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Essential Oil Constituents of *Salvia argentea* L. from Tunisia: Phenological Variations

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ABSTRACT

The essential oils (EOs) from the aerial parts of *Salvia argentea* L. were analyzed at three developmental stages (vegetative, flowering and fruiting stages). The highest content of oil (0.15%, w/w) was obtained at full flowering. The current study showed consistent compositional variations among the three studied stages. In fact, manool and manoyl oxide characterised the vegetative stage while viridiflorol, camphor, methyl eugenol and 1,8-cineole prevailed during flowering and the fruiting phase was marked by the prevalence of viridiflorol, α-humulene, β-ionone and methyl eugenol. Additionally, a wide array of bioactive terpenic compounds was commonly found at different stages, making *S. argentea* an advocated herb in pharmaceutical science.

Keywords: aerial parts, development stages, *Salvia argentea*, volatile oil constituents

INTRODUCTION

Since old times, species of the genus *Salvia* (Lamiaceae) have been extensively used in popular medicine. They also find use as condiment, food additive and herbal tea (Demirci *et al.* 2005). In addition to that, their extracts and essential oils (EO) are known to have relevant antibacterial (Peana *et al*. 1999), antioxidant (Lu and Foo 2001; Gülçin *et al.* 2004), antiviral (Tada *et al*. 1994), antifungal (Fraternale *et al*. 2005), and anti-inflammatory activities (Geschel *et al*. 1998; Baricevic *et al*. 2001).

In the flora of Tunisia, numerous wild-growing *Salvia* species are investigated among them *Salvia argentea* which is encountered in fields and pastures in the Tunisian Dorsale (NE Tunisia) (Pottier-Alapetite 1979). The development and the survival of *S. argentea* depend on the climatic conditions. In fact, high pluviometric precipitations and temperatures, above those characteristic of the native regions, are found to be unfavourable to the species growth and were found to be even deleterious (Mossi *et al*. 2011).

S. argentea also known as silver sage by reference to its woolly aspect and silver foliage due to the existence of dense hairs on the two sides of leaves (Hedge 1972). This hairy appearance has attracted the attention since ancient times to the folk medicinal use of this plant as haemostatic against wounds (Pieroni *et al*. 2004). The leaves of the plant collected in winter and spring are consumed stewed locally in Spain (Tardío *et al*. 2005). *S. argentea* plants with their large white and violet flowers and their rosette basal leaves can be also used in ornamental purposes (Nakipoğlu 1993). Fruit were mucilaginous on wetting. That mucilage is used for the treatment of eye diseases (Baytop 1999). Besides, the hexane extracts of the aerial parts of *S. argentea* collected from Antalya, Turkey, may show high larvicidal activity against the mosquito *Culex pipiens* L. (Diptera: Culicidae) under laboratory conditions (Gün *et al*. 2011).

Phytochemicals studies pointed out that the species possesses a significant array of secondary metabolites such as triterpenoids and diterpenoids (Michavila *et al*. 1986; Bruno *et al*. 1987). Moreover, previous investigations demonstrated the occurrence of exudate flavones and flavonols in this plant (Nikolova *et al*. 2006). These secondary metabolites in addition to EO are particularly prone to qualitative and quantitative changes according to abiotic conditions as well as to the analytical methods used. Another source of variability that considerably affects the production of these volatile metabolites is the phenological stage.

In Tunisia, *S. argentea* still unexplored as most of the wild-growing species; actually there is only a work performed by Salah *et al*. (2006) which investigated its antimicrobial and antioxidant activities. As for the chemical composition, the international literature survey showed also few works focused on the volatile oil of this species (Holeman *et al*. 1984; Couladis *et al*. 2001). We recently started detailed chemical studies of *Salvia* (Ben Taârit *et al.* 2010a, 2010b, 2010c) in order to give a scientific contribution to the distribution and characterization of Tunisian species. Yet, no reports have been carried out on EO of silver sage from Tunisia so far. So, following our research we proposed to investigate the chemical constituents of the EO of *S. argentea* with a special emphasis to the phenological variations.

MATERIALS AND METHODS

Plant material

S. argentea plants were harvested from a wild population in the locality of Sers (Northwestern Tunisia, latitude (36° 03′ 26″ N); longitude (36° 4′ 26″ E); altitude 111 m). The sampling was done by a randomised collection of plants at three stages of development (vegetative, full flowering and fruiting). At vegetative stage, rosette leaves were collected. At the full flowering stage, all the flowers on the shoots were opened. At the fruiting stage, shoots with dark brown developed nutlets were harvested from plants. The botanical identification was performed by Prof. Abderrazak Smaoui (Biotechnology Centre in Borj-Cedria Technopol, Tunisia) and according to the morphological description in Tunisian flora (Pottier-Alapetite 1979). A voucher specimen (Sa2007) was deposited in the herbarium of Biotechnological Centre of Borj-Cedria (Tunisia). The aerial parts of the harvested material were essayed for EO composition.

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Original Research Paper

Essential oil isolation

The aerial parts were subjected to conventional hydrodistillation for 90 min followed by a liquid-liquid extraction using diethyl ether and *n*-pentane mixture (v/v) as solvent. The concentration step was carried out at 35°C using a Vigreux column and the EOs obtained were dried over anhydrous sodium sulphate and stored in amber vials at -18°C until they were analyzed.

Chromatographic analysis

1. Gas chromatography (GC- FID)

The EOs were analysed by gas chromatography using a Hewlett-Packard 6890 gas chromatograph (Palo Alto CA, USA) equipped with a flame ionization detector (FID) and an electronic pressure control (EPC) injector. A polar HP Innowax (PEG) column and an apolar HP-5 column (30 m × 0.25 mm, 0.25 µm film thicknesses) were used. The carrier gas was N_2 with a flow rate of 1.6 ml/min; split ratio was 60:1. The analysis was performed using the following temperature program: oven temps isotherm at 35°C for 10 min, from 35 to 205°C at the rate of 3°C/min and isotherm at 205°C during 10 min. Injector and detector temperature were held, respectively, at 250 and 300°C.

2. Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was performed on a gas chromatograph HP 5890 (II) interfaced with a HP 5972 mass spectrometer with electron impact ionization (70 eV). A HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness) was used. The column temperature was programmed from 50°C to rise 240°C at a rate of 5°C/min. The carrier gas was helium with a flow rate of 1.2 ml/min; split ratio was 60:1. Scan time and mass range were 1s and 40-300 *m/z* respectively.

3. Identification of compounds

The identification of the EO constituents was based on the comparison of their retention indexes relative to (C_8-C_{22}) *n*-alkanes with those from the Mass Spectral Library "Terpenoids and Related Constituents of Essential Oils" using the Mass Finder 3 Software (http://www.massfinder.com) and from literature as well as those of authentic compounds available in our laboratory. Further identification was made by matching their recorded mass spectra with those stored in the Wiley/NBS mass spectral library of the GC-MS data system and other published mass spectra (Adams 2001).

Statistical analyses

Data were subjected to statistical analysis using "Statistica" statistical program package (StatSoft 1998). The percentages of volatile compounds are means of three experiments, the one-way analysis of variance (ANOVA) followed by Duncan's multiple range test were employed and the differences between individual means were deemed to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

The variations of the EO yields of *S. argentea* aerial parts are shown in **Table 1**. Significant ($P < 0.05$) changes were observed at the different growth stages. During the vegetative stage, *S. argentea* rosette leaves offered an EO yield of 0.08%. At the flowering and fruiting stages, the EO yields increased significantly and reached 0.15 and 0.11%, respectively. Firstly, we noticed that the flowering shoots of the studied plants of *S. argentea* from Tunisia appear to be the richest in EO in comparison with those from other localities namely Morocco (Holeman *et al.* 1984) and Serbia (Couladis *et al.* 2001) and that at the same stage of growth.

Moreover, it is well known that the production of volatile compounds depends not only on the environmental conditions (temperature, relative humidity, rainfall and the period of sun exposition) (Kastner 1969) but also on biotic factors such as the stage of growth. The current observed rise in EO biosynthesis of *S. argentea* during flowering and fruiting was also noticed in *Thymus vulgaris* plants (Özguven and Tansi 1998). Other species belonging to the Lamiaceae family mainly *Hyptis suaveolens* (Oliveira *et al.* 2005), *Mentha piperita* (Rohloff *et al*. 2005) and *Satureja rechingeri* (Sefidkon *et al.* 2007) exhibited a maximum EO yield during the flowering stage. Moreover, the survey of EO yield variations of *Salvia bracteata* revealed enrichment during flowering followed by a sharp decrease (Amiri 2007). Pitarevic *et al.* (1984) also recorded a variation in EO yield for *S. officinalis* collected in Yugoslavia over various seasons, with flowering period giving the highest yield. Likewise those *Salvia* species, *S. argentea* at full-flowering stage appears appropriate to harvesting in order to ensure maximum EO yield.

EO compounds identified in *S. argentea* aerial parts are listed in **Table 1** following their elution order on the HP-5 column. As observed, the EO was resolved into 28 components at the vegetative, flowering and fruiting stages. The common constituents to the three oil samples were manool, manoyl oxide, α-pinene, α-fenchone, camphor, viridiflorol, α-cadinol and T-cadinol. Although these constituents were found in the three studied stages of development, notable quantitative variations were observed. In fact, the rosette stage is marked by the prevalence of labdane type diterpenes. This class of compounds, albeit not frequent in aromatic plants, had an interesting pharmacological concern. Hence, an *in vitro* cytotoxic activity against lines of human leukemic cells was demonstrated (Dimas *et al.* 1998). Oxygenated sesquiterpenes mostly viridiflorol, α-cadinol and T-cadinol constitute a second main terpenic class at rosette stage. These compounds could attribute to the plant numerous biological activities during this phase of development. In fact, the viridiflorol is owing to the EO a camphorous odour note (Sefidkon and Mirza 1999) and an antifungal potential (Kordali *et al*. 2005). Currently, α-cadinol and T-cadinol showed an efficient antimite activity (Chang *et al.* 2001a). Moreover, α-cadinol is also advocated for its termiticidal property (Chang *et al.* 2001b). In addition to that, it possesses antifungal activities against a broad spectrum of tested plant pathogenic fungi and could be used as potential antifungal agent for the control of fungal diseases in plants (Chang *et al.* 2008).

At the flowering stage, the EO was found to be rich in oxygenated terpenic forms. Based on this finding, it seems that EO at this stage is of high quality since it is recognized that the high quality of oil is closely related to a substantially higher amounts of oxygenated components and lower amounts of hydrocarbons (Özel *et al.* 2006). Among the oxygenated compounds, viridiflorol was found to be the major component of the oil followed by the camphor. Accordingly, the EO of silver sage from Serbia during flowering contain a substantial amount of sesquiterpenes represented mostly by viridiflorol (32.4%) pursued by α-humulene (10.7%). Hence, viridiflorol is the pre-eminent constituent of the EO of silver sage from both of Serbia (Couladis *et al.* 2001) and Tunisia. Besides, a fair amount of the diterpeneol manool (14.6%) characterised the EO from Serbia. While the volatile profile of the plant from Morocco during the flowering phase belongs to the camphor chemotype (Holeman *et al.* 1984). These authors reported that EO is prevailed by monoterpenes (camphene, α-pinene, borneol and α-thujone) which is in clear contrast with our findings and those of Couladis *et al.* (2001).

Furthermore, it is worthy to note that the flowering stage is characterised by the occurrence of the non-terpenic compounds namely eugenol methyl and β-ionone. Of interest, these components have been advocated for their biological activities methyl eugenol, for example, constitutes an antifungal and an antibacterial agent (Wright 2007), besides its central nervous system depressant activity with anesthetic, hypothermic, myorelaxant and anticonvulsant properties (Emea 2004). β-Ionone is another compound

Table 1 Relative percentage (%) of the essential oil compounds from *S. argentea* at different phenological stages.

Compounds*	RI[a]	RI[b]	Rosette	Flowering	Fruiting
(Z)-3-Hexenol	855	1370	-	0.13 ± 0.03 b	1.14 ± 0.08 a
α-Pinene	939	1032	2.03 ± 0.03 b	3.15 ± 0.15 a	1.90 ± 0.08 c
1,8-Cineole	1033	1213	-	5.8 ± 0.05 a	2.8 ± 0.18 b
α-Fenchone	1087	1406	3.17 ± 0.15	-	-
α-Thujone	1089	1430	-	0.99 ± 0.18 b	1.59 ± 0.12 a
Linalool	1098	1553	1.07 ± 0.12 c	2.3 ± 0.15 a	1.30 ± 0.15 b
β-Thujone	1103	1451	-	0.79 ± 0.12 b	1.86 ± 0.15 a
Camphor	1143	1532	6.68 ± 0.15 b	9.02 ± 0.10 a	3.79 ± 0.10 c
Borneol	1165	1719	0.16 ± 0.09 c	2.1 ± 0.10 a	1.94 ± 0.15 b
Terpinen-4-ol	1178	1611	0.28 ± 0.09	-	-
α-Terpineol	1189	1706	0.66 ± 0.05	-	-
Linalyl acetate	1239	1565	1.27 ± 0.04 a	0.14 ± 0.12 c	1.02 ± 0.12 b
Bornyl acetate	1270	1590	0.22 ± 0.05 a	0.12 ± 0.09 b	0.13 ± 0.04 b
Geranyl acetate	1383	1765	1.58 ± 0.05 a	0.08 ± 0.15 b	0.07 ± 0.15 b
Neryl acetate	1385	1733	0.52 ± 0.15	-	-
Methyl eugenol	1402	2028	0.53 ± 0.04 b	6.87 ± 0.05 a	6.13 ± 0.05 a
β-Caryophyllene	1418	1612	1.85 ± 0.02	-	-
Aromadendrene	1443	1628	-	3.66 ± 0.10 b	3.96 ± 0.15 a
α-Humulene	1454	1687	2.24 ± 0.11 c	4.80 ± 0.07 b	8.83 ± 0.15 a
Germacrene-D	1480	1726	0.67 ± 0.02 a	0.30 ± 0.06 c	0.47 ± 0.15 b
β-Ionone	1482	1960	2.86 ± 0.15 c	3.87 ± 0.15 b	6.57 ± 0.15 a
Spathulenol	1572	2152	0.91 ± 0.02 b	4.36 ± 0.01 a	4.48 ± 0.05 a
Caryophyllene oxide	1580	2008	1.50 ± 0.08 c	3.05 ± 0.02 a	2.05 ± 0.15 b
Iso-Caryophyllene oxide	-	2008	0.99 ± 0.07	-	-
Viridiflorol	1592	2104	4.70 ± 0.18 c	15.9 ± 0.12 a	11.3 ± 0.08 b
Humulene epoxide I	1596	2042	2.88 ± 0.10 a	2.87 ± 0.18 a	2.40 ± 0.15 b
γ-Eudesmol	1618	2185	1.66 ± 0.10 b	2.14 ± 0.13 a	1.45 ± 0.05 c
T-Cadinol	1640	2187	7.00 ± 0.10 a	1.35 ± 0.05 c	1.58 ± 0.12 b
β-Eudesmol	1650	-	0.50 ± 0.10	-	-
α-Cadinol	1652	2255	5.29 ± 0.10 a	3.58 ± 0.02 b	1.58 ± 0.02 c
Tetradecanoic acid	1768	-	-	2.77 ± 0.20 b	3.71 ± 0.12 a
1-Octadecene	1793	-	2.25 ± 0.14 b	0.32 ± 0.18 c	2.68 ± 0.09 a
Manoyl oxide	1990	-	18.10 ± 0.05 a	1.12 ± 0.08 b	1.12 ± 0.08 b
Manool	2056	-	20.15 ± 0.32 a	2.47 ± 0.12 b	1.50 ± 0.20 c
Phytol	2111	-	-	1.05 ± 0.18 b	2.51 ± 0.14 a
Grouped compounds					
Monoterpene hydrocarbons			2.03	3.15	1.90
Oxygenated monoterpenes			12.02	21.00	13.28
Sesquiterpene hydrocarbons			4.76	8.76	13.26
Oxygenated sesquiterpenes			25.43	33.25	24.84
Diterpenes			38.25	4.64	5.13
Others			9.23	14.30	21.45
Essential oil yield (%, w/w)			0.08 ± 0.005 c	0.15 ± 0.06 a	0.11 ± 0.05 b

* Order of elution in apolar column (HP-5). Means (n = 3) in the same lines with a different letter (a–c) are significantly different at $P < 0.05$ according to DMRT. RI, retention indices on (a) the HP-5 and (b) HP Innowax columns; - : not detected.

with proved activity in rat mammals as potent anticancer both *in vitro* and *in vivo* (Liu *et al.* 2008). More compounds characterised the EO at this stage namely aromadendrene, spathulenol, caryophyllene oxide, α-cadinol, tetradecanoic acid and particularly 1,8-cineole.

As regards the fruiting phase, viridiflorol is a prevailing compound accompanied with α-humulene which may attributed to the plant EO a potent antimicrobial property (Shafi *et al.* 2002). Interestingly, we detected also some oxygenated sesquiterpenes apart from viridiflorol we cite mainly spathulenol, caryophyllene oxide and humulene epoxide I which could confer to the plant numerous biological activities. In fact, spathulenol is credited with a possible immunomodulatory activity according to Ziaei *et al.* (2011). Moreover, caryophyllene oxide is known as antimicrobial (Shafi *et al.* 2002). It was noticeable that the fruiting stage contained an important proportion of eugenol methyl and β-ionone as the flowering stage. Additional non-terpenic compounds namely (*Z*)-3-hexenol, octadecene and tetradecanoic acid were detected. The occurrence of these compounds can explain the abundance of waxes and resins in the fruit. Regarding the tetradecanoic acid, it accordingly occurs (3.6%) in the EO of the Serbian silver sage (Couladis *et al.* 2001). Generally, fatty acids are frequently found in the EO of the different *Salvia* species. In fact, an important amount of palmitic acid was detected in the EO of *S. pratensis* (14.3-19.1%), *S. nemorosa* (2.80-18.50%) and *S. bertoloni* (15.50%) originated from Serbia and Montenegro (Mimica-Dukić *et al.* 2002).

Furthermore the existence of the (*Z*)-3-hexenol, a C_6-alcohol biosynthesized in the lipoxygenase/HPL pathway, is in relation to the herbivore repellence/attraction, as well as the induction of gene expression in neighboring unattacked plants. This compound was credited with positive roles in the indirect defence. Therefore, this compound can be used to develop novel insect pest control strategies (Wei and Kang 2011). The very low percentage of this compound proves that silver sage plants were preserved from animal damage and cutting practises (Matsui 2006). Some sesquiterpenes namely spathulenol, caryophyllene oxide and aromadendrene are common to both of the flowering and fruiting stage.

The sharp variability of the observed EO composition through the different phases of herbal development is a common feature of *Salvia* spp. In fact, Pitarevic *et al.* (1984) reported a variation in EO composition of *S. officinalis* collected at various seasons of the year. Moreover, differences on EO composition of *S. officinalis* were afforded to phenological stages according to Mirjalili *et al.* (2006). Furthermore, Zawislak and Dyduch (2006) found that the oil components of *S. officinalis* varied quantitatively depending on the harvest time. According to Piccaglia *et al.* (1997), the oil produced from *S. officinalis* plants harvested at flowering stage differed from that of plants collected at the vegetative stage. Marked EO variations were also detected during the development phases of *Salvia fruticosa* (Müller-Riebau *et al.* 1997). As well as for *S. sclarea*, qualitative and quantitative changes occur during stages of inflorescence maturity according to Balinova-Tsvetkovaan and Tsankova (1992) and Lattoo *et al.* (2006). At full flowering and early seed ripeness stages Carrubba *et al.* (2002) and Lorenzo *et al.* (2004) reported differences in EO composition. Pešić and Banković (2003) also indicated some compositional fluctuations in oil at full blossom, at seed formation, and at full seed maturity. Qualitative variation in EO composition of *S. libanotica* was also demonstrated where the major components of the oil fluctuated from one season to another (Farhat *et al.* 2001). Other Lamiaceae members (*Thymbra spicata* var. *spicata*, *Satureja thymbra* and *Mentha pulegium*) presented also the same differential pattern (Müller-Riebau *et al.* 1997).

The observed quantitative and qualitative variations in EO of silver sage are likely due to morphologic differences which appear in course of the phenological cycle. In fact, according to the hypothesis of Kramer and Kozlowski (1979), the metabolites of photosynthesis are converted in course of the secondary metabolism into flowers and fruits following the ontogenesis which explain the increase in EO accumulation at flowering and fruiting phases. Luckner (1980) ascribed these EO variations to the biosynthesis or activation of particular enzymes during a specific stage of plant development. Such pattern could explain the higher EO yield obtained during silver sage flowering the period in which plants may produce substantial amounts of volatile compounds in order to attract more pollinators (Palá-Paúl *et al.* 2001).

CONCLUSIONS

In summary, our results indicate that silver sage is marked by a phenological regulation of its EO production. A remarkable rise of the EO accumulation has been observed during flowering indicating the optimum period of plant harvesting. This paper gives a better understanding of the dynamic of biosynthesis of these secondary metabolites in course of the plant development.

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