NPC Natural Product Communications

2010
Vol. 5
No. 9
1369 - 1374

Essential Oil Composition, Antimicrobial and Cytotoxic Activities of Two Endemic *Stachys cretica* Subspecies (Lamiaceae) from Turkey

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Received: May 21st, 2010; Accepted: June 21st, 2010

The chemical compositions of the water-distilled essential oil of *Stachys cretica* ssp. *lesbiaca* Rech. fil. and *S. cretica* ssp. *trapezuntica* Rech. fil. were determined by GC and GC–MS. Altogether 63 compounds were identified. The sesquiterpene hydrocarbon, germacrene D (20.3% and 12.9% respectively) was the main component identified in both oils. Furthermore, ethanol, light petroleum, dichloromethane, ethyl acetate and *n*-butanol extracts prepared from the aerial parts of the plants were tested for their antimicrobial activities against six bacterial strains and the yeast *Candida albicans*. The extracts exhibited no antibacterial activity, but the light petroleum and *n*-butanolic fractions showed low antifungal activities. Crude ethanolic extracts of the two subspecies were tested for their ability to inhibit the growth of HL-60 and Ishikawa human tumor cell lines. The IC_{50} values were 100 μg/mL for the HL-60 cell line and 200 μg/mL for the Ishikawa cell line.

Keywords: *Stachys cretica* ssp. *lesbiaca*, *Stachys cretica* ssp. *trapezuntica*, essential oil, antimicrobial, cytotoxicity.

The genus *Stachys* contains around 270 species that are spread in the northern hemisphere and tropical Australasia, with a center of biodiversity in the Mediterranean and Middle-East, including south and east Anatolia, Caucasia, north-west Iran, Iraq and the Balkan Peninsula. With 83 recorded species and a level of 48% endemism, Turkey is one of the richest countries in *Stachys* diversity. The genus has been separated morphologically into 15 sections, 12 subsections and two subgenera in the flora of Turkey [1a-1g].

Many members of this genus find use in traditional medicine of Anatolia and are known as Deli adaçayı or Dağ çayı. They are used for the same purpose as sage to treat skin infections, digestive problems and respiratory disorders [2a,2b]. Similar folkloric uses of many other species that possess antiphlogistic, cholagogic, sedative, and hypotensive properties and which are used for the treatment of coughs, kidney diseases, tumors, and throat pains have also appeared in the world literature [2c-2e]. The multiple diverse traditional uses of *Stachys* species can be explained by at least nine natural product chemical classes present in these plants including alkaloids [3a], iridoids [3b], terpenoids [3c,3d], steroids, flavonoids[3e-3g], and phenylpropanoid glycosides [3h], as well as carbohydrates [2e], essential oils [3i,3j] and lipids [3k]. Also, biological evaluation of essential oils, extracts and isolated metabolites have shown significant antioxidant [4a,4b], antibacterial [4c,4d], anti-inflammatory [4e] and antinephritic [4f] effects for some *Stachys* species.

While the number of *Stachys* species growing in Turkey is fairly high, a limited number of reports has appeared in the literature. Among 83 species, the volatile oil

Table 1: Chemical constituents of essentials oils of *Stachys cretica* ssp. *lesbiaca* (Scre-les) and *S. cretica* ssp. *trapezuntica* (Scre-tra) obtained by hydrodistillation.

	Compound	RI	Scre-les (%)	Scre-tra (%)
1	α-Pinene	1032	8.6	0.7
2	α-Thujene	1035	tr	-
3	β-Pinene	1118	6.2	1.5
4	Sabinene	1132	0.3	-
5	Myrcene	1174	0.2	tr
6	α-Phellandrene	1176	tr	-
7	Limonene	1203	1.0	tr
8	β-Phellandrene	1218	0.7	-
9	(*Z*)-β-Ocimene	1246	1.0	tr
10	(*E*)-β-Ocimene	1266	0.3	tr
11	Nonanal	1400	0.2	0.5
12	1-Octen-3-ol	1452	-	0.4
13	α-Cubebene	1466	-	1.1
14	α-Copaene	1497	0.7	-
15	Linalool	1553	-	2.6
16	Linalyl acetate	1565	-	5.2
17	*cis*-Chrysanthenyl acetate	1582	4.8	-
18	β-Ylangene	1589	0.3	-
19	β-Elemene	1600	0.7	0.5
20	β-Caryophyllene	1612	9.5	0.9
21	Octyl 2-methyl butyrate	1634	0.4	tr
22	(*Z*)-β-Farnesene	1668	3.1	4.0
23	Sesquisabinene	1669	2.1	-
24	α-Humulene	1687	1.8	-
25	α-Terpineol	1706	-	0.4
26	Germacrene D	1726	20.3	12.9
27	β-Bisabolene	1741	2.8	1.6
28	Bicyclogermacrene	1755	0.7	0.6
29	(*E*,*E*)-α-Farnesene	1758	0.8	tr
30	*cis*-Chrysanthenol	1764	1.6	-
31	Geranylacetate	1765	-	2.1
32	γ-Cadinene	1733	0.7	1.6
33	*(E)*-α-Bisabolene	1784	0.4	-
34	(*E*)-β-Ionone	1958	-	0.7
35	2-Phenylethyl-2-methylbutyrate	1988	0.6	-
36	Caryophyllene oxide	2008	2.9	0.5
37	(*E*)-Nerolidol	2050	0.6	0.4
38	Germacrene D-4β-ol	2069	0.5	-
39	*cis*-Sesquisabinene hydrate	2096	0.2	tr
40	Hexahydrofarnesylacetone	2131	0.3	1.0
41	Spathulenol	2144	0.7	0.6
42	Valeranone	2145	0.4	1.0
43	β-Bisabolol	2170	0.2	-
44	3,4-Dimethyl-5-pentylidene-2(5H)-furanone	2179	0.3	0.5
45	T-Muurolol	2209	0.2	-
46	α-Bisabolol	2237	1.7	1.7
47	α-Cadinol	2255	0.2	0.9
48	Tricosane	2300	-	1.3
49	9-Geranyl-*p*-cymene	2312	-	4.9
50	Farnesyl acetone	2384	-	0.8
51	Pentacosane	2500	-	2.5
52	Phytol	2622	0.2	0.9
53	Tetradecanoic acid	2670	0.2	-
54	Heptacosane	2700	0.3	4.8
55	Octacosane	2800	-	7.2
56	Nonacosane	2900	0.4	4.9
57	Hexadecanoic acid	2931	1.3	3.5
	Monoterpene Hydrocarbons		**18.3**	**2.2**
	Oxygenated Monoterpenes		**6.4**	**10.7**
	Sesquiterpene Hydrocarbons		**45.3**	**24.1**
	Oxygenated Sesquiterpenes		**7.6**	**5.9**
	Fatty acids+esters		**1.5**	**3.5**
	Diterpenes		**0.2**	**5.8**
	Others		**2.5**	**24.2**
	Identified compounds		**81.8**	**76.4**

Components listed in order of elution from an Innowax FSC column.
- , Not detected; tr, Trace amount (< 0.1%).
RI, experimental retention indices on the Innowax FSC column.

composition of *S. lavandulifolia* var. *lavandulifolia* [5a], *S. recta*, *S. balansae* [5b], *S. obliqua*, [5c], *S. athorecalyx* [5d], *S. iberica* subsp. *stenostachya* [5e], *S. aleurites* [5f,5g], *S. laetivirens* [5h] and *S. pinardii* [5g] have been defined. *S. cretica*, in subsection Creticae, possesses ten subspecies of which six are endemic [1a,1g]. Herbal tea prepared from the aerial parts of *S. cretica* subsp. *anatolica* and *S. cretica* subsp. *mersinaea* are used for the treatment of colds and stomach ailments in central Anatolia [6a]. The essential oil composition of three *S. cretica* subspecies, namely *S. cretica* ssp. *mersinaea* [5g], *S. cretica* ssp. *anatolica* [6b] and *S. cretica* ssp. *symrnaea* [6c], have previously been investigated.

S. cretica ssp. *lesbiaca* Rech. fil. and *S. cretica* ssp. *trapezuntica* Rech. fil. are two of these endemic subspecies and, to our knowledge, there is no report on either their essential oil composition, or their antimicrobial and cytotoxic activities. Thus, we aimed to analyze the essential oil composition and to evaluate the antimicrobial and antiproliferative activities of different extracts obtained from the aerial parts.

In total 63 compounds in the two subspecies essential oils were identified; 48 for the oil of *S. cretica* ssp. *lesbiaca* (Scre-les) and 41 for the oil of *S. cretica* ssp. *trapezuntica* (Scre-tra). The retention indices with the percentage compositions are given in Table 1. Germacrene D (20.3%) was the main constituent of Scre-les oil, together with β-caryophyllene (9.5%), α-pinene (8.6%), β-pinene (6.2%) and *cis*-chrysanthenyl acetate (4.8%). The most abundant components were sesquiterpenes (52.9%), particularly hydrocarbon sesquiterpenes (45.3%), represented principally by germacrene D, followed by monoterpene hydrocarbons (18.3%), among which α-pinene (8.6%) and β-pinene (6.2%) prevailed.

The essential oil of *S. cretica* ssp. *trapezuntica* also consisted mainly of sesquiterpenes hydrocarbons, but with a considerably reduced percentage (24.1%). The levels of several sesquiterpene hydrocarbons, such as germacrene D (12.9%) and (Z)-β-farnesene (4.0%) were significant. Linalool (2.6%), linalyl acetate (5.2%) and geranyl acetate (2.1%) were the main oxygenated monoterpenes, whereas α-bisabolol (1.7%) was the main oxygenated sesquiterpene. 9-Geranyl-*p*-cymene (4.9%) was identified as a major diterpene, followed by phytol (0.9%). Among other compounds, besides terpenoids, the oils also contained considerable amounts of acyclic and aromatic carbonylic compounds, fatty acids and alcohols (24.2%); octacosane (7.2%), nonacosane (4.9%), hexadecanoic acid (3.5%), and 1-octen-3-ol (0.4%) being the main components. An unidentified diterpenoid $C_{20}H_{32}$ (14.9%) was also

Table 2: Major constituents of the essential oils of *Stachys cretica* ssp. *lesbiaca* (Scre-les), *S. cretica* ssp. *trapezuntica* (Scre-tra), *S. cretica* ssp. *mersinaea* (Scre-mer) [5c], *S. cretica* ssp. *symrnaea* (Scre-sym) [5g], *S. cretica* ssp. *anatolica* (Scre-ana) [5f], *S. cretica* ssp. *cretica* (Scre-cre) [5h] and *S. cretica* ssp. *vacillans* (Scre-vac) [6a] obtained by hydrodistillation.

Compound	Scre-les	Scre-tra	Scre-mer	Scre-sym	Scre-ana	Scre-cre	Scre-vac
Germacrene D	**20.3**	**12.9**	2.14	**32.8**	*	**33.5**	**9.5**
β-Caryophyllene	9.5	-	-	-	*	-	-
α-Pinene	8.6	-	-	0.2	*	-	tr
β-Pinene	6.2	-	-	0.04	*	-	0.4
Octacosane	-	7.2	-	-	*	-	-
Linalyl acetate	-	5.2	-	-	*	-	-
Nonacosane	0.4	4.9	-	-	*	-	-
α-Curcumene	-	-	**34.1**	-	*	-	-
Tetradecanol	-	-	6.2	-	*	-	-
(*Z*)-β-Caryophyllene	-	-	4.8	-	*	-	-
Caryophyllene oxide	2.9	0.5	-	1.4	*	-	2.1
Caryophyllene dioxide	-	-	3.9	-	*	-	-
trans-β-Caryophyllene	-	-	-	**51.0**	*	-	-
α-Humulene	1.8	-	-	3.1	*	-	0.5
β-Elemene	0.7	0.5	-	2.1	*	1.1	-
δ-Cadinene	0.7	1.6	-	2.1	*	1.6	-
Carvacrol	-	-	-	-	**33.5**	-	2.2
Pimaradiene	-	-	-	-	*	**18.6**	-
Hexadecanoic acid	1.3	1.5	-	-	*	-	**17.2**
(Z,Z)-9,12-Octadecadienoic	-	-	-	-	*	-	8.1
Spathulenol	0.7	0.6	-	-	*	0.4	6.1
4-Vinylguaiacol	-	-	-	-	*	-	5.8
Pulegone	-	-	-	-	*	-	3.0
Monoterpene hydrocarbons	18.3	2.2	-	0.4	*	-	0.3
Oxygenated monoterpenes	6.4	10.7	-	0.2	33.5*****	0.3	4.6
Sesquiterpene hydrocarbons	45.3	24.1	42.1	92.3	*	49.9	21.3
Oxygenated sesquiterpenes	7.6	5.9	5.3	2.9	*	14.5	14.6
Total identified	**81.8**	**76.4**	**56.5**	**99.7**	**33.5 ***	**84.5**	**91.8**

* This subspecies has been investigated only for its carvacrol content

Table 3: Cytotoxic activities of ethanolic extracts prepared from two *Stachys* subspecies.

Plant extracts		Inhibition of cell proliferation %±SEM					
		HL-60			Ishikawa		
		24h	**48h**	**72h**	**24h**	**48h**	**72h**
Scre - les	**200 µg/mL**	23±0	26±0	40±1	46±0	55±1	56±1
	100 µg/ mL	59±0	62±1	83±0	66±0	64±1	64±1
	10 µg/ mL	77±1	62±0	69±1	104±0	96±0	93±0
	1 µg/ mL	86±1	93±0	97±1	114±1	103±0	97±1
Scre-tra	**200 µg/ mL**	33±1	52±0	19±1	41±0	52±0	54±1
	100 µg/ mL	53±1	61±1	34±0	61±1	60±0	60±1
	10 µg/ mL	86±1	111±1	80±2	101±0	92±1	89±1
	1 µg/ mL	137±0	152±1	92±1	111±1	98±1	93±1
LiCl	**8 µg/ mL**	53±1	52±1	41±1	51±1	50±1	49±1

HL-60: Human promyelocytic leukemia cell line, Ishikawa: Human endometrial adenocarcinoma Ishikawa. Data are expressed as mean (%) ±SEM of three independent experiments (n:6). LiCl (8 µg/mL) was used as positive control

abundant in the oil of *S. cretica* ssp. *trapezuntica*. The mass spectrum was consistent with that of pimaradiene, a compound found in *S. cretica* ssp. *cretica*, but the retention index (RI = 2183) was not in agreement with that reported in the literature (RI = 1942) [6d].

A summary of the main components found in essential oils of *S. cretica* subspecies is given in Table 2 in order to compare our results with those of previous studies. Sesquiterpene hydrocarbons were the main group of constituents in the different essential oils of the *S. cretica* subspecies. The concentrations of sesquiterpene hydrocarbons in *S. cretica* ssp. *cretica* (49.9%) from Greece [6d] and *S. cretica* ssp. *vacillans* (21.3%) from Italy [6e] were similar to that obtained for *S. cretica* ssp. *lesbiaca* (45.3%), *S. cretica* ssp. *trapezuntica* (24.1%) and *S. cretica* ssp. *mersinaea* (42.1%) from Turkey [5g]. Scre-les, Scre-tra, and Scre-cre essential oils have in common germacrene D as the major constituent, which is also present in low percentages in Scre-mer and Scre-vac oils. In addition, Scre-sym has a higher content of *trans*-β-caryophyllene and Scre-mer has α-curcumene as its most abundant compound. Scre-ana was investigated only for its carvacrol content, which separated it from other subspecies by its high content of this compound, which was also present in the oil of Scre-vac, but only in a small amount.The above mentioned differences in the volatiles from *S. cretica* subspecies could also be helpful in their taxonomic characterization. However, it should be considered that the chemical composition of essential oils may vary according to several environmental (climatic, seasonal, geographical) conditions, as well as genetic differences [7a]. The *in vitro* antibacterial effects of the extracts

towards the tested bacteria were studied via their MIC values. The ethanol, light petroleum, dichloromethane, ethyl acetate and *n*-butanol extracts prepared from the aerial parts of the two subspecies showed no activity against any of the bacteria tested. However, we have noted that the light petroleum and *n*-butanolic fractions showed weak activities against *Candida albicans* ATCC 10231 (MIC = 625 μg/mL). Similar results have been reported for different extracts of *Stachys* species. An example is the chloroform subfraction of *S. inflata*, which exhibiting low anticandidal activity (MIC = 250 μg/mL) against *C. albicans* [4b].

The ethanolic extracts of the *Stachys* species were tested for their ability to inhibit the growth of two human tumor cell lines. After every 24 h of treatment for 72 h, the cytotoxicity via counting total cell number was determined. The cytotoxic effects of these two cell lines on the growth of human tumor cell lines are given in Table 3. Scre-les and Scre-tra extracts modulated cell growth in concentration and cell type dependent manner. The IC_{50} values of Scre-les and Scre-tra were determined as 100 μg/mL for the HL-60 cell line and 200 μg/mL for the Ishikawa cell line. In previous studies, the methanol stem extract of *S. recta* has been found to be active against the MCF7 breast carcinoma cell line [7b]. The essential oils obtained from six different *Stachys* species also exerted cytotoxicity [6e]. Scre-tra exhibited weak cytotoxicity and/or increased cell proliferation on the HL-60 cell line at low concentrations (1 μg/mL and 10 μg/mL). The cytotoxicity was also determined at lower concentrations of Scre-les, especially at 1 μg/mL, but no cell proliferation was determined. Scre-les exhibited weak cytotoxicity on the Ishikawa cell line in comparison with the Scre-tra extract. Scre-les at 1 μg/mL and 10 μg/mL concentrations increased Ishikawa cell proliferation after 48 h and 24 h, respectively. In addition, Scre-tra extract at 1 μg/mL and 10 μg/mL increased Ishikawa cell proliferation only at the 24^{th} h. Both subspecies showed weak cytotoxicity towards the Ishikawa cell line in comparison with HL-60 cells. The results suggest moderate antiproliferative properties and support the ethnomedical claims for the genus.

Experimental

Plant materials: Aerial parts of *S. cretica* ssp. *lesbiaca* were collected in June 2009 in Çanakkale, Dağahmetçe village, and aerial parts of *S. cretica* ssp. *trapezuntica* in August 2009 in Rize, Ikizdere, Cimil region of Turkey by Dr Tuba Şerbetçi. Voucher specimens have been deposited in the Herbarium of the Faculty of Pharmacy (ISTE), Istanbul University (ISTE 86107 and ISTE 86110, respectively). The plants were identified by Assoc. Prof. Şükran Kültür, Department of Pharmaceutical Botany, Istanbul University.

Exract preparation: To evaluate their antimicrobial and cytotoxic activities crude extracts were prepared. Air-dried and powdered aerial parts of each plant material were mixed with ethanol and percolated for 2 days. The ethanolic solutions were filtered and evaporated under vacuum. The resulting extract was suspended in water and successively partitioned to provide light petroleum, dichloromethane, ethyl acetate and *n*-butanol fractions.

Essential oil isolation and identification: The essential oils from air-dried plant materials were isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus to produce a small amount of essential oil, which was trapped in *n*-hexane. The obtained oils were dried over anhydrous sodium sulfate and stored at +4°C in the dark until analyzed and tested. GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300°C. To obtain the same elution order for GC-MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms. The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. An Innowax FSC column (60 m x 0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 450. Identification of the essential oil components was carried out by comparison of their relative retention times with either those of authentic samples or by comparison of their relative retention index (RRI) with a series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, Adams Library, MassFinder 3 Library) [7c,7d], and in-house "Başer Library of Essential Oil Constituents" built up from genuine compounds and components of known oils, as well as MS literature data [8,9a,9b], was used for the identification.

Antimicrobial activity

Bacterial and fungal strains: The extracts obtained were tested against 6 standard bacteria and 1 standard yeast: Gram-negative bacteria were represented by *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153 and Gram-positive strains by *Staphylococcus aureus* ATCC 6538 and *S. epidermidis* ATCC 12228. The only fungal strain was *Candida albicans* ATCC 10231.

Minimum inhibitory concentration: The antibacterial and antifungal effects were determined by the microbroth dilution techniques using the Clinical Laboratory Standards Institute recommendations [9c,9d]. Serial two-fold dilutions ranging from 5000 μg/mL to 4.9 μg/mL of the extracts were prepared and the following media were used: Mueller-Hinton broth for growing *Staphylococcus aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153 and RPMI-1640 medium buffered to PH 7.0 with MOPS for *Candida albicans* ATCC 10231 in a 96 well microplate. The inoculum was prepared using a 4-6 h broth culture of each bacterium and 24 h culture of yeast strains adjusted to a turbidity equivalent to a 0.5 McFarland standard, diluted in broth media to give a final concentration of 5×10^5 CFU/mL for bacteria and 0.5×10^3 to 2.5×10^3 CFU/mL for yeast in the test microplates. The microplates were covered and placed in plastic bags to prevent evaporation. The microplates containing Mueller-Hinton broth were incubated at 35°C for18-20h and the trays containing RPMI-1640 medium were incubated at 35°C for 46-50 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of compound giving complete inhibition of visible growth. As control, antimicrobial effects of the solvents were investigated against test microorganisms. According to values of the controls, the results were evaluated.

Cytotoxicity assay

Cell culture: The human promyelocytic leukemia cell line HL-60 (ATCC No:CCL-240) and human endometrial adenocarcinoma Ishikawa (Sigma no: 99040201) cell lines were used in this experiment. The cell lines were cultured in RPMI 1640 medium and supplemented with 10% fetal bovine serum, 1% L-glutamine,1% non-essential amino acids and 100 units/mL penicillin and streptomycin (Sigma Chemical Co., St Louis, Missouri). Following trypan blue exclusion assay, 1×10^5 cells per well were seeded in 24-well microtiter plates. Ishikawa cells were incubated for 24 h to allow for cell attachment and HL-60 cells were seeded on the experiment day. The cells were treated with serial concentrations of the samples. Twenty μL per well of each concentration (n:6) was added to the plates in 3 replicates to obtain final concentrations of 1,10, 100, 200 μg/mL, and lithium chloride (LiCl) at 8 μg/mL as a positive control. By these serial dilutions, the final mixture used for treating the cells contained not more than 0.5% of the solvent (dimethyl sulfoxide), the same as in the solvent control wells. The culture plates were kept at 37°C with 5% (v/v) CO_2 for 72 h. After every 24 h of incubation, total cell numbers were counted using a cell counter, and recorded [9e].

Statistical analysis: All the results of cell proliferation were statistically analyzed using the independent Student's *t*-test. Data were represented as mean ± standard error mean (SEM) and at least in triplicate. Results were considered significant with $p < 0.05$. Cytotoxicity was expressed as the 50% inhibitory concentration (IC_{50}), which is the concentration needed to reduce the cell number of treated cells by 50% with reference to the control (untreated cells). IC_{50} was calculated from the Prism dose–response curve (statistical program) obtained by plotting the percentage of inhibition versus the concentrations.

Acknowledgments - This work was supported by the Scientific Research Foundation of Istanbul University Project no: 3784 and UDP 7069.

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