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Phytochemical composition, anti-inflammatory and antitumour activities of four *Teucrium* essential oils from Greece

Federica Menichini [a], Filomena Conforti [b,*], Daniela Rigano [c], Carmen Formisano [c], Franco Piozzi [d], Felice Senatore [c]

[a] *Pharmacognosy Research Laboratories, Department of Pharmacy, King's College London, Franklin–Wilkins Building, 150 Stamford St., London SE1 9NN, UK*
[b] *Department of Pharmaceutical Sciences, University of Calabria, via Pietro Bucci, I-87036 Rende (CS), Italy*
[c] *Department of Chemistry of Natural Compounds, University of Naples "Federico II", Via D. Montesano, 49, I-80131 Naples, Italy*
[d] *Department of Organic Chemistry, University of di Palermo, Viale delle Scienze, Parco d'Orleans II, I-90128 Palermo, Italy*

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ABSTRACT

The essential oils of four *Teucrium* species were studied and 150 components, in all, were identified. All oils were rich in sesquiterpenes (50.1–55.8%). Spathulenol and δ-cadinene were the main compounds of *Teucrium brevifolium* oil; caryophyllene and 4-vinyl guaiacol predominated in *Teucrium flavum*. Carvacrol and caryophyllene oxide predominated in *Teucrium montbretii* ssp. *heliotropiifolium*, while carvacrol and caryophyllene were the most abundant components in *Teucrium polium* ssp. *capitatum*. The oil which most effectively inhibited LPS-induced NO production in macrophage cell line RAW 264.7 was that from *T. brevifolium* (IC_{50} = 7.1 μg/ml), followed by *T. montbretii* ssp. *heliotropiifolium* and *T. polium* ssp. *capitatum* (IC_{50} = 16.5 and 29.4 μg/ml, respectively). The *in vitro* cytotoxic assay on three human cancer cell lines showed that the most antiproliferative oils were those from *T. polium* ssp. *capitatum* and *T. montbretii* ssp. *heliotropiifolium* on CACO-2 cell lines (IC_{50} = 52.7 and 92.2 μg/ml, respectively). The *T. brevifolium* oil showed a selective cytotoxicity on COR-L23 while significant activity was exerted by *T. polium* oil on C32.

1. Introduction

Many new drugs derived from plant secondary metabolites have been applied for the treatment and/or prevention of various diseases. Investigations about natural products have recently regained prominence with the increasing understanding of their biological significance and increasing recognition of their origin and structural diversity. Most clinically important medicines are steroidal or non-steroidal anti-inflammatory chemical therapeutics for treatment of inflammation-related diseases. Though these have potent activity, long-term administration is required for treatment of chronic disease. Furthermore, these drugs have various and severe adverse effects. Therefore, naturally occurring agents, with high effectiveness and very few side-effects, are desirable as substitutes for chemical therapeutics.

Nitric oxide (NO) is a diatomic free radical produced from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues. Nitric oxide (NO), superoxide (O^{2-}) and their reaction product peroxynitrite ($ONOO^-$) may be generated in excess during the host response against viral and antibacterial infections and contribute to pathogenesis by promoting oxidative stress, tissue injury and, even, cancer (Maeda & Akaike, 1998). Since 1990, there has been a 22% increase in cancer incidence and mortality, with over 10 million new cases and over six million deaths worldwide in 2000 (excluding non-melanoma skin). Important progress has been made in cancer chemotherapy, a considerable portion of which can be attributed to plant-derived drugs. The search for more effective and safer anti-inflammatory and cytotoxic compounds has continued to be an important area of active research and, after the recommendations made by WHO, investigation of anti-inflammatory and cytotoxic compounds from medicinal plants has become important.

Aromatic plants are frequently used in traditional medicine and essential oils and volatile constituents extracted from them are widely used as antioxidants and antidiabetic agents and for the prevention and treatment of different human diseases, such as cancer, cardiovascular diseases, including atherosclerosis and thrombosis, bacterial and viral infections (Edris, 2007).

Teucrium (Lamiaceae) is a cosmopolitan genus that differs from other related genera in that its corolla is formed of one lip. It

* Corresponding author. Tel.: +39 0984 493063; fax: +39 0984 493298.
E-mail address: filomena.conforti@unical.it (F. Conforti).

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comprises more than 300 species; almost 50 are known in Europe and are distributed mainly in the Mediterranean basin (Tutin et al., 1976). Plants of this genus have been used medicinally since ancient Greek times, when they were used for coughs and asthma; most of these are bitter, astringent, anti-rheumatic herbs that were used as an antispasmodic for gastric ulcer and intestinal inflammation, to stimulate the digestion, lower fever, and as diuretic, antiseptic, antipyretic and antihelmintic agents. Many species show interesting biological properties, e.g., analgesic, hypoglycaemic, hypolipidemic, antipyretic, anti-inflammatory, antiulcer, antioxidant and antibacterial (Abdollahi, Karimpour, & Monsef-Esfehani, 2003; Barrachina, Bello, Martinez-Cuesta, Esplugues, & Primo-Yufera, 1995; Bellomaria, Arnold, & Valentini, 1998; Panovska, Kulevanova, Gjorgoski, Bogdanova, & Petrushevska, 2007; Saracoglu, Calis, Inoue, & Ogihara, 1997; Tariq, Ageel, al-Yahya, Mossa, & al-Said, 1989; Ulubelen, Topcu, & Sonmez, 2000; Özkan, Kuleaşan, Çelik, Göktürk, & Ünal, 2007).

Maccioni, Baldini, Tebano, Cioni, and Flamini (2007) noted the importance of *Teucrium* species as alimentary plants, as some of them are currently used in the preparation of flavoured wines, herbal teas, bitters and liqueurs and infusions of leaves and flowers are used for flavouring beer in some areas. The importance of this genus in food industries lies also in the fact that many species show antimicrobial, antioxidant and antifungal activities, rendering them useful as natural preservative ingredients (Saroglou, Arfan, Shabir, Hadjipavlou-Litina, & Skaltsa, 2007; Ulubelen et al., 2000; Özkan et al., 2007).

The genus *Teucrium* is one of the richest sources of diterpenes, with a neoclerodane skeleton: more than 220 diterpenes have been described up to now, and many of these are particularly interesting because of their ecological role as antifeedants against different species of insects and for their role in the medicinal properties of the plants (Piozzi, Bruno, Rosselli, & Maggio, 2005). As in other Lamiaceae, the aerial organs of *Teucrium* spp. are covered by an indumentum of glandular and non-glandular trichomes. So far, essential oils have been reported from the aerial parts of several *Teucrium* spp. The oils have different yields in the various species, ranging between 0.5% and 1.5%, and the percentage of the major chemical constituents (mainly monoterpene/sesquiterpenes hydrocarbons and oxygenated sesquiterpenes) differs notably from species to species (Saroglou et al., 2007).

Teucrium brevifolium Schreber is 60 cm high and has small violet–purple flowers with a persistent aroma. Nine diterpenoids of the neoclerodane type (teubrevins A–I) have been isolated from the aerial parts of this plant (Piozzi et al., 2005) while the essential oil has never been studied.

Teucrium flavum L. is an evergreen perennial subshrub (up to 60 cm high), known in Greece as "Chamaidrya", "Moskhokhortaro" and "Dontokhorti". It is characterised by pubescent stems and yellow corolla assembled in terminal spikes. An infusion of the aerial parts has been used in folk medicine, orally as an antidiabetic and externally as an astringent, to heal skin eruptions and wounds (Bellomaria et al., 1998). Different extracts showed significant anti-inflammatory effects (Barrachina et al., 1995). The chemical composition of *T. flavum*, in particular the presence of diterpenoids, has been previously reported (Piozzi et al., 2005). The essential oils from plants growing in Croatia, Corsica, Serbia and Montenegro, Iran and Greece (East Thessaly) were also studied (Baher & Mirza, 2003; Bellomaria et al., 1998; Kovacevic, Lakusic, & Ristic, 2001; Stanic, Petricic, Blazevic, & Plazibat, 1993).

Teucrium montbretii Benth. ssp. *heliotropiifolium* (Barbey) Davis is an evergreen dwarf (5–20 cm high) semishrub–chasmophytes with lignified branches, opposite and ovate leaves. The different subspecies of *T. montbretii* show different qualitative contents of neoclerodane diterpenoids; the subspecies *heliotropifolium* contains 6β-hydroxyteuscordin, montanin D and two other neoclerodanes, namely, teugin and teucrin H_2 (Piozzi et al., 2005). The antioxidant and antimicrobial activities of *T. montbretii* Bentham subsp. *pamphylicum* P.H. Davis (Lamiaceae) methanolic extracts were recently investigated (Özkan et al., 2007). The essential oil has never been previously studied.

Teucrium polium L. ssp. *capitatum* (L.) Arcangeli (syn *Teucrium capitatum* L. or felty germander) belongs to the section *Polium* that is the largest and most morphogically diverse section of the genus (El Oualidi, Verneau, Puech, & Dubuisson, 1999). It is a perennial shrub, 20–50 cm high, widely distributed in the hills and deserts of Mediterranean countries. The bruised foliage releases a pleasant aromatic scent and the flowers are small, in clusters and range from pink to white. The plant is mixed with boiled water and sugar to form a refreshing beverage. The leaves are used in cooking as a spice (Facciola, 1990). *T. polium* has been used for over 2000 years in traditional medicine for various types of pathological conditions, such as gastrointestinal disorders, inflammations, diabetes and rheumatism (Abdollahi et al., 2003; Tariq et al., 1989). It is also used as antibacterial, antiulcer, hypotensive, antispasmodic, anorexic and antipyretic agents. The plant possesses hypoglycaemic and insulinotropic activities, reduces body weight and lowers high blood pressure and has hypolipidemic, antinociceptive and antioxidant properties, comparable to that of α-tocopherol (Panovska et al., 2007). Recently, the evaluation of cytotoxic effects of an ethanol extract of *T. polium*, on four cell lines, showed that the extract suppressed the growth of all tested cell lines (Nematollahi-Mahani, Rezazadeh-Kermani, Mehrabani, & Nakhaee, 2007). Phytochemical investigations have shown that *T. polium* contains various compounds, such as iridoids, flavonoids and diterpenoids (Piozzi et al., 2005). The composition of the essential oil of *T. polium* has been the subject of several investigations, recently summarised by Cozzani et al. (2005). Some chemical differences in the oil compositions were reported, probably related to the different subspecies and/or the geographical origin of the plants. As specifically regards the subspecies *capitatum*, two reports describe the composition of essential oils from plants of Corsica (Cozzani et al., 2005) and Portugal (Antunes, Sevinate-Pinto, Barroso, Cavaleiro, & Salgueiro, 2004).

Since the chemical composition of volatile oils from *Teucrium* spp. depends on various environmental conditions, the aim of this work was to investigate the volatiles of four *Teucrium* species from Greece by analysing their essential oils. Furthermore, a survey of the literature revealed that no studies on the potential anti-inflammatory and cytotoxic activity of essential oils of *Teucrium* spp. had yet been undertaken, so in this study we also investigated the plant essential oils for their biological properties.

2. Materials and methods

2.1. Plant material

Aerial parts of *T. brevifolium* were collected in July, 2006, on the road between the Ankassa and Menetes villages, 269 m above sea level (ASL), Karpathos Island, Greece. Aerial parts of *T. flavum* were collected in July, 2006, near Milopotamos, on the Pileon Mountain, Greece. The aerial parts of *T. montbretii* ssp. *heliotropiifolium* were collected in June, 2006, at Menetes, on limestone cliffs at Linoseli (Kakiskala), on the Karpathos Island of Greece. Aerial parts of *T. polium* ssp. *capitatum* were collected in June, 2006, at Gournes, about 15 km east of Heraklion-Crete. The identification was done by Prof. N.A. Arnold, University of Saint Esprit, Lebanon. Voucher specimens (No. PAL 232/06 for *T. brevifolium*, 233/06 for *T. flavum*, 234/06 for *T. montbretii* and 235/06 for *T. polium*) were deposited in the Herbarium of the Department of Organic Chemistry, University of Palermo, Italy.

2.2. Isolation of the essential oils

The oil from air-dried and ground aerial parts of plants was isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method recommended in the European Pharmacopoeia (2004). The oil was dried over anhydrous sodium sulphate and stored under N_2 at +4 °C in the dark until tested and analysed. All the oils were yellow with a pleasant smell. The yields (w/w) were as follows: 0.14% of for *T. brevifolium* (**B**); 0.10% for *T. flavum* (**F**); 0.10% for *T. montbretii* (**M**) and 0.35% for *T. polium* (**P**).

2.3. GC and GC–MS analyses

Analytical gas chromatography was carried out on a Perkin–Elmer Sigma 115 gas chromatograph fitted with a HP-5 MS capillary column (30 m × 0.25 mm id; 0.25 μm film thickness). Helium was the carrier gas (1 ml min^{-1}). Column temperature was initially kept at 40 °C for 5 min, then gradually increased to 250 °C at 2 °C min^{-1}, held for 15 min and finally raised to 270 °C at 10 °C min^{-1}. Diluted samples (1/100 v/v, in *n*-hexane) of 1 μl were injected manually at 250 °C, and in the splitless mode. Flame ionisation detection (FID) was performed at 280 °C. Analysis was also run by using a fused silica HP Innowax polyethyleneglycol capillary column (50 m × 0.20 mm id; 0.20 μm film thickness).

GC–MS analysis was performed on an Agilent 6850 Ser. II apparatus, fitted with a fused silica HP-1 capillary column (30 m × 0.25 mm id; 0.33 μm film thickness), coupled to an Agilent Mass Selective Detector MSD 5973; ionisation voltage 70 eV; electron multiplier energy 2000 V. Gas chromatographic conditions were as reported above; transfer line temperature was 295 °C. Analysis was also done by using a fused silica HP Innowax polyethyleneglycol capillary column (60 m × 0.25 mm id; 0.33 μm film thickness).

2.4. Qualitative and quantitative analyses

Most constituents were identified by gas chromatography by comparison of their retention indices (*I*) with either those of the literature (Davies, 1990; Jennings & Shibamoto, 1980) or with those of authentic compounds available in our laboratories. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8–C_{24}) under the same operating conditions. Further identification was done by comparison of their mass spectra on both columns with either those stored in NIST 02 and Wiley 275 libraries or with mass spectra from the literature (Adams, 2001; Jennings & Shibamoto, 1980) and our home-made library. Component relative concentrations were calculated from GC peak areas without using correction factors.

2.5. Cell line and cell culture

The murine monocytic macrophage cell line RAW 264.7 (ECACC No.: 91062702) was grown in plastic culture flask in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic/antimitotic solution (penicillin/streptomycin) under 5% CO_2 at 37 °C. After 4–5 days, cells were removed from the culture flask by scraping and centrifuging for 10 min at 1500 rpm. The medium was then removed and the cells were resuspended with fresh DMEM. Three cancer cell lines, namely large lung cell carcinoma COR-L23 (ECACC No.: 92031919), colorectal adenocarcinoma CACO-2 (ATCC No.: HTB-37), and amelanotic melanoma C32 (ATCC No.: CRL-1585), were used in this experiment. The COR-L23, C32 cells were cultured in RPMI 1640 medium, while CACO-2 cells were cultured in DMEM medium. Both media were supplemented with 10% foetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin.

Cell counts and viabilities were determined by using a standard trypan blue cell counting technique. The cell concentration was adjusted to 1×10^6 cells/ml for RAW 264.7 and 2×10^4 for COR-L23, C32 and CACO-2 in the same medium. About 100 μl of the above concentration were cultured in 96-well plate for one day until nearly confluent. Concentrations, ranging from 5 to 200 μg/ml of the samples, were prepared from the stock solutions by serial dilution in DMEM, to give a volume of 100 μl in each well of a microtitre plate (96-well). Then, cells were cultured with vehicle, essential oils for 48 h for human tumour cells and for 24 h in the presence of 1 μg/ml LPS for RAW 264.7.

2.6. Inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells

The presence of nitrite, a stable oxidised product of NO, was determined in cell culture media by Griess reagent (1% sulfanamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4) (Prakash, Ali, Bala, & Goel, 2005). About 100 μl of cell culture supernatant was removed and combined with 100 μl of Griess reagent in a 96-well plate, followed by spectrophotometric measurement at 550 nm using a microplate reader (GDV DV 990 B/V, Roma, Italy). Nitrite concentration in the supernatants was determined by comparison with a sodium nitrite standard curve.

2.7. Assay for cytotoxic activity

Cytotoxicity was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Italy) assay reported by Tubaro et al. (1996) with some modification. The assay for each concentration of samples was performed in triplicate and the culture plates were kept at 37 °C with 5% (v/v) CO_2 for one day. After incubation, 100 μl of medium were removed from each well. Subsequently, 100 μl of 0.5% w/v MTT, dissolved in phosphate-buffered saline, were added to each well and allowed to incubate for a further 4 h. After 4 h of incubation, 100 μl of DMSO were added to each well to dissolve the formazan crystals. Absorbance values at 550 nm were measured with a microplate reader (GDV DV 990 B/V, Roma, Italy). Cytotoxicity was expressed as IC_{50}, which is the concentration to reduce the absorbance of treated cells by 50% with reference to the control (untreated cells).

2.8. Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed by using the Student's *t*-test. Differences were considered significant at $P \leqslant 0.05$. The inhibitory concentration 50% (IC_{50}) was calculated from the Prism dose–response curve (Prism Graphpad, Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA), obtained by plotting the percentage of inhibition versus the concentration.

3. Results and discussion

3.1. Composition of the essential oils

In the four oils, 150 compounds in all were identified (Table 1): 61 for **B** (92.1% of the total oil), 58 for **F** (95.7% of the oil), 56 for **M** (91.7% of the oil) and 70 for **P** (92.5% of the oil). The retention indices, percentage composition and identification methods are given in Table 1, where the components are listed in order of elution from a HP-5MS column.

In **B**, the most abundant compounds were spathulenol (9%) and δ-cadinene (4.2%). On the whole, the oil was constituted mainly of

Table 1
Chemical composition of the essential oils of *Teucrium brevifolium* (**B**), *Teucrium flavum* (**F**), *Teucrium montbretii* (**M**) and *Teucrium polium* (**P**).

R_i[a]	R_i[b]	Compound	Identif[c]	% **B**[d]	% **F**[d]	% **M**[d]	% **P**[d]
938	1076	α-Pinene	R_i, MS, Co-GC	2.8			0.2
973	1132	Sabinene	R_i, MS				0.5
977	1452	1-Octen-3-ol	R_i, MS	0.7	0.3		
978	1118	β-Pinene	R_i, MS, Co-GC	3.6			0.2
993	1174	Myrcene	R_i, MS, Co-GC	0.7			
1012		α-Terpinene	R_i, MS, Co-GC				t
1025	1278	*p*-Cymene	R_i, MS, Co-GC			t	0.3
1029	1218	β-Phellandrene	R_i, MS, Co-GC	2.8			
1030		Limonene	R_i, MS, Co-GC			t	
1038		(*Z*)-β-Ocimene	R_i, MS				t
1048		Phenylacetaldehyde	R_i, MS, Co-GC			t	
1062	1450	*cis*-Linalool oxide, furanoid	R_i, MS				0.4
1063	1555	*cis*-Sabinene hydrate	R_i, MS				0.3
1076		*trans*-Linalool oxide, furanoid	R_i, MS			t	0.2
1086	1265	Terpinolene	R_i, MS		1.3		
1093		*trans*-Sabinene hydrate	R_i, MS				0.2
1098	1553	Linalool	R_i, MS		3.4	3.5	0.8
1099		Isoamyl isovalerate	R_i, MS		0.6		
1113		Phenyl ethyl alcohol	R_i, MS		0.1		
1128	1489	α-Campholenal	R_i, MS	0.4			
1132	1653	*cis*-Pinocarveol	R_i, MS		1.2		
1135	1789	*cis*-Sabinol	R_i, MS				1.3
1136	1597	Nopinone	R_i, MS				0.5
1138	1654	*trans*-Pinocarveol	R_i, MS	3.8	0.9		
1144	1663	*cis*-Verbenol	R_i, MS		0.8		2.1
1148	1737	Veratrole	R_i, MS		2.5		
1155	1652	Sabina ketone	R_i, MS				0.6
1165		4-Vinyl anisole	R_i, MS		0.7		
1165	1587	Pinocarvone	R_i, MS	1.2			
1167	1719	Borneol	R_i, MS, Co-GC	0.2			0.1
1175	1656	Umbellulone	R_i, MS				0.6
1176	1611	Terpineol-4	R_i, MS, Co-GC		0.3	t	0.7
1189	1706	α-Terpineol	R_i, MS, Co-GC				0.6
1193	1648	Myrtenal	R_i, MS	2.7			t
1196	1804	Myrtenol	R_i, MS	1.4	1.4		1.0
1204	1723	*cis*-Verbenone	R_i, MS	0.9		0.1	3.7
1208		α-Ionene	R_i, MS			0.1	
1217	1845	*trans*-Carveol	R_i, MS	0.6	0.4		
1223		β-Cyclocitral	R_i, MS			t	
1226	1878	*cis*-Carveol	R_i, MS				0.6
1232		*p*-Anisaldehyde	R_i, MS, Co-GC		0.3		
1232	1804	Cumin aldehyde	R_i, MS				0.2
1241	1752	Carvone	R_i, MS	0.4	0.3		0.3
1249		Isoamyl hexanoate	R_i, MS		0.8		
1255	1857	Geraniol	R_i, MS, Co-GC			0.5	
1275	1741	Phellandral	R_i, MS	0.8			
1285	2115	*p*-Cymen-7-ol	R_i, MS	0.4	0.4		1.0
1290	2198	Thymol	R_i, MS, Co-GC			0.6	0.1
1297	2239	Carvacrol	R_i, MS, Co-GC			13.9	10.1
1302	1797	*p*-Methoxyacetophenone	R_i, MS, Co-GC	0.8			2.2
1313	2180	4-Vinyl guaiacol	R_i, MS		9.7		
1335	1476	δ-Elemene	R_i, MS	0.7			
1351		α-Longipinene	R_i, MS				0.1
1352	1466	α-Cubebene	R_i, MS	0.3	0.3	0.4	
1353	2186	Eugenol	R_i, MS, Co-GC	0.8	0.8	0.7	0.4
1363		Cyclosativene	R_i, MS				t
1372	1493	α-Ylangene	R_i, MS	0.3		0.1	
1377	1497	α-Copaene	R_i, MS	0.7	2.8		0.7
1380	1835	(*E*)-β-Damascenone	R_i, MS	1.9	0.7	0.2	
1382	1547	β-Cubebene	R_i, MS			0.2	0.1
1385	1535	β-Bourbonene	R_i, MS	1.8	3.1	3.5	0.3
1387	1600	β-Elemene	R_i, MS	0.9			0.3
1391		Cyperene	R_i, MS	0.4			
1396		α-Elemene	R_i, MS			0.2	
1408	1529	α-Gurjunene	R_i, MS	1.5			1.4
1409	1510	α-Funebrene	R_i, MS				0.2
1418	1612	Caryophyllene	R_i, MS		12.2	7.8	9.8
1422		$C_{10}H_{14}$	R_i, MS			0.8	
1423	1612	β-Gurjunene (Calarene)	R_i, MS	0.5			0.2
1424	1638	β-Cedrene	R_i, MS			0.6	
1435	1573	(*Z*)-α-*trans*-Bergamotene	R_i, MS		2.1		
1436	1650	γ-Elemene	R_i, MS				0.4
1437	1674	β-Humulene	R_i, MS				0.4
1437	1628	Aromadendrene	R_i, MS	1.7			
1455	1689	α-Humulene	R_i, MS		6.0	2.4	3.8

Table 1 (*continued*)

R_i[a]	R_i[b]	Compound	Identif[c]	% **B**[d]	% **F**[d]	% **M**[d]	% **P**[d]
1463	1661	*allo*-Aromadendrene	R_i, MS	0.1	1.5	3.0	0.4
1473	16,876	γ-Gurjunene (5,11-Guaiadiene)	R_i, MS	0.4			
1475	1715	β-Selinene	R_i, MS	1.7		0.3	
1477	1726	Germacrene D	R_i, MS		2.8	2.4	3.1
1478	1704	γ-Muurolene	R_i, MS	2.5	1.5	0.8	
1482	1957	(*E*)-β-Ionone	R_i, MS, Co-GC		0.8		
1483	1784	*ar*-Curcumene	R_i, MS				0.5
1485	1675	Epizonarene	R_i, MS	2.7			
1486	2355	Dihydroactinidiolide	R_i, MS			1.0	
1487	1679	α-Amorphene	R_i, MS	0.3		0.4	3.0
1490	1694	*cis*-β-Guaiene	R_i, MS				1.2
1490		Valencene	R_i, MS			0.2	0.2
1491	1756	Bicyclogermacrene	R_i, MS			0.4	0.7
1498		*epi*-Bicyclosesquiphellandrene	R_i, MS		0.6	1.6	0.5
1503	1740	α-Muurolene	R_i, MS		0.3	0.2	
1506	1760	(*E*, *E*)-α-Farnesene	R_i, MS		0.9		
1510	1743	β-Bisabolene	R_i, MS		0.7		
1513	1709	Ledene	R_i, MS		0.3		
1515	1776	γ-Cadinene	R_i, MS	0.6	0.4		
1520		1-*endo*-Bourbonanol	R_i, MS			0.4	
1526	1773	δ-Cadinene	R_i, MS	4.2	2.9	2.0	2.9
1538	1799	Cadina-1,4-diene	R_i, MS				0.4
1541	1918	α-Calacorene	R_i, MS	1.8	0.6		0.3
1550	1942	β-Calacorene	R_i, MS	1.2			
1553	2076	*cis*-α-Copaen-8-ol	R_i, MS	0.7			
1554	1856	Germacrene B	R_i, MS	0.3			0.7
1565	2057	Ledol	R_i, MS	0.7		1.2	
1577	2069	Germacrene D 4-ol	R_i, MS				2.7
1578	2150	Spathulenol	R_i, MS	9.0			
1580	2008	Caryophyllene oxide	R_i, MS, Co-GC	3.8	7.9	12.7	
1588	2098	Globulol	R_i, MS			1.5	0.3
1591	2104	Viridiflorol	R_i, MS	3.4	2.2	0.2	
1598	2108	Guaiol	R_i, MS		1.0		
1605	2071	Humulene epoxide II	R_i, MS		2.4		1.4
1606	2133	Cedrenol	R_i, MS	0.5			0.1
1608	2098	β-Oplopenone	R_i, MS		0.4		
1635		Isospathulenol	R_i, MS	2.5			
1640	2316	Caryophylladienol I	R_i, MS		0.5	3.7	1.3
1642	2209	T-Muurolol	R_i, MS			1.0	
1645		Torreyol	R_i, MS			0.1	7.6
1650	2258	β-Eudesmol	R_i, MS	3.4			
1652	2255	α-Cadinol	R_i, MS		1.0	0.6	4.5
1653	2252	α-Eudesmol	R_i, MS	2.5			
1653		Ledene alcohol	R_i, MS	0.7			
1664		Patchoulol	R_i, MS			0.4	
1669	2219	α-Bisabolol	R_i, MS, Co-GC	1.3			
1675		(*Z*)-α-Bisabolene epoxide	R_i, MS	1.6			
1677	2256	Cadalene	R_i, MS	3.4		0.2	1.1
1689		Isolongifolen-5-one	R_i, MS	0.3			
1691		Vulgarol B	R_i, MS		0.1	1.6	
1698		*cis*(*Z*)-α-Bisabolene-epoxide	R_i, MS				0.2
1723		Tetradecanoic acid methyl ester	R_i, MS, Co-GC		0.1		
1762	2655	Benzyl benzoate	R_i, MS, Co-GC		0.8		
1766	2287	Aristolone	R_i, MS	0.5			
1815		Hexadecanal	R_i, MS	0.7			
1845	2131	Hexahydrofarnesylacetone	R_i, MS	1.8	2.6	1.5	0.6
1923	2814	Phenanthrene	R_i, MS, Co-GC	1.5			
1929	2208	Hexadecanoic acid methyl ester	R_i, MS, Co-GC		1.9		
1950	2622	(*Z*)-Phytol	R_i, MS		1.9		
1957	2931	Hexadecanoic acid	R_i, MS, Co-GC		1.2	9.8	
1963		13-*epi*-Manoyl oxide	R_i, MS			t	
2037	2388	Octadecanal	R_i, MS	0.9			
2048	2399	Kaurene	R_i, MS				0.6
2092		(*Z*)-9-Octadecenoic acid methyl ester	R_i, MS, Co-GC		0.6		
2122	3157	(*Z*, *Z*)-9,12-Octadecadienoic acid	R_i, MS, Co-GC			1.9	
2300	2300	Tricosane	R_i, MS, Co-GC		0.5		
2400	2400	Tetracosane	R_i, MS, Co-GC		0.4		
2500	2500	Pentacosane	R_i, MS		1.8	0.7	0.8
2700	2700	Heptacosane	R_i, MS		1.0	1.5	2.1
2800	2800	Octacosane	R_i, MS			0.7	
2829		Squalene	R_i, MS, Co-GC		0.7		
2900	2900	Nonacosane	R_i, MS			1.9	2.6
3100	3100	Hentriacontane	R_i, MS			1.6	1.4
		Total 150 compounds		92.1	91.7	90.0	93.5
		Monoterpene hydrocarbons		9.9	1.3	0.1	1.2
		Oxygenated monoterpenes		12.8	9.1	4.7	13.6

(*continued on next page*)

Table 1 (*continued*)

R_i[a]	R_i[b]	Compound	Identif[c]	% **B**[d]	% **F**[d]	% **M**[d]	% **P**[d]
		Sesquiterpene hydrocarbons		24.6	39.0	26.7	32.7
		Oxygenated sesquiterpenes		30.9	15.5	23.4	23.1
		Phenols		0.9	13.7	15.2	11.6

[a] R_i, retention index on a HP-5MS column.
[b] R_i, retention index on a Innowax column.
[c] R_i, retention index identical to bibliography; MS, identification based on comparison of mass spectra; and Co-GC, retention time identical to authentic compounds.
[d] t, Trace; less than 0.05%.

sesquiterpenes (55.5%) and monoterpenes (22.7%). In the first fraction, oxygen-containing sesquiterpenes (30.9%) prevailed over sesquiterpene hydrocarbons (24.6%). In particular, 14 oxygen-containing sesquiterpenes were present in the oil, with a prevalence of spathulenol (9%), caryophyllene oxide (3.8%), viridiflorol (3.4%) and β-eudesmol (3.4%). Among the 21 sesquiterpene hydrocarbons, the most abundant was δ-cadinene (4.2%). As regards monoterpenes, 11 oxygen-containing monoterpenes accounted for 12.8% of the total oil, with *trans*-pinocarveol (3.8%) as the main compound, while β-pinene (3.6%) prevailed among the four monoterpene hydrocarbons.

In **F**, the main components were caryophyllene (12.2%), 4-vinyl guaiacol (9.7%), caryophyllene oxide (7.9%) and α-humulene (6.0%). Sesquiterpenes constituted the most abundant fraction of the oil (54.5%), with a prevalence of sesquiterpene hydrocarbons (39%), among which caryophyllene (12.2%) and α-humulene (6.0%) predominated. Among the eight oxygen-containing sesquiterpenes (15.5%), caryophyllene oxide (7.9%) was the most abundant. As regards monoterpenes, nine oxygen-containing monoterpenes accounted for 9.1% of the total oil, with linalool (3.4%) as the main compound, while terpinolene (1.3%) was the only monoterpene hydrocarbon. Also, the phenolic fraction was noteworthy (13.7%), with 4-vinyl guaiacol (9.7%) being the main compound. Caryophyllene, the main compound in **F**, was abundant also in the oils of *T. flavum* from Croatia (15.8%) (Stanic et al., 1993), Greece (East Thessaly) (22.2%) (Bellomaria et al., 1998) and Iran (30.6%) (Baher & Mirza, 2003), while it was present in minor concentrations in *T. flavum* from Serbia and Montenegro (5.4%) (Kovacevic et al., 2001). As regards the other compounds, there is a great variability in the compositions of the oils, depending on the subspecies and the geographical position.

In **M**, the main compounds were carvacrol (13.9%), caryophyllene oxide (12.7%), hexadecanoic acid (9.8%) and caryophyllene (7.8%). On the whole, the main fraction was constituted of sesquiterpenes (50.1%). Among these, sesquiterpene hydrocarbons (26.7%) and oxygen-containing sesquiterpenes (23.4%) were present in similar amounts. In the first fraction, caryophyllene (7.8%), β-bourbonene (3.5%) and alloaromadendrene (3.0%) predominated while, among the 11 oxygen-containing sesquiterpenes, the most abundant compounds were caryophyllene oxide (12.7%) and caryophyllenol II (3.7%). Phenols were quite abundant (15.2%) and were constituted almost entirely of carvacrol (13.9%). Fatty acids (11.7%) were represented by hexadecanoic acid (9.8%) while, among monoterpenes (4.8%), linalool (3.5%) was the main constituent.

Carvacrol (10.1%), caryophyllene (9.8%), torreyol (7.6%) and caryophyllene oxide (5.0%) were the main compounds of **P**. As in the other oils studied, sesquiterpenes constituted the main fraction and accounted for 55.8% of the total oil with a prevalence of sesquiterpene hydrocarbons (32.7%) over oxygen-containing sesquiterpenes (23.1%). Among the 26 sesquiterpene hydrocarbons, caryophyllene (9.8%), α-humulene (3.8%), germacrene D (3.1%), α-amorphene (3.0%) and δ-cadinene (2.9%) were the most abundant. In the other fraction, torreyol (7.6%) and caryophyllene oxide (5.0%) prevailed. Monoterpenes contributed 14.8% of the oil, with a predominance of oxygen-containing monoterpenes (13.6%), particularly *cis*-verbenone (3.7%) and *cis*-verbenol (2.1%). Phenols (11.6%) were represented almost entirely by carvacrol (10.1%). The oils from *T. capitatum*, collected in Portugal (Antunes et al., 2004) and in Corsica (Cozzani et al., 2005), showed different compositions. As noted by Antunes et al. (2004), there is a great polymorphism in the volatile oils of *T. capitatum*, probably due to genetic factors. As a matter of fact, even for plants collected at the same developmental stage and in very close localities with similar ecological features, the oils notably differ.

All four oils have, in common, a high percentage of sesquiterpenes and a scarce amount of monoterpenes. Previous papers on the analysis of the essential oils of *Teucrium* spp. showed that the sesquiterpenes group is usually dominant, although the main component may vary. Our results concur with these findings.

3.2. Effects of the essential oils on NO production in LPS-stimulated RAW 264.7 cells

The anti-inflammatory activity of essential oils from *Teucrium* spp. was studied *in vitro*, analysing their inhibitory effects on chemical mediators released from macrophages. Once activated by inflammatory stimulation, macrophages produce a large number of cytotoxic molecules. Treatment of RAW 264.7 macrophages with LPS (1 μg/ml) for 24 h induces NO production, as assessed by measuring the accumulation of nitrite, a stable metabolite of NO, in the media, based on the Griess reaction. NO, a macrophages-derived mediator, is considered to play a key role in inflammatory response, based on its occurrence at inflammatory sites and its ability to induce many of the hallmarks of the inflammatory response. The beneficial effect of essential oils from *Teucrium* spp. on the inhibition of production of inflammatory mediators in macrophages may be mediated by oxidative degradation of products of phagocytes, such as O^{2-} and HOCl. As shown in Table 2, incubation of RAW 264.7 cells with essential oils from *Teucrium* spp. induced a significant inhibitory effect on the LPS-induced nitrite production, with IC_{50} values lower than those of the positive control, indomethacin. The oil which most effectively inhibited LPS-induced NO production was that from *T. brevifolium*, with an IC_{50} value of 7.1 μg/ml, followed by *T. montbretii* ssp. *heliotropiifolium* and *T. polium* ssp. *capitatum* with IC_{50} values of 16.5 and 29.4 μg/ml, respectively (Table 2).

These activities are significant if compared with other essential oils; for example, the essential oil from *Farfugium japonicum* produced a 25% of inhibition of NO production at a concentration of 100 μg/ml (Kim et al., 2008) while the oil from *Cinnamomum osmophloeum* produced a 100% of inhibition of NO production at a concentration of 50 μg/ml (Tung, Chua, Wang, & Chang, 2008). The ethanolic extract of *T. polium*, at a dose of 500 mg/kg body weight, produced significant inhibition of carrageenan-induced inflammation and cotton-pellet granuloma (Tariq et al., 1989) and the essential oil of the same species was found to be responsible for analgesic properties of the plant when studied by a writhing test, a visceral pain model in mice (Abdollahi et al., 2003). The less active oil was that from *T. flavum* (IC_{50} = 41.4 μg/ml), although MeOH and CH_2Cl_2 extracts of this species showed a significant anti-inflammatory effect when tested in the carrageenan-induced paw oedema in rats (Barrachina et al., 1995). A survey of literature

Table 2
Inhibition of NO production in LPS-induced RAW 264.7 macrophages by *Teucrium* species.

Compound	Concentration (μg/ml)	% Inhibition	IC_{50} (μg/ml)
Teucrium flavum	200	92 ± 1.4**	41.4 ± 1.11**
	100	79 ± 1.4**	
	50	56 ± 0.8**	
	25	38 ± 0.7**	
	10	28 ± 0.5**	
Teucrium montbretii ssp. *heliotropiifolium*	200	93 ± 1.4**	16.5 ± 0.82**
	100	92 ± 1.8**	
	50	61 ± 0.8**	
	25	58 ± 0.7**	
	10	42 ± 0.4**	
Teucrium polium ssp. *capitatum*	200	100 ± 2.0**	29.4 ± 0.91**
	100	96 ± 1.9**	
	50	80 ± 0.8**	
	25	46 ± 0.6**	
	10	44 ± 0.3**	
Teucrium brevifolium	200	100 ± 2.0**	7.1 ± 0.32**
	100	100 ± 2.0**	
	50	94 ± 1.0**	
	25	84 ± 0.7**	
	10	69 ± 0.6**	
	5	34 ± 0.5**	
Indomethacin	200	95 ± 2.1**	52.8 ± 1.2**
	100	66 ± 1.3**	
	50	46 ± 1.1**	
	25	5 ± 0.3**	
	12.5	2.5 ± 0.2**	

Values are means ± SD ($n = 3$); multicomparison by Dunnett's test: **p (probability) < 0.01. Indomethacin, a known anti-inflammatory drug, was used as positive control.

reveals that other *Teucrium* spp. were previously demonstrated to have potent anti-inflammatory activity against both the acute and delayed phases of inflammation (Fernández Puntero, Iglesias Peinado, & Villar del Fresno, 1997).

Some essential oils, containing major constituents similar to those in the oil of *T. brevifolium*, have shown anti-inflammatory activity. For example, the essential oil of *Garcinia brasiliensis*, which contains spathulenol (8.7%) as a major constituent (Martins et al., 2008), exhibited anti-inflammatory activity, using the rat-paw oedema model induced by carrageenan.

The highest anti-inflammatory activities of *T. polium* ssp. *capitatum* and *T. montbretii* ssp. *heliotropiifolium* were probably due to their major components, caryophyllene and carvacrol, which showed anti-inflammatory activity in previous studies (Passos et al., 2007; Sosa et al., 2005).

Cytotoxic effect, on RAW 264.7 macrophages, of all samples in the presence of LPS (10 μg/ml) was also evaluated. Essential oils from *Teucrium* spp. did not show any cytotoxicity at concentrations of 10–100 μg/ml.

3.3. Effects of the essential oils on cytotoxicity of CACO-2, COR-L23 and C32 cells

The four essential oils from Greece were evaluated for their *in vitro* cytotoxic properties on three human cancer cell lines: colon adenocarcinoma CACO-2, large lung carcinoma COR-L23 and amelanotic melanoma C32. All three human tumour cell lines used in this assay were capable of attachment to form a homogeneous monolayer on the plastic substratum of the culture wells, which is ideal for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT test is a simple bioassay used for the primary screening of crude plant extracts and isolated compounds. For each cell line, there was a linear relationship between

Table 3
Cytotoxic activities against human tumour cell lines of essential oils from *Teucrium* species.

Essential oil	IC_{50} (μg/ml)		
	CACO-2	C32	COR-L23
Teucrium flavum	>200	>200	104 ± 2.1*
Teucrium montbretii ssp. *heliotropiifolium*	92.2 ± 2.1*	135 ± 2.1*	143 ± 2.1*
Teucrium polium ssp. *capitatum*	52.7 ± 2.1*	91.2 ± 2.1*	104 ± 2.1*
Teucrium brevifolium	164 ± 2.1*	>200	80.7 ± 2.1*

Exposure time 48 h ± SEM ($n = 3$). CACO-2, colon adenocarcinoma; C32, amelanotic melanoma cells; and COR-L23, large lung carcinoma. Vinblastine (2 μg/ml) was used as positive control.
* $p < 0.05$.

cell number and absorbance, measured at 550 nm in both control and drug-treated wells. After 48 h of treatment, the cytotoxicity of essential oils was determined. The cytotoxic effects of essential oils on the growth of human tumour cell lines are given in Table 3. The most antiproliferative oils were those from *T. polium* ssp. *capitatum* and *T. montbretii* ssp. *heliotropiifolium* on CACO-2 cell lines, with IC_{50} values of 52.7 and 92.2 μg/ml, respectively. The oil from *T. brevifolium* showed a selective cytotoxicity on large lung carcinoma (IC_{50} value of 80.7 μg/ml) while significant activity was exerted by *T. polium* ssp. *capitatum* oil on melanoma (IC_{50} value of 91.2 μg/ml). Some caffeic acid-containing phenylpropanoid glycosides extracted from *T. polium* were previously shown to have cytotoxic and cytostatic activity against several kinds of cancer cells (Saracoglu et al., 1997), while the ethanol extract of the same plant suppressed the growth of four tested cell lines effectively (Nematollahi-Mahani et al., 2007).

An important finding of the present study is that pure essential oils are responsible for anti-inflammatory and cytotoxic activity of the four *Teucrium* species analysed. Cell type cytotoxic specificity is observed in some plant extracts. This specificity of plant extracts is likely to be due to the presence of different classes of compounds in the extract, as has been documented in the case of known classes of compounds (Cragg et al., 1994). Previous pharmacological studies show that, generally, terpenic compounds and volatile oils are responsible for the pharmacological activity of *Teucrium* species (Ulubelen et al., 2000). Sesquiterpenes, which are the most representative components in the oils investigated, were previously reported to exhibit cytotoxic activity against different cell lines (Sylvestre, Pichette, Lavoie, Longtin, & Legault, 2007) and also anti-inflammatory activity (Medeiros et al., 2007).

4. Conclusion

The anti-inflammatory and cytotoxic activities of *Teucrium* essential oils were evaluated to obtain an insight of the beneficial effects of this plant species in conditions related to inflammation, reduced risk for cardiovascular diseases and cancer prevention by acting as anti-inflammatory agents. The results clearly show that the oils present strong anti-inflammatory and cytotoxic activity against the three human tumour cell lines tested. The biological activity could be partly explained by the presence of sesquiterpenes, such as spathulenol, δ-cadinene, caryophyllene, caryophyllene oxide, α-humulene, and torreyol. Further studies are warranted to confirm the pharmacological activity of these oils in more detail in animal models of inflammation.

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