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# Comparative chemical composition, free radical-scavenging and cytotoxic properties of essential oils of six *Stachys* species from different regions of the Mediterranean Area

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ABSTRACT

The chemical composition of essential oils of six *Stachys* species, *S. cretica* L. ssp. *vacillans* Rech. fil., *S. germanica* L., *S. hydrophila* Boiss., *S. nivea* Labill., *S. palustris* L. and *S. spinosa* L., obtained by hydrodistillation, was studied by GC and GC–MS. All the oils have in common a great percentage of fatty acids and esters (24.2–58.5%) and a high amount of sesquiterpenes (16–35.9%), with the exception of the oil from *S. palustris*, which consisted mainly of carbonylic compounds (25.4%). The antioxidant activity by DPPH test and the antiproliferative activity on a series of human cancer cell lines (C32, amelanotic melanoma and ACHN, renal cell adenocarcinoma) were investigated for all the oils. *S. palustris, S. cretica* and *S. hydrophila* showed the highest antiradical effect, with $IC_{50}$ values of 0.482, 0.652 and 0.664 mg/ml, respectively. The most antiproliferative essential oil against C32 cell line was the oil of *S. germanica* with a 77% of inhibition at a concentration of 100 μg/ml. *S. germanica*, *S. palustris* and *S. spinosa* showed the most antiproliferative activity on ACHN cell line, at a concentration of 100 μg/ml,with 81%, 77% and 73% inhibition, respectively.


## 1. Introduction

Spices and their products have been used in the food industry for their flavouring and biological activities since ancient times. The shelf life and stability of food products are important issues for the industry. Oxidation, deterioration, and microbial reactions occurring in food products may cause economic loss. Deterioration of lipids is catalysed by different internal and external factors, such as free radicals, metal ions, light, and heat. (Yanishlieva, Marinova, & Pokorny, 2006).

The Lamiaceae family is well-known because of the antioxidant properties of its taxa. *Stachys* L., or woundwort, is a subcosmopolitan genus of herbs and shrubs that comprises more than 270 species (Ball, 1972) and is considered as one of the largest genera of the Lamiaceae. This genus is distributed in temperate and tropical regions of the world, with the exception of Australasia, and it is widespread in mountainous and moist places (Ball, 1972). Plants of this genus have been used medicinally for centuries to treat genital tumours, sclerosis of the spleen, inflammatory diseases, cough, ulcers and infected wounds (Hartwell, 1982). Tea made from the whole plant or leaves is used on account of its sedative, antispasmodic, diuretic and emmenagogic activity. It is also known in traditional medicine for the treatment of fevers, diarrhoea, sore mouth and throat, internal bleeding, and weaknesses of the liver and heart (Grieve, 1971). In recent years, investigations on different taxa of this genus have showed that extracts or components of *Stachys* species exert various pharmacological effects: anti-inflammatory, (Khanavi, Sharifzadeh, Hadjiakhoondi, & Shafiee, 2005), antitoxic, antibacterial, antioxidant (Bilušić Vundać, Brantner, & Plazibat, 2007; Grujic-Jovanovic, Skaltsa, Marin, & Sokovic, 2004; Salehi, Sonboli, & Asghari, 2007) and cytotoxic (Haznagy-Radnai et al., 2008). The antimicrobial, antioxidant and antifungal activities showed by plants of this genus render them particularly useful as natural preservative ingredients and are the cause of the importance of this genus in the food industry. Many *Stachys* species are besides known as

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alimentary plants, as some of them are currently used for making herbal teas, in the preparation of foods such as yoghurt or jelly to improve the taste and are used traditionally as flavourings and seasonings (De Vincenzi, Mancini, & Dessi, 1997; Hou et al., 2007; Yi et al., 2007).

Like many other representatives of the family Lamiaceae, *Stachys* species produce essential oils, but in spite of the large size of this genus, the composition of volatile compounds is known only in a small number of species (Grujic-Jovanovic et al., 2004; Kirimer, Baser, & Tumen, 1995; Ozkan, Gokturk, Unal, & Celik, 2006; Radulovic, Lazarevic, Ristic, & Palic, 2007; Salehi et al., 2007; Senatore, Formisano, Rigano, Piozzi, & Rosselli, 2007; Skaltsa, Demetzos, Lazari, & Sokovic, 2003). There are some differences amongst *Stachys* taxa, indicating the existence of a chemical polymorphism.

*S. cretica* L. ssp. *vacillans* Rech. fil. is also called Cretan hedgenettle. Methanolic extracts of leaves and flowers of *S. cretica* ssp. *anatolica* from Turkey were found to be effective against *Bacillus subtilis* (Benli, Güney, Bingöl, Geven, & Yiğit, 2007). Essential oils of *S. cretica* ssp. *mersinaea* (Ozkan et al., 2006) and *S. cretica* ssp. *anatolica* (Kirimer et al., 1995) from Turkey and the oil of *S. cretica* ssp. *cretica* from Greece (Skaltsa et al., 2003) have been previously analysed, whilst a literature search revealed no references to previous work on the essential oil of *S. cretica* ssp. *vacillans*. *S. germanica* L. (downy woundwort) has leaves densely covered with long, white, silky hairs (Grieve, 1971). The essential oils of these plants growing in the Balkan Peninsula, in Canada, in Hungary (Grujic-Jovanovic et al., 2004; Radulovic et al., 2007) and in Greece (Skaltsa et al., 2003) have been studied; the oils principally contain hydrocarbon sesquiterpenes and monoterpenes. A 80% aqueous MeOH extract of inflorescences of *S. germanica* collected in Hungary exerted over 25% inhibition on the MCF7 cell line (Haznagy-Radnai et al., 2008). *S. hydrophila* Boiss. (water woundwort) grows in shady places near streams and hedges. *S. nivea* Labill. (snowy woundwort) or *S. discolor*, called Ghebri in Lebanon, is white, woolly, covered with stellate hairs. Although this plant is important for bees, villagers use it as a mild laxative when boiled as the whole plant. There is no report of any thorough phytochemical investigation on *S. hydrophila* and *S. nivea* available to date. *S. palustris* L. is considered a wholesome and nutritious food; the edible parts of the plant are leaves, roots and seeds. Tubers are consumed raw or cooked, and they have a pleasant, mild, nutty flavour. The tubers, harvested in autumn, can be dried and ground into a powder that is used in making bread. The young shoots can be used as an asparagus substitute, as they have a pleasant taste (Senatore et al., 2007). From the aerial parts of the plant was isolated an essential oil rich in carbonylic compounds (Senatore et al., 2007). Methanolic extract of *S. palustris* collected in Croatia was showed to be an effective DPPH radical scavenger with a $IC_{50}$ value of 2.67 μg/ml (Bilušić Vundać et al., 2007). In addition, an 80% aqueous MeOH extract of stem, leaf and inflorescences of *S. palustris* collected in Hungary exerted over 25% inhibition on different tumour cell lines (Haznagy-Radnai et al., 2008). *S. spinosa* L. was previously investigated by Kotsos, Aligiannis, and Mitakou (2007), and by Kotsos, Aligiannis, Mitaku, Skaltsounis, and Charvala (2001), who showed the presence of flavonoids and iridoids. The essential oil has not been studied previously.

To our knowledge, there are no published reports on the chemical composition, cytotoxic and antioxidant activity of the essential oils of the six *Stachys* species here described. For this reason, the chemical composition of these oils was analysed and their antiproliferative activity on a series of human cancer lines was investigated. The antioxidant activity of the oils was also evaluated by DPPH assay.

## 2. Experimental

### 2.1. Plant material

Aerial parts of *S. cretica* ssp. *vacillans* (***Sc***) and *S. nivea* (***Sn***) were collected in July 2006 in Lebanon. Aerial parts of *S. hydrophila* (***Sh***) were collected in July 2006 in Creta (Greece). The aerial parts of *S. spinosa* (***Ss***) were collected in June 2006 on the Karpathos island of Greece. Aerial parts of *S. germanica* were collected in two different localities of Southern Italy: in the Parco Nazionale del Pollino between Calabria and Basilicata (***SgP***) and in Piano Battaglia (Sicily), in the Parco Nazionale delle Madonie (***SgPB***). Aerial parts of *S. palustris* (***Sp***) were collected in Campania (Southern Italy) in June 2006. Voucher specimens (respectively PAL 2006-323, PAL 2006-324, PAL 2006-325, PAL 2006-326, PAL 2006-327, PAL 2006-328) have been deposited in the Herbarium of the Department of Organic Chemistry, University of Palermo, Italy.

### 2.2. Isolation procedure

The essential oils from air-dried and ground aerial parts of plants were isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method recommended in the *European Pharmacopoeia* (2004). The oils were dried over anhydrous sodium sulphate and stored under $N_2$ at +4 °C in the dark until tested and analysed. All the oils were yellowish with a pleasant smell. The yields (w/w) were the following: 0.18% for *S. cretica* ssp. *vacillans* (***Sc***); 0.15% for *S. germanica* collected in the Parco Nazionale del Pollino (***SgP***); 0.10% for *S. germanica* collected in Piano Battaglia (***SgPB***); 0.27% for *S. hydrophila* (***Sh***); 0.20% for *S. nivea* (***Sn***); 0.21% for *S. palustris* (***Sp***); 0.10% for *S. spinosa* (***Ss***).

### 2.3. GC and GC–MS analysis

Analytical gas chromatography was carried out on a Perkin–Elmer Sigma 115 gas chromatograph fitted with an HP-5MS capillary column (30 m × 0.25 mm i.d.; 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 splitless mode. Flame ionisation detection was performed at 280 °C. Analysis was also carried out using a fused silica HP Innowax polyethylene glycol capillary column (50 m × 0.20 mm i.d.; 0.20 μm film thickness).

GC–MS analysis was performed on an Agilent 6850 series II apparatus, fitted with a fused silica HP-1 capillary column (30 m × 0.25 mm i.d.; 0.33 μm film thickness), coupled to an Agilent 5973 MSD; ionisation voltage 70 eV; electron multiplier energy 2000 V. Gas chromatographic conditions were as reported above; transfer line temperature, 295 °C. Analysis was also performed using a fused silica HP Innowax capillary column (60 m × 0.25 mm i.d.; 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 (Jennings & Shibamoto, 1980; Davies, 1990) or with those of authentic compounds available in our laboratories. The retention indices were determined in relation to a homologous series of *n*-alkanes ($C_8$–$C_{24}$) run under the same operating conditions. Further identification was made 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 (Jennings &

**Table 1**
Essential oil composition (%) of aerial parts of *Stachys cretica* ssp. *vacillans* (***Sc***), *S. germanica* from Pollino (***SgP***), *S. germanica* from Piano Battaglia (***SgPB***), *S. hydrophila* (***Sh***), *S. nivea* (***Sn***), *S. palustris* (***Sp***) and *S. spinosa* (***Ss***).

| $K_i$[a] | $K_i$[b] | Component | Identification[c] | *Sc*%[d] | *SgP*%[d] | *SgPB*%[d] | *Sh*%[d] | *Sn*%[d] | *Sp*%[d] | *Ss*%[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| 854 | 1209 | (*E*)-2-Hexenal | I, MS | | 0.1 | 0.2 | 0.1 | t | 0.2 | |
| 900 | 1195 | Heptanal | I, MS | | | | t | t | t | |
| 901 | | Hexyl valerate | I, MS | | 0.2 | | | | | |
| 906 | 1395 | (*E*,*E*)-2,4-Hexadienal | I, MS | | | | | | 0.1 | |
| 936 | 1075 | α-Pinene | I, MS, Co-GC | t | | | 0.6 | 0.5 | t | |
| 959 | 1541 | Benzaldehyde | I, MS, Co-GC | 0.2 | | 0.4 | 0.5 | 0.4 | 0.1 | 0.7 |
| 969 | 1294 | 1-Octen-3-one | I, MS | | | | | | 0.8 | 0.1 |
| 973 | 1132 | Sabinene | I, MS | 0.6 | | | | | | |
| 977 | 1254 | 1-Octen-3-ol | I, MS | | 0.3 | 0.2 | 0.7 | | 0.5 | t |
| 978 | 1118 | β-Pinene | I, MS, Co-GC | 0.4 | 0.5 | 0.8 | 0.4 | 0.3 | 0.3 | |
| 990 | 1244 | 2-Pentylfuran | I, MS | | | | 0.4 | t | 0.1 | 1.1 |
| 996 | 1233 | Ethyl hexanoate | I, MS | | | | | | t | |
| 1002 | | δ2-Carene | I, MS | 0.8 | | | | | | |
| 1008 | 1506 | (*E*,*E*)-2,4-Heptadienal | I, MS | | | | | | t | |
| 1012 | 1157 | δ3-Carene | I, MS | t | | | | | | |
| 1023 | 1893 | Benzyl alcohol | I, MS | | | | | | 0.3 | |
| 1024 | 1278 | *p*-Cymene | I, MS, Co-GC | | | | | | 0.2 | |
| 1029 | 1218 | β-Phellandrene | I, MS | 0.4 | | 0.5 | | | | |
| 1030 | 1203 | Limonene | I, MS, Co-GC | | | | 0.6 | | | |
| 1041 | 1663 | Phenylacetaldehyde | I, MS, Co-GC | 0.5 | 0.4 | 0.4 | 0.5 | 0.5 | 1.3 | |
| 1058 | 1657 | Acetophenone | I, MS | | | 0.2 | | | | |
| 1076 | | α-Thujone | I, MS | | | | | 0.7 | | |
| 1086 | 1265 | Terpinolene | I, MS | | | | 0.4 | | | 1.1 |
| 1087 | | *trans*-Linalool oxide, furanoid | I, MS | t | | | | 0.5 | | |
| 1088 | 1553 | Linalool | I, MS, Co-GC | 1.6 | 0.4 | 1.6 | t | 1.3 | 1.5 | 2.5 |
| 1102 | | Nonanal | I, MS | 0.2 | | 0.6 | t | 0.7 | | |
| 1113 | 1925 | 2-Phenyl ethyl alcohol | I, MS | | | | t | 0.6 | 0.1 | 0.5 |
| 1142 | 1721 | *trans*-Sabinol | I, MS | | | 0.2 | | | | |
| 1148 | | (*E*,*E*)-2,6-Nonadienal | I, MS | 0.2 | | | 0.3 | | 0.3 | |
| 1163 | 1502 | Isomenthone | I, MS | | | | | | | t |
| 1167 | 1719 | Borneol | I, MS | | | | | | | 1.0 |
| 1169 | 2088 | Octanoic acid | I, MS | 0.2 | | | | | | |
| 1170 | 1450 | *cis*-Linalool oxide, pyranoid | I, MS | | | 0.3 | | | | 1.1 |
| 1176 | 1611 | 4-Terpineol | I, MS | | | 0.4 | | | | |
| 1180 | 1763 | Naphthalene | I, MS | 0.2 | | | 0.2 | | | 0.1 |
| 1184 | | Methylacetophenone | I, MS | | | 0.2 | | | | |
| 1185 | 1856 | *p*-Cymen-8-ol | I, MS | | | | 0.8 | | | |
| 1187 | 1706 | α-Terpineol | I, MS, Co-GC | | | 0.8 | 0.9 | 0.6 | 0.6 | |
| 1189 | 1798 | Methyl salicylate | I, MS | | | | | | 0.2 | |
| 1201 | | Safranal | I, MS | | | | | | 0.4 | |
| 1204 | | Decanal | I, MS | | | 0.2 | | 0.5 | | |
| 1208 | | α-Ionene | I, MS | | | | | | | 1.1 |
| 1217 | | 2,3-Dihydrobenzofuran | I, MS | | | | 0.5 | | | |
| 1224 | | Benzothiazole | I, MS, Co-GC | 0.2 | | | 0.2 | 0.6 | | 0.2 |
| 1232 | | *p*-Anisaldehyde | I, MS | | | | 0.4 | | 0.2 | 0.9 |
| 1234 | 1662 | Pulegone | I, MS | 3.0 | | | 2.5 | 1.2 | 0.3 | 4.1 |
| 1241 | | (*Z*)-3-Hexyl-2-methylbutanoate | I, MS | | | | | | t | |
| 1249 | | Benzylacetone | I, MS | | | 0.1 | | | | |
| 1255 | 1857 | Geraniol | I, MS, Co-GC | | | 0.2 | 0.4 | | 0.4 | |
| 1260 | 1655 | (*E*)-2-Decenal | I, MS | | | 0.3 | t | 0.7 | t | |
| 1267 | | (*Z*)-Chrysanthenyl acetate | I, MS | | | | | | t | |
| 1278 | 2190 | Nonanoic acid | I, MS, Co-GC | | | | 0.4 | 0.7 | | |
| 1287 | | Dihydroedulan II | I, MS | | | *1.0* | | | | |
| 1290 | 2198 | Thymol | I, MS, Co-GC | 1.8 | | | 3.5 | 2.2 | 5.8 | 4.5 |
| 1291 | 2471 | Indole | I, MS, Co-GC | 0.3 | | 0.8 | 0.5 | 1.5 | 0.6 | |
| 1297 | 2239 | Carvacrol | I, MS, Co-GC | 2.2 | | | 1.0 | 2.0 | 1.2 | 27.9 |
| 1302 | 1797 | *p*-Methoxyacetophenone | I, MS, Co-GC | t | | 0.4 | | | 5.1 | 0.8 |
| 1313 | 2180 | 4-Vinylguaiacol | I, MS | 5.8 | 3.4 | 2.4 | 6.4 | 6.1 | 3.8 | |
| 1315 | 1827 | (*E*,*E*)-2,4-Decadienal | I, MS | t | | | t | 0.4 | t | t |
| 1348 | 1466 | α-Cubebene | I, MS | 0.6 | | | | t | t | 0.7 |
| 1351 | 1579 | α-Longipinene | I, MS | 0.7 | | 0.6 | | | | |
| 1353 | 2186 | Eugenol | I, MS, Co-GC | 0.7 | | 0.8 | 1.2 | 1.0 | 0.4 | 0.7 |
| 1358 | 2298 | Decanoic acid | I, MS, Co-GC | | | 0.3 | 0.8 | 0.1 | | |
| 1360 | | γ-Nonalactone | I, MS | | | 0.4 | | 0.1 | | |
| 1361 | 1787 | (*E*)-β-Damascenone | I, MS | 0.5 | | | | | 3.0 | |
| 1368 | | Longicyclene | I, MS | 0.4 | | | | | | |
| 1368 | | Geranyl acetate | I, MS | 0.2 | | 1.5 | | | | |
| 1373 | 1493 | α-Ylangene | I, MS | | | 0.8 | | | | |
| 1377 | 1497 | α-Copaene | I, MS | 1.3 | 2.4 | 1.7 | 0.5 | 1.4 | | |
| 1381 | 1835 | (*Z*)-β-Damascenone | I, MS | | 0.8 | 0.5 | 2.6 | 1.5 | | 1.8 |
| 1382 | 1547 | β-Cubebene | I, MS | | 1.3 | | | | | |
| 1385 | 1535 | β-Bourbonene | I, MS | 0.7 | 1.2 | 1.9 | 0.6 | 0.5 | | |
| 1387 | 1598 | β-Elemene | I, MS | | 1.3 | | | | 0.5 | |
| 1394 | 2050 | Methyl cinnamate | I, MS | | | | | | 1.0 | |

(*continued on next page*)

**Table 1** (*continued*)

| $K_i$[a] | $K_i$[b] | Component | Identification[c] | *Sc%*[d] | *SgP%*[d] | *SgPB%*[d] | *Sh%*[d] | *Sn%*[d] | *Sp%*[d] | *Ss%*[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1404 | 1666 | (*Z*)-Caryophyllene | I, MS | | | | | | 0.2 | 1.0 |
| 1405 | | α-Cedrene | I, MS | 0.4 | | | | | t | |
| 1405 | 1724 | Dodecanal | I, MS | | | | | 0.2 | | |
| 1406 | | α-Cadinene | I, MS | t | 0.2 | | | | | |
| 1418 | 1612 | (*E*)-Caryophyllene | I, MS | 1.7 | | 2.4 | 3.6 | | 3.6 | |
| 1431 | | Thujopsene | I, MS | | | | 0.6 | | | |
| 1436 | 1650 | γ-Elemene | I, MS | | | | 0.6 | | | |
| 1440 | 1868 | (*E*)-2-Dodecenal | I, MS | | | | | 0.7 | 0.3 | |
| 1442 | 1498 | *Epi*-Bicyclosesquiphellandrene | I, MS | | 6.1 | | | | | |
| 1446 | | Nerylacetone | I, MS | | | *0.1* | | | | |
| 1450 | | Geranylacetone | I, MS | | | | 0.5 | | 0.4 | |
| 1452 | 1673 | *cis*-β-Farnesene | I, MS | 0.6 | 3.0 | 0.8 | | | | |
| 1455 | 1689 | α-Humulene | I, MS | 0.5 | | 0.5 | 0.5 | | | |
| 1472 | 1709 | Dodecanol | I, MS | | | | | | 0.3 | |
| 1475 | 1715 | β-Selinene | I, MS | | | | | 1.0 | | |
| 1477 | 1726 | Germacrene D | I, MS | 9.5 | 0.7 | | 0.7 | 2.5 | | |
| 1478 | 1704 | γ-Muurolene | I, MS | 0.8 | | | | | | |
| 1483 | 1784 | *ar*-Curcumene | I, MS | 0.9 | | | 0.5 | | | |
| 1484 | 1958 | β-Ionone | I, MS, Co-GC | 0.6 | | | 0.8 | 1.3 | 3.3 | 0.8 |
| 1486 | 2354 | Dihydroactinidiolide | I, MS | 1.6 | | 0.7 | 1.3 | 2.1 | 2.0 | 2.1 |
| 1490 | | Valencene | I, MS | 0.4 | | | | | | |
| 1491 | 1756 | Bicyclogermacrene | I, MS | | | | | 1.4 | | |
| 1497 | 1744 | α-Selinene | I, MS | | 0.4 | | | | | |
| 1499 | | β-Himachalene | I, MS | | | | | | 1.0 | |
| 1500 | 1500 | Pentadecane | I, MS | | | | 0.2 | 0.3 | | t |
| 1502 | | α-Farnesene | I, MS | 0.6 | | | | | | |
| 1503 | 1740 | α-Muurolene | I, MS | t | | | | 0.6 | | |
| 1505 | 1822 | Cuparene | I, MS | | | | 0.4 | | | |
| 1506 | 1760 | (*E*,*E*)-α-Farnesene | I, MS | | 0.4 | | | | | |
| 1510 | 1743 | β-Bisabolene | I, MS | | 2.4 | 0.4 | 0.7 | | | |
| 1513 | | Pentadecanal | I, MS | | | | | 0.1 | | |
| 1515 | 1776 | γ-Cadinene | I, MS | | | 1.2 | 0.4 | | | |
| 1520 | 1839 | Calamenene[e] | I, MS | | | | | t | 0.1 | |
| 1523 | | Megastigmatrienone[e] | I, MS | 0.5 | | | | 0.8 | 2.0 | t |
| 1526 | 1773 | δ-Cadinene | I, MS | 1.7 | 1.2 | | | 0.8 | | |
| 1541 | 1918 | α-Calacorene | I, MS | 0.5 | | | | | | |
| 1553 | 2076 | α-Copaen-8-ol | I, MS | | | | 0.6 | | | |
| 1560 | 2050 | (*E*)-Nerolidol | I, MS,Co-GC | t | | 0.7 | | t | 1.4 | |
| 1566 | 2503 | Dodecanoic acid | I, MS, Co-GC | 0.2 | 0.4 | 2.6 | 1.2 | 0.3 | t | 0.2 |
| 1575 | | Longipinanol | I, MS | | | | | | 0.1 | |
| 1580 | 2150 | Spathulenol | I, MS | 6.1 | | | 3.7 | 6.7 | | |
| 1581 | 2008 | Caryophyllene oxide | I, MS, Co-GC | 2.1 | | 0.5 | 2.7 | 1.3 | 7.8 | 4.4 |
| 1584 | | *epi*-Globulol | I, MS | 0.5 | | | | | | |
| 1588 | 2098 | Globulol | I, MS | | 0.3 | | 0.5 | | | |
| 1590 | 2512 | Benzophenone | I, MS | 0.3 | | | | 0.9 | | t |
| 1591 | 2104 | Viridiflorol | I, MS | | | | | 0.6 | | |
| 1598 | 2108 | Guaiol | I, MS | | | | 0.5 | | | |
| 1599 | 2120 | α-Cedrol | I, MS | 0.7 | | | | | 0.4 | |
| 1600 | | Widdrol | I, MS | 0.7 | | | 0.7 | 0.5 | 0.3 | |
| 1606 | 2133 | Cedrenol | I, MS | 0.5 | | | | | 0.2 | |
| 1607 | 2098 | β-Oplopenone | I, MS | | | | | | 0.1 | |
| 1608 | | *p*-Mentha-3,8-diene | I, MS | | | | | 0.2 | | |
| 1624 | 1935 | Tetradecanal | I, MS | | 0.2 | | | 0.6 | | |
| 1635 | | Isospathulenol | I, MS | | | | | 0.3 | | |
| 1636 | 2158 | τ-Cadinol | I, MS | 1.5 | | | | 0.7 | 0.3 | 0.8 |
| 1640 | 2316 | Caryophylladienol | I, MS | 0.6 | | | 0.6 | | t | |
| 1641 | 2209 | τ-Muurolol | I, MS | | | | | 0.6 | 0.1 | |
| 1646 | | Torreyol | I, MS | 0.4 | | | | | t | |
| 1652 | 2255 | α-Cadinol | I, MS | 1.5 | 0.6 | | 0.4 | | | |
| 1671 | | Valeranone | I, MS | | | | | | 0.1 | |
| 1672 | | *cis*-(*Z*)-α-Bisabolene epoxide | I, MS | | | | | | | 0.8 |
| 1677 | 2256 | Cadalene | I, MS | | | | | 0.7 | | |
| 1686 | 2219 | α-Bisabolol | I, MS | | 3.0 | 0.6 | | | | |
| 1724 | | (*Z*,*E*)-Farnesol | I, MS | | 0.2 | 0.2 | | | | |
| 1743 | | (*E*,*E*)-Farnesol | I, MS | | 0.4 | 0.9 | | | | |
| 1762 | 2655 | Benzyl benzoate | I, MS, Co-GC | | | | 0.3 | | 0.5 | |
| 1769 | 2713 | Tetradecanoic acid | I, MS, Co-GC | 0.9 | 2.5 | 6.0 | 2.5 | 1.8 | 3.8 | t |
| 1772 | | Guaiazulene | I, MS | 0.9 | | | | | | |
| 1794 | | 1-Octadecene | I, MS | 0.1 | | | | | | |
| 1828 | 2099 | Methyl pentadecanoate | I, MS | | | | | | 0.2 | |
| 1843 | | (*E*,*E*)-Farnesyl acetate | I, MS | | | | | | t | |
| 1845 | 2131 | Hexahydrofarnesyl acetone | I, MS | 1.7 | 1.7 | 3.4 | 2.6 | 1.7 | 7.4 | 2.8 |
| 1870 | 2822 | Pentadecanoic acid | I, MS, Co-GC | 0.4 | | 0.6 | 2.0 | 0.3 | 0.3 | |
| 1875 | | Cyclohexadecane | I, MS | | | | 0.1 | | | |
| 1892 | | 1-Nonadecene | I, MS | | | | | 0.4 | 0.4 | |

**Table 1** (*continued*)

| $K_i$[a] | $K_i$[b] | Component | Identification[c] | *Sc%*[d] | *SgP%*[d] | *SgPB%*[d] | *Sh%*[d] | *Sn%*[d] | *Sp%*[d] | *Ss%*[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1895 | | 1-Oxaspiro[4.5]deca-6,9-diene-2, 8-dione, 7,9-bis(1,1-dimethylethyl)- | I, MS | | | | | | | 0.7 |
| 1895 | | 7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione | I, MS | | | | 0.2 | | | |
| 1923 | 2814 | Phenanthrene | I, MS | | | | 0.2 | | | |
| 1925 | 2208 | Methyl hexadecanoate | I, MS, Co-GC | | | | | 1.2 | 0.2 | |
| 1942 | | *ent*-Pimara-8,15-diene | I, MS | | | | | | 0.1 | |
| 1949 | 2622 | (*Z*)-Phytol | I, MS | 0.2 | | | t | t | 6.4 | |
| 1961 | | 13-*epi*-Manoyl | I, MS | | | | | | t | |
| 1972 | 2931 | Hexadecanoic acid | I, MS, Co-GC | 17.2 | 22.1 | 37.4 | 17.7 | 17.1 | 6.8 | 10.6 |
| 1994 | | 1-Eicosene | I, MS | | | 0.1 | | 0.8 | | |
| 2011 | 2380 | 13-*epi*-Manoyl oxide | I, MS | | | | | | t | |
| 2074 | 2975 | Heptadecanoic acid | I, MS, Co-GC | 0.1 | | 0.2 | 0.7 | 0.6 | 0.4 | |
| 2100 | 2100 | Heneicosane | I, MS | | | | | | | 0.1 |
| 2111 | | 9,12,15-Octadecatrienal[e] | I, MS | | 1.4 | 0.9 | | | | |
| 2122 | 3157 | (*Z*)-9-Octadecenoic acid | I, MS, Co-GC | 0.5 | | | 0.6 | | | |
| 2122 | 3157 | (*Z,Z*)-9,12-Octadecadienoic acid | I, MS, Co-GC | 8.1 | 0.2 | 6.6 | 7.2 | 14.1 | 2.8 | 16.3 |
| 2132 | 2625 | (*E*)-Phytol | I, MS | 0.2 | 1.0 | 0.5 | 0.6 | 0.1 | 0.4 | |
| 2140 | 3193 | (*Z,Z,Z*)-9,12,15-Octadecatrienoic acid | I, MS | 1.0 | 33.3 | | 3.8 | | 6.7 | |
| 2172 | 3402 | Octadecanoic acid | I, MS, Co-GC | 1.4 | | 1.8 | 2.0 | 0.8 | 1.2 | |
| 2363 | | Docosanoic acid | I, MS | | | | | | 0.1 | |
| 2460 | | Eicosanoic acid | I, MS | | | | | | t | |
| | | **Total compounds 169** | | **72** | **34** | **54** | **71** | **68** | **92** | **38** |
| | | **Oxygenated monoterpenes** | | **4.6** | **0.6** | **5.1** | **4.6** | **4.3** | **3.1** | **8.7** |
| | | **Monoterpene hydrocarbons** | | **2.2** | **0.5** | **1.3** | **2.0** | **1.0** | **0.5** | **2.2** |
| | | **Oxygenated sesquiterpenes** | | **14.6** | **4.5** | **2.9** | **9.7** | **10.7** | **10.6** | **6.0** |
| | | **Sesquiterpene hydrocarbons** | | **21.3** | **20.6** | **10.3** | **9.1** | **8.9** | **5.4** | **1.7** |
| | | **Carbonylic compounds** | | **5.8** | **3.2** | **6.9** | **9.8** | **13.1** | **25.4** | **9.9** |
| | | **Phenols** | | **10.5** | **3.4** | **3.2** | **12.1** | **11.3** | **11.2** | **33.1** |
| | | **Fatty acids and esters** | | **30.2** | **58.5** | **55.5** | **39.2** | **37.0** | **24.2** | **27.1** |
| | | **Total identified** | | **91.8** | **94.0** | **89.8** | **90.1** | **90.7** | **93.6** | **91.5** |

[a] Retention index on a HP-5MS column.
[b] Retention index on a Innowax column.
[c] Ri = retention index identical to literature value; MS = identification based on comparison of mass spectra; Co-GC = retention time identical to authentic compounds.
[d] t = Trace, <0.05%.
[e] Correct isomer not identified.

Shibamoto, 1980; Adams, 2001) and our home-made library. Component relative concentrations were calculated based on GC peak areas without using correction factors.

### 2.5. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

This experimental procedure was adapted from Wang et al. (1998), modified as reported in Conforti, Marrelli, Statti, and Menichini (2006). The absorbance was measured using a Perkin–Elmer Lambda 40 UV/Vis spectrophotometer at 517 nm against a blank, which was without DPPH. All tests were run in triplicate and averaged. Ascorbic acid was used as a positive control. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical-scavenging activity. This activity is given as % DPPH radical-scavenging that is calculated in the equation:

$$100 \times \left[1 - \left(\frac{\text{sample absorbance with DPPH} - \text{sample absorbance without DPPH}}{\text{control absorbance}}\right)\right] \quad (1)$$

### 2.6. Cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reported by Tubaro et al. (1996), was used in this study for *in vitro* antitumour screening to estimate cell number indirectly. The amelanotic melanoma C32 (ATCC No.: CRL-1585) and renal cell adenocarcinoma ACHN (ATCC No.: CRL-1611) cell lines were used in this experiment. The cell lines were cultured in RPMI 1640 medium and supplemented with 10% foetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. For the assay, a final concentration of $2 \times 10^4$ cells/ml was used. One hundred microlitres per well of cell suspension were seeded in 96-well microtitre plates and incubated to allow for cell attachment. After 24 h the cells were treated with serial concentrations of the samples. One hundred microlitres per well of each concentration were added to the plates in three replicates to obtain final concentrations of 2.5, 5, 10, 25, 50, 100 μg/ml. By these serial dilutions, the final mixture used for treating the cells contained not more than 0.5% of the solvent (DMSO), the same as in the solvent control wells. The culture plates were kept at 37 °C with 5% (v/v) $CO_2$. After 48 h of incubation, 100 μl of medium were removed from each well. Subsequently, 100 μl of 0.5% w/v MTT (Sigma, Italy), dissolved in phosphate-buffered saline, was 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 $LD_{50}$, which is the concentration needed to reduce the absorbance of treated cells by 50% with reference to the control (untreated cells).

### 2.7. Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed by using Student's *t*-test. Differences were considered significant at $p \leqslant 0.05$. The 50% inhibitory concentration ($IC_{50}$) was calculated from the Prism dose–response curve (statistical

programme) obtained by plotting the percentage of inhibition versus the concentrations.

## 3. Results and discussion

### 3.1. Chemical composition of the essential oils

All the investigated *Stachys* taxa contained essential oils in the range 0.10–0.27%, based on dry weight. In the seven oils, 169 compounds in all were identified: 72 for the oil of *S. cretica* (***Sc***) (91.8% of the total oil), 34 for *S. germanica* from Pollino (***SgP***) (94.0% of the oil), 54 for *S. germanica* from Piano Battaglia (***SgPB***) (89.8% of the oil), 71 for *S. hydrophila* (***Sh***) (90.1% of the oil), 68 for *S. nivea* (***Sn***) (90.7% of the oil), 92 for *S. palustris* (93.6% of the oil) and 38 for *S. spinosa* (***Ss***) (91.5% 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 an HP-5MS column. For ***Sc*** the main compounds were found to be hexadecanoic acid (17.2%), (*Z,Z*)-9,12-octadecadienoic acid (8.1%), germacrene D (9.5%), spathulenol (6.1%), 4-vinylguaiacol (5.8%) and pulegone (3.0%). On the whole, the most abundant components were sesquiterpenes (35.9%), particularly hydrocarbon sesquiterpenes (21.3%) represented principally by germacrene D, followed by fatty acids and esters (30.2%), among which prevailed hexadecanoic and (*Z,Z*)-9,12-octadecadienoic acids. Also the phenolic fraction was noteworthy (10.5%), with 4-vinylguaiacol (5.8%) being the main compound.

The essential oils from *S. cretica* L. ssp. *mersinaea* (Boiss.) Rech. from Turkey (Ozkan et al., 2006) and *S. cretica* L. ssp. *cretica* from Greece (Skaltsa et al., 2003) have in common with *S. cretica* ssp. *vacillans* that sesquiterpene hydrocarbons are the main group of constituents. As well, in *S. cretica* ssp. *cretica* the most abundant compound is germacrene D (33.5%), present in high percentages also in ***Sc***, whilst *S. cretica* ssp. *mersinaea* is rich of α-curcumene (34.1%), not very abundant in ***Sc***. The essential oil of *S. cretica* ssp. *anatolica* from Turkey is instead carvacrol-rich (Kirimer et al., 1995), whilst carvacrol is scarce in ***Sc***. In ***SgP*** and ***SgPB*** there was a clear predominance of fatty acids and esters that accounted for the 58.5% in the first case and for the 55.5% in the second. The main compounds of both the oils fell within this category of compounds, as they were hexadecanoic acid (22.1%) and (*Z,Z,Z*)-9,12,15-octadecatrienoic acid methyl ester (33.3%) for ***SgP*** and hexadecanoic acid (37.4%), (*Z,Z*)-9,12-octadecadienoic acid (6.6%) and tetradecanoic acid (6.0%) for ***SgPB***. Sesquiterpene hydrocarbons accounted for 20.6% in ***SgP*** and for 10.3% in ***SgPB***; in this class of compounds *epi*-bicyclosesquiphellandrene (6.1%) and *cis*-β-farnesene (3.0%) prevailed in the first oil whilst caryophyllene (2.4%) prevailed in the second. Radulovic et al. (2007) summarise data on the essential oil composition of the taxa belonging to *S. germanica* complex studied since 1972. Hexadecanoic acid is not particularly representative in the oils cited in the paper, but it appears to be the main compound in *S. persica* and in *S. inflata* oils (Khanavi et al., 2004; Morteza-Semnani, Akbarzadeh, & Changizi, 2006), where it accounts respectively for 27.2% and 9.1%. Fatty acids prevailed also in ***Sh*** where they constituted 39.2% of the total oil with a prevalence of hexadecanoic acid (17.7%), (*Z,Z*)-9,12-octadecadienoic acid (7.2%) and (*Z,Z,Z*)-9,12,15-octadecatrienoic acid (3.8%). Also sesquiterpenes were quite abundant in the oil as they accounted for 18.8% and among these the percentages of sesquiterpene hydrocarbons (9.1%) and oxygenated sesquiterpene (9.8%) were similar. Phenols (12.1%), with 4-vinylguaiacol (6.4%) and thymol (3.5%) dominating, were also a well-represented fraction. Also ***Sn*** was constituted mainly by fatty acids and esters (37.0%), with hexadecanoic acid (17.1%) and (*Z,Z*)-9,12-octadecadienoic acid (14.1%) prevailing. As in the other oils sesquiterpenes were quite abundant (19.6%), above all oxygenated sesquiterpenes (10.7%) and among these spathulenol (6.7%) prevailed. Carbonylic compounds (13.1%) were also well represented. There is no report on any thorough phytochemical investigation on *S. hydrophila* and *S. nivea* available to date. As described previously (Senatore et al., 2007), ***Sp*** consisted mainly of carbonylic compounds (25.4%), fatty acids and their esters (24.2%), accompanied by sesquiterpenoidic compounds (16.0%) and phenols (11.2%). The main compounds were found to be caryophyllene oxide (7.8%), hexahydrofarnesyl acetone (7.4%), hexadecanoic acid (6.8%) and (*Z,Z*)-9,12-octadecadienoic acid (6.7%). Phenols (33.1%) were the most abundant components in ***Ss***, particularly carvacrol (27.9%) and thymol (4.5%). Fatty acids (27.1%) were also well represented with (*Z,Z*)-9,12-octadecadienoic acid (16.3%) and hexadecanoic acid (10.6%) being the most representative compounds of this fraction. Also monoterpenes (10.9%), particularly oxygenated monoterpenes (8.7%), with pulegone (4.1%) and linalool (2.5%) prevailing, were quite abundant.

Comparing in short the six *Stachys* species reported in this paper, it can be noted that all have in common a great percentage of fatty acids and esters (24.2–58.5%) and a high amount of sesquiterpenes (16–35.9%), with the exception of ***Ss***, in which they account for only 7.7%. Monoterpenes are scarce for all the samples (1.1–10.9%) whilst carbonylic compounds are particularly abundant only in ***Sp*** (25.4%) and vary from 3.2 to 13.1% for the other oils. Phenols clearly prevail in ***Ss*** (33.1%), whilst their concentration is low for ***Sc***, ***Sh***, ***Sn*** and ***Sp*** (10.5–12.1%) and scarce for ***SgP*** and ***SgPB*** (3.2–3.4%). Hexadecanoic acid (6.8–37.4%) is the only compound abundant for all the *Stachys* species studied. It is known that many factors influence the chemical constitution of *Stachys* spp. oils. The differences in the quantity or quality of the oils' composition in the present and previous studies may be correlated to the collection time, chemotypes, drying conditions, mode of distillation and geographical and climatic factors, or probably may originate as a result of pollination caused by genetic differentiations (infraspecific or intrapopulation cross-pollination). 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 noticeably differ.

### 3.2. Radical scavenging activity

The antioxidant potential of essential oils from *Stachys* species was determined by DPPH test. The reduction of DPPH absorption is indicative of the capacity of the oils to scavenge free radicals, independently of any enzymatic activity. The scavenging effects of essential oils on DPPH were examined at different concentrations (0.250, 0.500, 1, 2.5 and 5 mg/ml). The absorbance decreases as a result of a colour change from purple to yellow as the radical is scavenged by antioxidants. All essential oils were able to reduce the stable free radical DPPH to the yellow-coloured 1,1-diphenyl-2-picrylhydrazyl (Table 2). ***Sp***, ***Sc*** and ***Sh*** showed the highest antiradical effect, with $IC_{50}$ values of 0.482, 0.652 and 0.664 mg/ml, respectively, whilst ***SgPB*** and ***Sgp*** showed similar lower activity ($IC_{50}$ of 1.26 and 1.12 mg/ml, respectively) (Fig. 1).

**Table 2**
Radical-scavenging activity of essential oils from *Stachys* species.

| Essential oil | $IC_{50}$ (mg/ml) |
|---|---|
| ***Sc*** | 0.652 ± 0.09 |
| ***SgPB*** | 1.26 ± 0.17 |
| ***SgP*** | 1.12 ± 0.16 |
| ***Sh*** | 0.664 ± 0.08 |
| ***Sn*** | 0.804 ± 0.07 |
| ***Sp*** | 0.482 ± 0.05 |
| ***Ss*** | 1 ± 0.12 |

Data are mean ± S.D. ($n$ = 3). Ascorbic acid (2 μg/ml) was used as positive control.

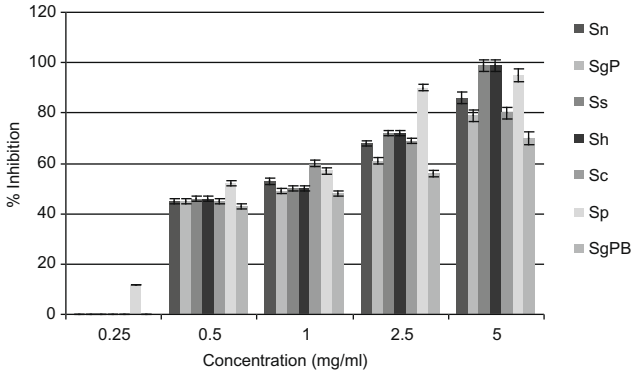


**Fig. 1.** Free radical-scavenging activity of essential oils from *Stachys* species.

The antioxidant activities of *Stachys* essential oils were evaluated, to obtain an insight into the beneficial effects of this plant species in conditions related to inflammation, reduced risk for cardiovascular diseases and cancer prevention. Previous pharmacological studies show that different *Stachys* species, such as *S. acerosa*, *S. alpina*, *S. officinalis*, *S. palustris*, *S. recta* subsp. *recta*, *S. recta* subsp. *subcrenata*, *S. salviifolia* and *S. sylvatica*, are relevant as DPPH scavengers, suggesting that they possess high radical-scavenging activity (Bilušić Vundać et al., 2007; Salehi et al., 2007). Essential oils are known to possess potential as natural antioxidants. Ruberto and Baratta (2000) have tested about 100 pure components of essential oils for their antioxidant effectiveness. The oils from *S. cretica*, *S. hydrophila* and *S. palustris*, that showed the highest antiradical effect in our study (Table 2), contain many of the compounds showed to be active. Sabinene and δ2-carene are present in ***Sc*** (0.6 and 0.8% respectively), guaiol in ***Sh*** (0.5%), whilst ***Sh*** and ***Sp*** contain 1-octen-3-ol (0.7 and 0.5%), α-terpineol (0.9 and 0.6%) and geraniol (0.4% for both). Other active components present in ***Sc***, ***Sh*** and ***Sp*** are pulegone (3.0, 2.5 and 0.3% respectively), thymol (1.8, 3.5 and 5.8%), carvacrol (2.2, 1.0 and 1.2%), eugenol (0.7, 1.2 and 0.4%) and (*E*)-phytol (0.2, 0.6 and 0.4%). We must remark, however, that essential oils are, from the chemical point of view, quite complex mixtures and this complexity makes it often difficult to explain the biological activities (Ruberto & Baratta, 2000).

### 3.3. Cytotoxic activity

The essential oils from *Stachys* species were tested for their ability to inhibit the growth of human tumour cell lines. 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. ***Sh*** exhibited weak cytotoxicity with 30% of inhibition on ACHN cell line and no activity on C32 cell line at a concentration of 100 μg/ml ($LD_{50}$ > 100 μg/ml). The most antiproliferative essential oil against C32 cell line was ***SgP***, followed by ***Ss*** with $LD_{50}$ values of 75 μg/ml and 83.5 μg/ml, respectively. ***SgP***, ***Sp*** and ***Ss*** showed the most antiproliferative activity on ACHN cell line with $LD_{50}$ values of 74.5 μg/ml, 83.9 μg/ml and 83.0 μg/ml, respectively.

**Table 3**
Cytotoxic activities of essential oils from *Stachys* species.

| Essential oil (100 μg/ml) | $LD_{50}$ (μg/ml) | |
|---|---|---|
| | **ACHN** | **C32** |
| ***Sc*** | >100 | >100 |
| ***SgPB*** | >100 | 87.2 |
| ***SgP*** | 74.5 | 75 |
| ***Sh*** | >100 | >100 |
| ***Sn*** | 90.8 | 93.2 |
| ***Sp*** | 83.9 | 90.5 |
| ***Ss*** | 83.0 | 83.5 |

Exposure time 48 h ± S.E.M. ($n$ = 3). ACHN: renal cell adenocarcinoma; C32: amelanotic melanoma cells. Vinblastine (2 μg/ml) was used as positive control.

Different *Stachys* species were found to be cytotoxic when their 80% aqueous MeOH extracts were tested on different tumour cell lines (Haznagy-Radnai et al., 2008). In the present study we show that also essential oils can be responsible for cytotoxic activity of *Stachys* species. Previous studies show that, generally, terpenic compounds are responsible for the pharmacological activity of essential oils (Ulubelen, Topcu, & Sonmez, 2000). Sesquiterpenes, which are a class of compounds well represented in the oils investigated, were previously reported to exhibit cytotoxic activity against different cell lines (Sylvestre, Pichette, Lavoie, Longtin, & Legault, 2007). Other compounds proved to be active on tumour cells are present in our oils. For instance some authors (Ait M'Barek et al., 2007) show that essential oils which contains carvacrol as the major component have an important *in vitro* cytotoxic activity against tumour cells resistant to chemotherapy, as well as a significant antitumour effect in mice. Carvacrol is present in high amounts (27.9%) in *S. spinosa* oil. Also palmitic acid, well represented in all the oils tested (6.8–37.4%), showed cytotoxicity *in vitro* and also *in vivo* antitumour activity in mice (Harada et al., 2002).

## 4. Conclusion

The results clearly show that the *Stachys* oils analysed in our study present strong antioxidant and cytotoxic activity against the two human tumour cell lines tested. All the plants of *Stachys* taxa seem to be promising as natural sources to develop free radical scavengers and cytotoxic compounds, and so could be proposed as potential substitutes of synthetic antioxidants in specific sectors of food preservation where their use is not in contrast with their aroma. An important finding of the present study is that pure essential oils are responsible for antioxidant and cytotoxic activity in *Stachys* species analysed. The biological activity could be explained partly by the presence of sesquiterpenes. Further investigations are needed to isolate and identify active components of the studied extracts and to confirm the pharmacological activity of these oils in more detail in animal models.

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