	1.52m 1.52m	1.55m 1.83m	2.35ddd (13/13/3)
5	1.69dd (4.2/4.3)	1.31m	1.58m	4.01s	2.10d
6 α **6** β	1.83 m 2.19 m	1.88 bd (13.7) 2.41 m	2.30m -	9.59d -	4.26dd (4.22/4.20)
7 α **7** β	5.41dd (7.3/7.5) -	2.81 m 2.85 m	2.85m 0.96m	9.70s -	5.16d (4.7) -
14	6.69 s	6.52 s	6.52s	7.33s	6.65s
15	3.25 m	3.21 m	3.15septet(6.7)	3.27septet (7.0)	3.19septet
16	1.18d (6.7)	1.20 d (7.2)	1.21d (6.7)	1.22d (7.0)	1.17d (7.0)
17	1.20d (6.9)	1.21 d (7.3)	1.19d (6.7)	1.22d (7.0)	1.18d (7.0)
18	0.89 s	0.97 s	0.88s	1.43s	0.79s
19	0.90 s	0.90 s	0.97s	1.26s	0.92s
OCH_3	-	-	3.73s	-	-

*Compound 5 in d_6-acetone; Since C-4, C- (8–13), and C-20 do not contain any proton they were not included in the table

Carnosic acid (2): Crystal, mp: 190 °C. ^{1}H and ^{13}C NMR data in Tables 6 and 7. ESI-MS m/z (%) = 332 (calcd. for $C_{20}H_{28}O_4$: 332.1988); $[M]^+$ (6), 286 (100), 271 (16), 243 (17), 230 (34), 218 (18), 215 (15), 149 (11).

Carnosic acid 12-methyl ether (3): Amorphous. ^{1}H and ^{13}C NMR data in Tables 6 and 7. ESI-MS (-) m/z (%) = 345 [M - 1] or 346 $[M]^+$ (6), 302 [M - CO_2] (1), 280 (8), 252 [M - CO_2 - OCH_3 - OH]$^+$ (100), 236 (7), 218 (14), 206 (6), 189 (27).

Rosmadial (4): Crystal, mp: 225 °C. ^{1}H and ^{13}C NMR data in Tables 6 and 7. ESI-MS

(-) m/z (%) = 343 [M - H]$^+$ (9), 315 [M - CHO]$^+$ (100), 299 [M - CHO - OH + 1]$^+$ (12), 287 [M^{-2} CHO^{+1}]$^+$ (1), 256 (0.5), 219 (0.25).

Isorosmanol (5): Crystal, mp: 227 °C. ^{1}H and ^{13}C NMR data in Tables 6 and 7. ESI-MS (-) m/z (%) = 690 [dimeric M]$^+$ (79), 346.3 [M + 1]$^+$ (1), 345 $[M]^+$ (100), 301 [M - CO_2]$^+$ (2), 299 [301 - 2H]$^+$ (12), 283 [M - CO_2 - H_2O]$^+$ (0.5).

Ferruginol (6): Crystal, mp: 56–57 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 6.63 (1H, s, H-14), 6.83 (1H, s, H-11), 3.12 (1H, septet, J =7.1, H-15), 2.81 (1H, brdd, J = 7.5/14, H-7eq), 1.62 (1H, m, H-7ax), 2.17 (1H, brd, J =12.8, H-1eq), 1.42 (1H, m, H-1ax), 1.22–1.24 (6H, d, J =7.0, H-16 and H-17), 1.61 (2H, m, H-6), 0.91 (3H, s, H-19), 0.94 (3H, s, H-20), 1.17 (3H, s, H-18), 4.53 (Ar - OH); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 150.6 (C-12), 148.4 (C-9), 131.1 (C-13), 127.0 (C-8), 126.3 (C-14), 110.0 (C-11), 50.2 (C-5), 41.5 (C-3), 38.7 (C-1), 37.4 (C-10), 33.2 (C-4), 29.6 (C-7), 33.1 (C-18), 26.5 (C-15), 24.7 (C-20), 22.6 (C-17), 22.4 (C-16), 19.2

(C-2), 19.1 (C-6), 21.5 (C-19). ESI-MS (-) m/z (%) = 286.2278 (calcd for $C_{20}H_{30}O$, 286) [M -] (100), 270 $[M - 16]^+$ (60), 243 (15), 228 (10), 189 (36), 115 (55), 91 (7.8), 69 (6.7).

Table 7. ^{13}C NMR data for compounds **1**–**5** (in $CDCl_3$).*

	δ				
Position	1	2	3	4	5
1	31.0	34.5	34.8	33.8	29.9
2	22.1	23.6	17.5	18.8	18.3
3	43.2	46.1	41.7	42.4	40.8
4	37.6	38.0	34.6	37.2	34.4
5	nd	53.2	52.8	66.4	58.2
6	34.3	59.1	17.7	202.8	78.8
7	82.1	36.2	31.2	191.2	69.4
8	138.1	133.7	134.6	127.3	128.3
9	121.8	135.2	125.3	139.1	123.4
10	nd	nd	46.5	47.3	48.9
11	148.2	148.5	145.9	143.3	147.3
12	147.7	144.2	141.1	143.2	143.8
13	138.5	132.1	137.7	131.3	138.1
14	114.8	120.1	117.5	134.4	115.9
15	26.9	29.1	26.7	29.7	28.3
16	25.2	25.2	23.5	22.5	23.4
17	24.6	26.3	22.9	26.4	23.6
18	33.2	34.8	18.9	34.6	32.1
19	21.5	23.4	32.5	26.2	21.4
20	181.2	184.1	179.8	178.0	176.7
OCH_3	-	–	61.8	–	-

*Compound **5** in d_6-acetone; nd: not detected

Manool (7): Crystal, mp: 49–52 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.86 (lH, dd, J = 11/17.5 Hz, H-14), 5.22 (1H, dd, J = 2/17.5 Hz, H-15), 5.07 (1H, J = 2/11 Hz, H-15′), 4.75 (1H, brs, H-17), 4.43 (1H, brs, H-17′), 1.22 (3H, s, Me-16), 0.83 (3H, s), 0.76 (3H, s), 0.62 (3H, s) (Me-18, Me-19, Me-20); ^{13}C NMR (150 MHz, $CDCl_3$) δ_C: 38.2 (C-1), 19.6 (C-2), 43.7 (C-3), 34.4 (C-4), 54.7 (C-5), 25.3 (C-6), 39.4 (C-7), 150.6 (C-8), 56.2 (C-9), 37.6 (C-10), 16.9 (C-11), 42.4 (C-12), 72.9 (C-13), 146.5 (C-14), 113.0 (C-15), 28.2 (C-16), 105.9 (C-17), 34.7 (C-18), 23.6 (C-19), 15.2 (C-20); ESI-MS m/z (%) = 290 [M +] (2), 272 (14), 257 (60), 244 (10), 229 (9), 215 (5), 204 (23), 189 (44), 177 (26), 161 (24), 149 (23), 137 (100), 121 (53).

α-Amyryltetracosanoate (8): Amorphous, ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 0.79 (3H, s, H-28), 0.86 (3H, d, J = 6.5 Hz, H-29), 0.87 (3H, t, J = 7.0, H-24′), 0.92 (3H, d, J = 6.8 Hz, H-30), 0.81 (3H, s, H-23), 0.91 (3H, s, H-24), 0.95 (3H, s, H-25), 1.01 (3H, s, H-26), 1.07, (3H, s, H-27), 4.44 (1H, dd, J = 5.6/10.2 Hz, H-3), 5.06 (1H, t, 2.5 Hz, H-12). ^{13}C NMR (150 MHz, $CDCl_3$) δ_C: 14.12, (C-24′), 16.87 (C-24), 15.73 (C-25), 17.51 (C-26), 17.51 (C-28), 17.51 (C-29), 18.25 (C-6), 21.4 (C-30), 23.24 (C-27), 23.38 (C-11), 25.18 (C-3′), 26.61 (C-2), 26.61 (C-15), 28.09 (C-23), 28.75 (C-16), 29.15–29.7 (10C, C-4′ - C-13′), 31.30 (C-21), 32.87 (C-7), 34.9 (C-2′), 33.76 (C-17), 36.79 (C-10), 37.76 (C-4), 38.45 (C-1), 39.62 (C-20), 39.66 (C-19), 40.04 (C-8), 41.53 (C-22), 42.08 (C-14), 47.63 (C-9), 55.27 (C-5), 59.07 (C-18), 80.60 (C-3), 124.33 (C-12), 139.63 (C-13), 173.71 (C-1′); APCI - MS (+)m/z (%) = 777 $[M + H]^+$ (3), 734 $[(M + H) - (C_3H_7)]^+$ (5), 695 (9), 680 (20), 664 (20), 442 (10), 424 (12), 410 $[(M + H) - (C_{24}H_{47}O_2)]^+$ (100), 284 (5), 171 (2).

3.18. Statistical analysis

The results were mean ±SD of 3 parallel measurements. Analysis of variance was performed using ANOVA procedures. Significant differences between means were determined by Student's t-test, and P values <0.05 were regarded as significant.

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