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Cytotoxic Activity of Some Anatolian *Salvia* Extracts and Isolated Abietane Diterpenoids

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Abstract

Salvia hypargeia Fisch. et Mey. (Lamiaceae) root extract, among 16 *Salvia* extracts, showed the highest activity against the human ovarian cancer cell line. Bioactivity-guided fractionation of this plant extract has yielded four abietane-type diterpenes [5,6-didehydro-7-hydroxytaxodone (**1**), 14-deoxycoleon U (= 6-hydroxysalvinolone) (**2**), demethylcryptojaponol (**3**), and salvicanaric acid (**4**)] two triterpenes (lupeol and lupeol-3-acetate), and a fatty acid mixture consisting mainly of palmitic acid (51.6%) and palmitoleic acid (6.4%). Compounds **2** and **3** were found to be active against A2780 human ovarian cancer cell line with IC_{50} values of 3.9 and 1.2 μg/mL, respectively, and the fatty acid composition was the most active part of the extract ($IC_{50} = 0.6$ μg/mL).

Keywords: Abietane diterpenes, cytotoxic activity, Lamiaceae, *Salvia* extracts, *Salvia hypargeia*.

Introduction

The plant name *Salvia* came from Latin word *salvare* ("healer"), and the plant has been used against cold and menstrual disorders since ancient times. The genus *Salvia* L. (Lamiaceae), with more than 900 species, is distributed all around the world. In Turkey, *Salvia* plants are represented by 86 species and 92 taxa (Davis, 1982), half of which are endemic.

Salvia species are a rich source of abietane diterpenes (Rodriguez-Hahn et al., 1992; Nagy et al., 1999) and possess a number of biological activities including antiseptic, antibacterial (Yang et al., 2001), astringent, anti-inflammatory (Baricevic et al., 2001), antiviral (Tada et al., 1994), cytotoxic (Ryu et al., 1996), cardiovascular, sedative, tranquilizing (Chang & But, 1986), spasmolitic, anticonvulsant (Coelho de Souza & Elisabetsky, 1998), and carminative. In China, *Salvia miltiorrhiza* Bunge has been used in the treatment of coronary heart diseases, particularly angina pectoris and myocardial infarction (Chang et al., 1990). Ethnobotanical, phytochemical, and pharmacological studies showed that *Salvia* species are considered "cure-all" type plants (Harley et al., 1998; Foster & Tyler, 2000).

In Turkish folk medicine, especially in rural areas, *Salvia* species have been used as stomachic, diuretic, hemostatic, spasmolitic, carminative, and in the treatment of colds and mouth and throat irritations because of its antibacterial and wound healing properties (Baytop, 1984). More than 50 *Salvia* species have been investigated by our group for their chemical constituents (Ulubelen & Topcu, 1998; Topcu & Ulubelen, 2007) and when possible for their biological activities (Ulubelen & Topcu, 1998; Topcu, 2006). We have isolated several antibacterial (Ulubelen et al., 2000) and antituberculous active diterpenes (Ulubelen et al., 1997) and some cytotoxic abietane and rearranged abietane diterpenes (Ulubelen et al., 1999*; Topcu et al., 2003) and triterpenes (Topcu et al., 2004) as well as cardiovascular compounds (Ulubelen, 2003) from endemic Turkish *Salvia* species.

Since ancient times, some *Salvia* species have been used to treat menstrual disorders, and, in Turkey, a few *Salvia* species, such as *S. virgata* Jacq. have been used in the treatment of vaginitis, uterus cancer, and leukemia

*The structure of 6-hydroxysalvinolone was incorrectly drawn without a keto group at C-7 in the paper.

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Table 1. Cytotoxicity of some *Salvia* extracts against the A2780 human ovarian cancer cell line[a].

Plant extract	IC_{50} (μg/mL)
S. amplexicaulis Lam. (root-EtOH)	21.0
S. aucheri Benth. (root-EtOH)	39.6
S. bracteata Banks & Sol. (root-EtOH)	38.2
S. candidissima Vahl (root-EtOH)	31.9
S. cassia G. Samuelsson ex Rech. f. (aerial parts-EtOH)	29.4
S. eriophora Boiss. & Kotschy ex Boiss. (aerial parts-EtOH)	NA
S. heldreichiana Boiss. ex Benth. (root-EtOH)	36.0
S. hypargeia Fisch. & Mey. (root-acetone)	15.5
S. napifolia Jacq. (root-EtOH)	35.6
S. pilifera Mont. & Auch. ex Benth. (aerial parts-EtOH)	33.3
S. recognita Fisch. & Mey. (aerial parts-acetone)	29.7
S. staminea Mont. & Auch. ex Benth. (whole plant-MeOH)	36.2
S. staminea Mont. & Auch. ex Benth (whole plant-CH_2Cl_2)	38.8
S. syriaca L. (whole plant-EtOH)	41.7
S. tomentosa Mill. (root-EtOH)	36.3
S. triloba L. (whole plant-CH_2Cl_2)	17.2

[a] The tests were carried out as dose-dependent assay starting from 50 μg/mL doses.

(Tuzlaci & Aymaz, 2001). Therefore, in this study, a series of extracts of Anatolian *Salvia* species were screened for their cytotoxic activity against a human ovarian cancer cell line (A2780) (Table 1), and the most active extract was found to be the acetone extract of *S. hypargeia* Fisch. et Mey., which is an endemic perennial plant, growing wild in the higher elevations of southern Turkey (Adana). We had previously investigated *S. hypargeia* and its closely related species *S. montbretii*. The former afforded 14 diterpenoids, and they were tested against a group of cancer cell lines, except ovarian cancer: BC1 (human breast cancer), COL2 (human colon cancer), KB (derived from human nasopharyngeal cancer), KB IV (multidrug-resistant KB), LNCaP (human prostate cancer), P388 (mouse lymphocytic leukemia). Among the isolated 14 diterpenoids, taxodione, 6-hydroxysalvinolone, and saprorthoquinone were found to be active (Ulubelen et al., 1999). In the current study, the ovarian cytotoxic activity–guided isolation was carried out, and four active abietane diterpenoids were isolated, and their structures were established by spectral methods, namely based on 1D and 2D NMR experiments.

Materials and Methods

Plant material

All *Salvia* species were collected from different regions of Anatolia in their flowering season, especially between June and July (related studies can be found in our previously published studies, some given in the references). The roots of *S. hypargeia* Fisch. et Mey. (Labiatae = Lamiaceae) were collected from a highland area of Adana (Tekir-Bürücek) in southern Turkey at an altitude 1850 m and identified by Prof. Dr. Kerim Alpinar, and a voucher specimen is deposited in the herbarium of the Faculty of Pharmacy, University of Istanbul (ISTE 68316).

Equipment

Optical rotations were determined using an Optical Activity Ltd AA-5 polarimeter (Huntington, UK) UV with a Shimadzu 1601 spectrometer (Kyoto, Japan), IR with a Perkin-Elmer model 983, and ^{1}H and ^{13}C NMR on Varian 400 (USA) and Jeol Eclipse 500 spectrometers (Tokyo, Japan). Mass spectra were obtained on a VG-Zabspec (Micromass-VG Analytical, Manchester, UK) instrument. HPLC was performed on a Shimadzu LC-10AT instrument (Japan).

Extraction and isolation

In this study, fifteen *Salvia* species, growing in Turkey, were exhausted by maceration in ethanol to study their ovarian cytotoxic activity while a few in other solvents. We used the roots of the plants (*S. amplexicaulis* Lam., *S. aucheri* Benth., *S. bracteata* Banks & Sol., *S. candidissima* Vahl, *S. heldreichiana* Boiss. ex Benth., *S. hypargeia* Fisch. et Mey., *S. napifolia* Jacq., *S. tomentosa* Mill.) or the aerial parts (*S. cassia* G. Samuelsson ex Rech. f., *S. eriophora* Boiss. & Kotschy ex Boiss., *S. pilifera* Montb. & Auch., and *S. recognita* Fisch. & Mey.) for the extraction. However, the whole plant extracts were prepared from *S. staminea* Montb. & Auch. ex Benth., *S. triloba* L. (*S. fruticosa* Mill.) and *S. syriaca* L. (Table 1) .

For the most active plant, *S. hypargeia*, the air-dried and powdered roots (1 kg) were extracted with acetone in a Soxhlet apparatus. The solvent was removed *in vacuo*. The crude acetone extract (58 g) was fractionated on a silica gel (70–200 mesh) column (5 × 75 cm) eluting with *n*-hexane (10 × 200 mL) followed by a gradient CH_2Cl_2 with 5% increments reaching 100%, and followed by a gradient EtOH. The eluted fractions were checked by TLC, and similar fractions were combined to give six fractions. The first five of these fractions have been previously studied (Ulubelen et al., 1999), so the final polar fraction was purified in a bioactivity-guided way to afford the four diterpenes **1–4** (Fig. 1), the two triterpenes **5** and **6**, and a mixture of fatty acids. Compound **1** was purified by preparative TLC (Si-gel F254 plates) using a $CHCl_3$:MeOH (95:05) solvent system, and a $CHCl_3$:MeOH (90:10) solvent mixture was used for compounds **2** and **3**. Compound **4** was purified by HPLC on a reversed-phase C-18 column using the solvent system MeOH:H_2O (7:3). All six compounds were identified by comparision of their ^{1}H and ^{13}C NMR data with literature data (Kupchan et al., 1968; Hueso-Rodriguez et al., 1983; Gonzalez et al., 1987; Luis & Grillo, 1993; Misra et al., 1993; Marques et al., 2002). The compounds

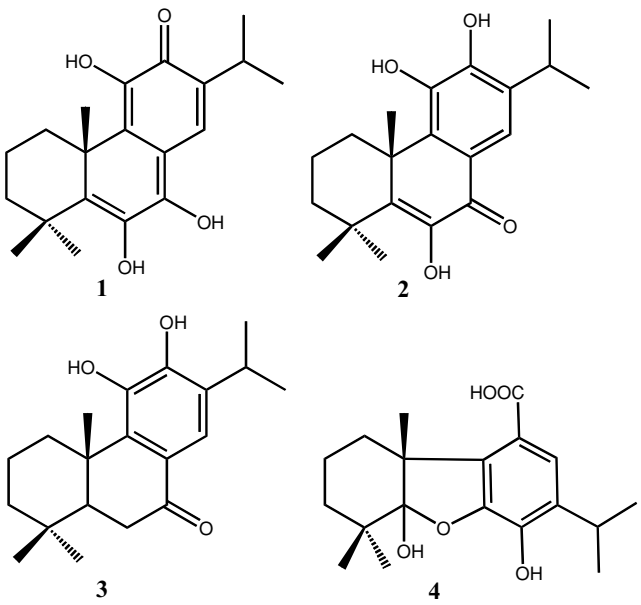

Figure 1. Abietane diterpenoids isolated from the polar fraction of *Salvia hypargeia*

were identified as 5,6-didehydro-7-hydroxytaxodone (**1**) (20 mg) $[\alpha]_D = 0^\circ$ (MeOH, *c* 0.1), 14-deoxycoleon U (6-hydroxysalvinolone) (**2**) (50 mg) $[\alpha]_D = 42^\circ$ ($CHCl_3$, *c* 0.1), demethylcryptojaponol (**3**) (60 mg) $[\alpha]_D = +31.2^\circ$ ($CHCl_3 c$ 0.6), salvicanaric acid (**4**) (13 mg) (acetate methyl ester of **4**) $[\alpha]_D = 72.2^\circ$, ($CHCl_3$, *c* 0.072), lupeol (34 mg) $[\alpha]_D = +28^\circ$ ($CHCl_3$,*c* 0.3), and lupeol acetate (45 mg) $[\alpha]_D = +45^\circ$ ($CHCl_3$, *c* 0.5). GC-MS analysis of the fatty acid mixture indicated the presence of 51.6% hexadecanoic acid (palmitic acid) and 6.4% 9-hexadecenoic acid (palmitoleic acid); the remaining material was composed of more than 20 unidentified fatty acids.

Cytotoxicity assay

Cytotoxicity was determined against A2780 human ovarian cancer cells using a microtiter plate assay. The plates were seeded with cells, and the extracts or compounds (dissolved in 1:1 DMSO:H_2O) were added to the cells at specific concentrations. The plates were incubated at 37°C and 5% CO_2 for 48 h. Then Alamar blue (Biosource International, Camarillo, CA, USA) was added to the cells, and the plates were incubated for 3 h. During this time, the Alamar blue is taken up by the live cells and reduced. The reduced form of Alamar blue is stable and fluorescent. The fluorescence of each well in the plate was measured. The fluorescence is directly proportional to the percent inhibition of the growth of the cells. The IC_{50} value was determined by plotting the data on a dose-response curve of percent inhibition versus concentration. The IC_{50} value is defined as the concentration of sample necessary to produce 50% inhibition of the growth of the cells. Lower IC_{50} indicate greater activity of values compounds (Louie et al., 1985; Guza, 2004).

Results and Discussion

In this study, a number of crude extracts of Turkish *Salvia* species including *S. hypargeia* (Table 1) have been screened in a dose-dependent manner against the A2780 human ovarian cell line, and *S. hypargeia* extract was found to be the most active one among them. Isolation of cytotoxic constituents from this extract yielded four abietane diterpenoids [5,6-didehydro-7-hydroxytaxodone (**1**) (Luis & Grillo, 1993), 14-deoxycoleon U (= 6-hydroxysalvinolone) (**2**) (Hueso-Rodriguez et al., 1983; Marques et al., 2002), demethylcryptojaponol (**3**) (Hueso-Rodriguez et al., 1983), and salvicanaric acid (**4**) (Gonzalez et al., 1987) (Fig. 1)], and two triterpenoids (lupeol and lupeol-3-acetate) (Misra et al., 1993), as well as a fatty acid mixture. The activity of the diterpenoids against ovarian cancer cell lines was determined, and the results are given in Table 2. The data of Table 2 indicate that demethylcryptojaponol (**3**) and 14-deoxycoleon U (**2**) are highly active, and compounds **1** and **4** are not as active as **2** and **3.** Interestingly, the most active component was found to be the fatty acid mixture. Its high cytotoxic activity can be attributed to the presence of its main constituents palmitic and palmitoleic acid, which were found in high percentages in this mixture. In a recent study on melanoma cells, palmitic acid was the most toxic against B16F10 murine melanoma cells, including cell death by apoptosis and necrosis. In fact, the human melanoma cell lines were more resistant to the toxic effect of fatty acids. In SK-Mel 23 cells, cytotoxicity was detected after 48-h treatment with arachidonic, linoleic, palmitic, and palmitoleic acids at 200 μM, and linoleic acid was found to be the most toxic fatty acid, whereas in SK-Mel 28 human cells, only palmitic acid caused a significant decrease of the number of viable cells inducing DNA fragmentation after 24-h and 48-h treatments at the same concentration (Andrade et al., 2005).

Compound **4** (salvicanaric acid) is a rearranged abietane diterpenoid, and its formation through demethylcryptojaponol has been postulated previously (Gonzalez et al., 1987). It was also isolated from a few *Salvia* species including *S. montbretii* (Topcu & Ulubelen, 1996) and *S. munzii Epl.* (Luis et al., 1993).

The chemical constituent profiles of *S. hypargeia* and *S. montbretii* were found to be very similar. 14-Deoxycoleon U (6-hydroxysalvinolone), demethylcryptojaponol, salvicanaric acid, taxodione, ferruginol, microstegiol, and saprorthoquinone were isolated from both species (Topcu & Ulubelen, 1996; Ulubelen et al., 1999), which verified both plants are close congeners, as mentioned in *Flora of Turkey* (Davis, 1982). Since the 1960s, taxodione has been known as a potential anticancer agent, which was first presented by Kupchan et al. (1968). 14-Deoxycoleon U (**2**) and its 14-hydroxylated derivative "coleon U" were also found to

Table 2. Cytotoxicity of the isolated abietane diterpenoids and fatty acid mixture against the A2780 cell line.[a]

Compound	IC_{50} (μg/mL)
S. hypargeia [fraction (125–211)]	13.4
1	18.8
2	3.9
3	1.2
4	15.0
5	34.2
6	9.0
Fatty acid mixture	0.6

1 = 6,7,11-trihydroxy-12-oxo-abieta-5,7,9,13-tetraen, **2** = 6-hydroxysalvinolone (14-deoxycoleon U), **3** = demethylcryptojaponol, **4** = salvicanaric acid, (**5** = cryptojaponol and **6** = taxodione were used for comparison).

[a]The test was carried out as dose-dependent assay starting from 50 μg/mL doses.

be highly active against several cancer cell lines (Marques et al., 2002; Cerquira et al., 2004) by other researchers, but these diterpenoids have not been previously tested against ovarian cell lines. Furthermore, demethylcryptojaponol and taxodione showed potent antimicrobial activity with MIC values of 4–10 μg/mL against methicillin-resistant *Staphylococcus aureus* (Yang et al., 2001). In a recent study, 14-deoxycoleon U and demethylcryptojaponol were investigated for their antifeedant activity. The former showed strong antifeedant activity against *Leptinotarsa decemlineata*, whereas the latter was toxic to this insect, but they had no antifeedant or negative effects on *Spodoptera littoralis* (Boisd.) (Fraga et al., 2005).

On the other hand, in our previous study, most abietane diterpenoids isolated from *S. hypargeia* (Ulubelen et al., 1999) have been tested against a series of cell lines, except for ovarian cells, and they were found more or less active, and the highest activity was obtained by taxodione against most tested cell lines. However, in the current study, taxodione did not show high activity against the A2780 ovarian cell line. In contrast, demethylcryptojaponol (**3**) and 14-deoxycoleon U (**2**) were found to be highly active ovarian cytotoxic compounds with IC_{50} values of 1.2 and 3.9 μg/mL, respectively.

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