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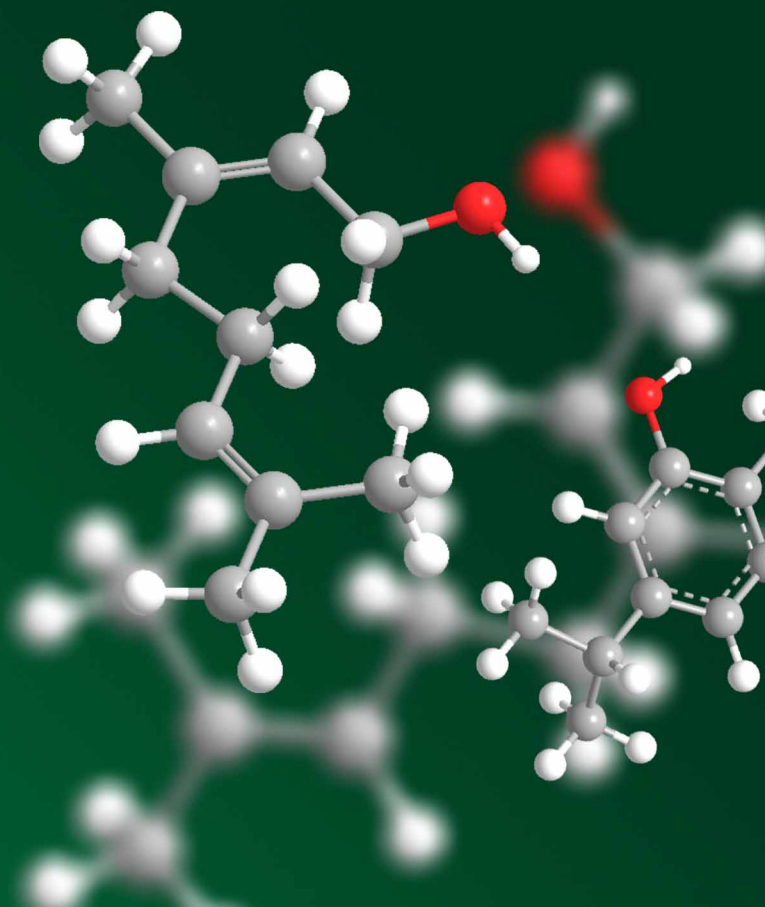

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REVIEW

A review on the essential oil chemical profile of *Salvia* genus from Iran

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Abstract

Iran is one of the major centres of diversity for *Salvia*, with 58 species of which 25% are endemic. A literature-based survey of the chemical profile of *Salvia* species essential oil found in Iran was carried out. As a result, 46 species belonging to *Salvia* genus were identified for their essential oil composition. A concise review of the scientific literature pertaining to components of *Salvia* essential oils and volatile fractions is presented. The present review showed that the Iranian *Salvia* species have been classified into four main categories based on the GC/MS and GC-FID analyses of their essential oils. monoterpene chemotype, mono and sesquiterpene chemotype, sesquiterpene chemotype and GLV chemotype which stands for green leaf volatiles. It was indicated the most of the studied *Salvia* species were considered as sesquiterpene chemotype. Among the sesquiterpenes, β-caryophyllene followed by germacrene D, spathulenol and caryophyllene oxide were the most common and abundant in Persian *Salvia* species. The β-caryophyllene and germacrene D were reported from 27 and 22 *Salvia* species in Iran, respectively.

Keywords: *Salvia*, Lamiaceae, Essential oil

Introduction

Aromatic plants are at present widely studied for their large therapeutic potential and benefits. These benefits depend largely on essential oils which, in general terms, occur in many herbs. Essential oils are used to give flavour to foods and drinks and as fragrances in the food and cosmetics industries, where numerous herbal plant and spice ingredients are components in the manufacture of skin creams, lip balms, shampoos, soaps and perfumes (de Almeida et al., 2011).

The largest genus of the Lamiaceae family, the genus *Salvia* L. represents an enormous and cosmopolitan assemblage of nearly 1000 species displaying remarkable variation. It has undergone marked species radiations in three regions of the world: Central and South America (500 spp.), Central Asia/ Mediterranean (250 spp.) and Eastern Asia (90 spp.) (Walker et al., 2004, Claßen-Bockhoff, 2017). Iran, particularly, is one of the centers of origin of the genus with 58 species, here called with the common Persian name of "Maryam-Goli" and about 25% of endemics (Mozaffarian, 1996.)

Several species of *Salvia* are cultivated for their aromatic characteristics and are used as flavourings, food condiments, cosmetics, and perfume additives (Firdous et al., 1999). Additionally, *Salvia* species have commonly been widely used as folk medicines as antibacterial, antiviral, antitumor, spasmolytic, antioxidant and anti-inflammatory treatments and have further been used in the treatment of nervous and gastrointestinal conditions (Lu & Foo, 2002; Tepe et al., 2004)

The diversity, species richness, and variation, as well as chemical properties have led to much research on the genus *Salvia*. Diterpenoids of the abietane, icetexane, labdane, neoclerodane and phenalenone types (Kusumi et al., 1985; Nieto et al., 2000), triterpenes and sterols (Rauter et al., 2007), anthocyanins, coumarins, polysaccharides, flavonoids and phenolic acids and their derivatives (Lu & Foo, 2002) were reported as major

constituents of *Salvia* species. Most *Salvia* species are rich in essential oils, and various biologically active monoterpene/sesquiterpene have been reported in them possessing diverse biological activities such as antioxidant (Tepe et al., 2004; Kabouche et al., 2007; Guy et al., 2010), anti-inflammatory [11,12], analgesic and antipyretic [13], antiepileptic, anti-ulcerogenic, tranquillizing activities [14] and (Guy et al., 2010; El-Sayed et al., 2006) antimicrobial activities (Tepe et al., 2004; Tepe et al., 2005; Usama & Mohamed, 2006). Furthermore, the *Salvia* species, often pleasantly aromatic plants of potential economic interest, comprise most of the essential oil rich genera of the Lamiaceae, and particularly tend to accumulate monoterpenoid-rich essential oils.

There are several reports on the chemical composition of the essential oils from the members of the genus *Salvia* found in Iran. With the purpose of giving an overview of the structural complexity and interesting chemical diversity of the essential oil composition of the genus *Salvia*, here we review systematically the articles reported over the past decades, concerning the isolation and structural elucidation of Persian *Salvia* sp. essential oil components. Particularly, we report on the essential oil composition referring to 46 native and endemic species of *Salvia* grow wild in Iran.

Materials and Methods

The present study was carried out based on the literature review of the essential oil chemical composition of native or endemic *Salvia* species found in Iran. The data presented in this work was collected using scientific data retrieved from encyclopaedia books, journals, articles, and websites including Pubmed, Scopus and Google Scholar.

Results and Discussion

In this review the essential oil chemical profile of 46 species including the native and endemic *Salvia* species found in Iran have been reported. All the native and endemic species which grow wild in Iran are given in Table 1.

Table 1. The native and endemic *Salvia* species found in Iran (Mozaffarian, 1996).

No.	Scientific Name	Persian Name	Synonyms	N/E	[Ref.]
1	*S. aegyptiaca* L.	Mesri Maryam-goli	*S. multicaulis* Vahl.	Native	(Jassbi et al., 2012)
2	*S. aethiopis* L.	Pashmalu Maryam-goli	*S. sclarea* L.	Native	(Rajabi et al., 2014; Salimpour et al., 2011)
3	*S. aristata* Aucher ex Benth.	Sikhak-dar Maryam-goli	-	Native	(Emadipoor et al., 2016)
4	*S. artopatana* Bunge	Azerbayjani Maryam-goli	-	Native	(Jassbi et al., 2012; Salimpour et al., 2011; Mirza & Ahmadi, 2000)
5	*S. bazmanica* Rech. f. & Esfand	Bazmani Maryam-goli	*S. multicaulis* Vahl.	Endemic	(Akhgar et al., 2011)
6	*S. brachyantha* (Bordz.) Pobed	Forutan Maryam-goli	*S. indica* L.	Native	(Jamzad et al., 2012)
7	*S. bracteata* Banks & Soland.	Barge-dar Maryam-goli	-	Native	(Amiri, 2077; Sefidkon et al., 2007)
8	*S. ceratophylla* L.	Shakh-gavazni Maryam-goli	-	Native	-
9	*S. choloroleuca* Rech. f. & Aell.	Sefid Maryam-goli	-	Native	(Jassbi et al., 2012; Khalilzadeh et al., 2011)
10	*S. chorassanica* Bunge.	Khorassani Maryam-goli	*S. limbata* C. A. Mey., *S. splendens* Ker. Gawl.	Endemic	(Ebrahimi et al., 2014)
11	*S. compressa* Vent.	Marmarashk	*S. macrosiphon* Boiss., *S. spinosa* L., *S. hydrangea* DC. ex Benth.	Native	(Mirza & Bahernik, 2007)

12	*S. eremophila* Boiss.	Biabani Maryam-goli	-	Endemic	(Jassbi et al., 2012; Rajabi et al., 2014; Salehi et al., 2014)
13	*S. frigida* Boiss.	Yakhchali Maryam-goli	-	Native	-
14	*S. glutinosa* L.	Jangali Maryam-goli	-	Native	(Tavassoli et al., 2009)
15	*S. grossheimii* Sosn.	Nakhjavani Maryam-goli	*S. viridis* L.	Native	(Mirza & Bahernik, 2010)
16	*S. hydrangea* DC.	Aghraban	-	Native	(Sonbili et al., 2006; Ebrahimi & Ranjbar, 2016)
17	*S. hypochionaea* Boiss.	Khoee Maryam-goli	-	Endemic	-
18	*S. hypoleuca* Benth.	Boland Maryam-goli	-	Endemic	(Jassbi et al., 2012; Sonbili et al., 2016; Nichavar et al., 2005)
19	*S. indica* L.	Hendi Maryam-goli	-	Native	(Jamzad et al., 2011; Bahernik & Mirza, 2010)
20	*S. jamzadii* Mozaff.	Bakhtiari Maryam-goli	-	Endemic	-
21	*S. kermanshahensis* Rech. f.	Kermanshahi Maryam-goli	*S. atropatana* Bunge., *S. staminea* Montbr. & Auch. ex Benth.	Endemic	-
22	*S. lanchnocalyx* Hedge.	Eghlidi Maryam-goli	-	Endemic	-
23	*S. lanigera* Poir.	Ilami Maryam-goli	-	Native	-
24	*S. leriifolia* Benth.	Kaboli Maryam-goli	-	Native	(Attaran et al., 2016; Monfared & Ghorbanli, 2010; Yousefi et al., 2015)
25	*S. limbata* C. A. Mey.	Labeh-dar Maryam-goli	-	Native	(Sajjadi & Shahpiri, 2004; Rajabi et al., 2014; Rustaiyan et al., 2005; Morteza-Semnani et al., 2014; Mirza et al., 2005)
26	*S. macilenta* Boiss.	Masghati Maryam-goli	-	Native	(Sonbili et al., 2005)
27	*S. macrochlamys* Boiss. & Kotschy	Gol-dorosht Maryam-goli	-	Native	(Kazemizadeh et al., 2010)
28	*S. macrosiphon* Boiss.	Lule-e Maryam-goli	-	Native	(Jassbi et al., 2012; Rajabi et al., 2014; Mirza et al., 2005; Sefidkon et al., 2013; Kariminik et al., 2019; Salimpour et al., 2011)
29	*S. mirzayanii* Rech. f. & Esfand.	Morpojo	*S. brachyantha* (Bordz.) Pobed.	Endemic	(Sonboli et al., 2006; Yamini et al., 2008; Mirza et al., 2003; Asadipour et al., 2013; Haghighat et al., 2012; Javidnia et al., 2002)
30	*S. multicaulis* Vahl.	Arghavani Maryam-goli	-	Native	(Ahmadi & Mirza, 1999)
31	*S. nemorosa* L.	Mazrae-rui Maryam-goli	-	Native	(Rajabi et al., 2014; Mirza & Sefidkon, 1999)
32	*S. officinalis* L.	Daruee Maryam-goli	-	Native	(Kazemi et al., 2015; Alizadeh & Shaabani, 2012)
33	*S. oligophylla* Auch. ex Benth.	Rudbari Maryam-goli	-	Endemic	(Salimpour et al., 2011)
34	*S. pachstachys* Trautv.	Kutah-sonbol Maryam-goli	-	Native	(Amiri, 2007; Shakeri et al., 2018)
35	*S. palaestina* Benth.	Felestini Maryam-goli	-	Native	(Rustaiyan et al., 2005; Salehi et al., 2005)
36	*S. persepolitana* Boiss.	Perspolisi Maryam-goli	*S. aristata* Aucher. ex Benth.	Endemic	-
37	*S. plebeia* R. Br.	Baluchestani Maryam-goli	-	Native	-
38	*S. poculata* Nab.	Fenjani Maryam-goli	-	Native	-
39	*S. reuterana* Boiss.	Isfahani Maryam-goli	-	Native	(Jassbi et al., 2012; Rajabi et al., 2014; Mirza & Sefidkon, 1999; Batooli et al., 2013; Salimpour et al., 2011; Lari Yazdi et al., 2005)
40	*S. rhytidea* Benth.	Taftani Maryam-goli	-	Native	(Rustayian et al., 2005; Sajjadi & Ghanndi, 2005; Habibi et al., 2008)
41	*S. russellii* Benth.	Halabi Maryam-goli	-	Native	-

42	*S. sahendica* Boiss. & Buhse.	Sahandi Maryam-goli	-	Endemic	(Salehi et al., 2004)
43	*S. santolinifolia* Boiss.	Khaliji Maryam-goli	-	Native	(Jassbi et al., 2012; Sonboli et al., 2006; Rustayian et al., 2005; Bahadori et al., 2016; Salehi et al., 2014)
44	*S. sclarea* L.	Maryam-goli	-	Native	(Rajabi et al., 2014; Batooli et al., 2013; Salimpour et al., 2011)
45	*S. sclareopsis* Bornm. ex Hedge.	Karandi Maryam-goli	*S. ceratophylla* L.	Endemic	(Hemmati et al., 2018)
46	*S. sharifii* Rech. f. & Esfand.	Jonubi Maryam-goli	*S. hydrangea* DC. ex Benth.	Endemic	(Zare & Jassbi, 2014; Asgarpanah et al., 2017)
47	*S. spinosa* L.	Khar-dar Maryam-goli	-	Native	(Salehi et al., 2014; Bahernik & Mirza, 2005)
48	*S. staminea* Montbr. & Auch. ex Benth.	Parcham-boland Maryam-goli	-	Native	(Salehi et al., 2013)
49	*S. suffruticosa* Montbr. & Auch. ex Benth.	Bute-e Maryam-goli	*S. aristata* Aucher. ex Benth.	Native	(Jamzad et al., 2011; Norouzi-Arasi et al., 2005)
50	*S. syriaca* L.	Suri Maryam-goli	-	Native	(Lari Yazdi et al., 2005; Sefidkon & Mirza, 1999)
51	*S. tebesana* Bunge.	Tabasi Maryam-goli	-	Native	(Goldansaz et al., 2017)
52	*S. trichoclada* Benth.	Shakhe-korki Maryam-goli	-	Native	-
53	*S. urumiensis* Bunge.	Urumie-e Maryam-goli	-	Endemic	(Khalilzadeh et al., 2011)
54	*S. verticillata* L.	Banafsh Maryam-goli	-	Native	(Rajabi et al., 2014)
55	*S. virgata* Jacq.	Harz Maryam-goli	-	Native	(Rajabi et al., 2014; Sefidkon & Mirza, 1999)
56	*S. viridis* L.	Sabz Maryam-goli	-	Native	-
57	*S. wendelboi* Hedge.	Sanandaji Maryam-goli	-	Endemic	-
58	*S. xanthocheila* Boiss. ex Benth.	Alborzi Maryam-goli	-	Native	(Khalilzadeh et al., 2011; Salehi et al., 2005)

The essential oils of the Iranian *Salvia* species are the best-studied *Salvia* products. To the best of our knowledge, the essential oils from 46 species of sage, have been analysed by gas chromatography (GC) and gas chromatography/Mass (GC/MS) spectroscopy. Publications on *Salvia* essential oils clearly demonstrate that chemical polymorphism is characteristic of this genus, and the oil composition depends on variety, growing site, climatic conditions, and analysis method. However, the composition of volatile compounds is known for most of the species, few data are found in chemotaxonomic studies of this genus.

The Iranian *Salvia* species have been classified into four main categories based on the GC/MS and GC-FID (flame ionization detector) analyses of their essential oils (Jassbi et al., 2012).

The first category (monoterpene chemotype) includes the *Salvia* species which contain monoterpene-rich essential oils with pinane, *p*-menthane, isocamphane, and bornane C-skeletons. The bornane-type monoterpenes are mostly in the form of borneol or its acetate ester. α-pinene, β-pinene, linalool and the related ester linalyl acetate, 1,8-cineole and borneol are characterized as the main chemical markers of the first category. Camphene and sabinene are the other major monoterpenes in some species in this chemotype. Most of the oils in this category contain α- and β-pinene as the monoterpene hydrocarbons, but the single-isomer is usually in higher concentration.

The second category (mono and sesquiterpene chemotype) consists of the *Salvia* species which contain almost equal quantities of monoterpenes with 2,6-dimethyl octane, pinane, *p*-menthane, and bornane C-skeleton, and sesquiterpenes with caryophyllane, aromadendrane, germacrane, or cadinane C-skeleton. β-caryophyllene, germacrene D, bicyclogermacrene, spathulenol, and caryophyllene oxide are identified as the major chemical markers of this category.

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The third category of the *Salvia* species (sesquiterpene chemotype) contains sesquiterpenes with caryophyllane-type C-skeleton as the predominant compounds, germacrane, aromadendrane, cadinane, and other sesquiterpenes as the other major constituents. The monoterpene fractions of these oils are in lower levels compared to the sesquiterpene.

The last category of the *Salvia* species (GLV chemotype) is rich in green leaf volatiles (GLVs; C6 alcohols, aldehydes, and their ester derivatives), aldehydes, esters and other fatty acids or low-molecular-weight acid derivatives, aromatic phenylpropanoids, and finally non-phenylpropanoid volatiles, which are synthesized from amino acid pathways (Baldwin, 2010). This group is called GLV producers.

Among the terpenes, the most abundant components determined are the sesquiterpenes that frequently appear as the main constituents and most of the studied *Salvia* species are considered as sesquiterpene chemotype. Sesquiterpenes are a class of terpenes that consist of three isoprene units and often have the molecular formula $C_{15}H_{24}$. They may be acyclic or contain rings.

The biosynthesis of the sesquitepenes is described as production of sesquiterpene precursor farnesyl diphosphate (FDP) from coupling dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Degenhardt et al., 2009). Then, these precursors are taken as substrates by different sesquiterpene synthases, many of which are multi-product enzymes. Biochemical modifications such as oxidation or rearrangement produce the related sesquiterpenoids.

Among the sesquiterpenes, β-caryophyllene followed by germacrene-D, spathulenol and caryophyllene oxide are the most common and abundant in Persian *Salvia* species studied. The β-caryophyllene and germacrene D have been reported from 27 and 22 *Salvia* species in Iran, respectively. β-Caryophyllene and germacrene D are representative of the sesquiterpenes derived from an initial macrocyclic intermediate formed by a C_1–C_{11} ring closure. These sesquiterpenes are distinguished by being directly derived from the trans FPP substrate and not requiring isomerization to the Cis conformation or nerolidyl diphosphate intermediate and in this regard these components might be considered the simplest of sesquiterpenes.

According to the Table 2, *S. aethiopis, S. atropatana, S. bracteata, S. compressa, S. grossheimii, S. hypoleuca, S. indica, S. nemorosa, S. palaestina, S. reuteriana, S. syriaca, S. verticillata, S. verbascifolia, S. virgata*, *and S. xanthocheilacan* are include the third category of the *Salvia* classification while *S. brachysiphon*, S*. chloroleuca, S. hydrangea, S. hypoleuca, S. lachnocalyx, S. limbata, S. mirzayanii*, and *S. virgata* can be classified in the second category of mono and sesquiterpene chemotype in which the *Salvia* species produce essential oils with almost equal quantities of mono and sesquiterpenes.

α-Pinene constituted the most abundant compound in the oils of the *Salvia* species in the first category of monoterpene chemotype.

Salvia species, which the essential oils contain monoterpenes include *S. aegyptiaca, S. brachycalyx, S. bracteata, S. eremophila, S. limbata, S. leriifolia, S. macilenta, S. mirzayanii, S. multicaulis, S. rhytidea, S. sahendica, S. santolinifolia*, and *S. suffruticosa*.

S. atropatana, S. macrosiphon, S. persepolitana, S. reuteriana, S. sclareopsis, and *S. spinosa* are the species which the essential oils are rich in GLVs and other low-molecular-weight acid derivatives. Greenleaf volatiles are biosynthesized via different pathway. They are synthesized from fatty acid hydroperoxides (Dewick, 1997).

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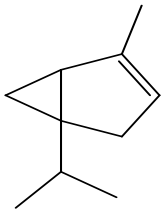
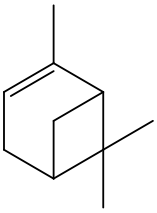
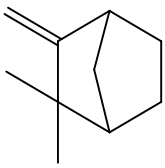
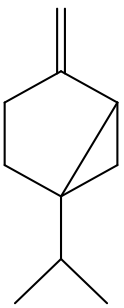
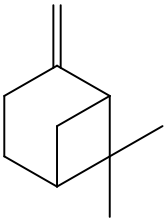
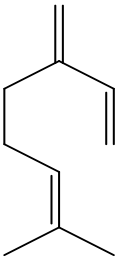
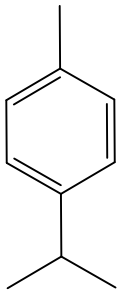
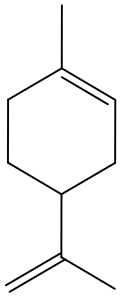
Table 2. Components of the essential oil of *Salvia* species found in Iran (5% <).

No.	Compound	Species	Percentage (%)	References
1	α-Thujene 	*S. bazmanica*	9.5	(Akhgar et al., 2011)
2	α-Pinene 	*S. aegyptiaca*	8.3	(Jassbi et al., 2012)
		S. aristata	6.7	(Emadipour et al., 2016)
		S. bazmanica	8.9	(Akhgar et al., 2011)
		S. bracteata	28.9	(Amiri, 2007)
		S. chloroleuca	11.4	(Khalilzadeh et al., 2011)
		S. eremophila	31.5	(Jassbi et al., 2012)
		S. hydrangea	5.5	(Sonboli et al., 2006)
		S. hypoleuca	5.8, 29.3, 5.9	(Jassbi et al., 2012; Sonboli et al., 2016; Nickavar et al., 2005)
		S. indica	17.0	(Jamzad et al., 2011)
		S. leriifolia	15.1, 16.5	(Attaran et al., 2016; Monfared & Ghorbanli, 2010)
		S. limbata		(Sajjadi & Shahpiri, 2004; Rajabi et al., 2014; Rustaiyan et al., 2005)
		S. macilenta	15.5, 17.1, 23.7	(Sonboli et al., 2005)
				(Ahmadi & Mirza, 1999)
		S. multicaulis	60.0	(Shakeri et al., 2018)
		S. pachystachys	15.6	(Salehi et al., 2004)
		S. sahendica	8.0	(Jassbi et al., 2012; Sonbili et al., 2006; Bahadori et al., 2016)
		S. santolinifolia	14.2	
		S. tebesana	52.5, 49.3, 72.4	(Khalilzadeh et al., 2011)
				(Goldansaz et al., 2017)
		S. urumiensis	7.5	
		S. tebesana	14.0	
3	Camphene 	*S. eremophila*	13.7	(Salehi et al., 2014)
		S. leriifolia	10.9	(Yousefi et al., 2015)
		S. pachystachys	11.7	(Amiri, 2007)
		S. santolinifolia	8.1, 7.8	(Rustaiyan et al., 2005; Salehi et al., 2014)
				(Jamzad et al., 2011)
		S. suffruticosa	7.9	
4	Sabinene 	*S. chloroleuca*	9.6	(Khalilzadeh et al., 2011)
		S. limbata	8.3, 10.6, 14.5	(Sajjadi & Shahpiri, 2004; Rustaiyan et al., 2005; Morteza-Semnani et al., 2014)
				(Rustayian et al., 2005; Sajjadi & Ghannadi, 2005; Habibi et al., 2008)
		S. rhytidea	5.8, 13.5, 17.5	(Salehi et al., 2004)
		S. sahendica	8.5	

5	β-Pinene 	*S. bazmanica*	37.5	(Akhgar et al., 2011)
		S. bracteata	7.9	(Amiri, 2007)
		S. chloroleuca	9.7	(Khalilzadeh et al., 2011)
		S. hydrangea	11.6	(Ebrahimi & Ranjbar, 2016)
				(Sonboli et al., 2016; Nickavar et al., 2005)
		S. hypoleuca	29.8, 7.2	(Jamzad et al., 2011)
				(Attaran et al., 2016)
		S. indica	11.3	(Sajjadi & Shahpiri, 2004; Rajabi et al., 2014; Rustaiyan et al., 2005)
		S. leriifolia	24.3, 26.0	
		S. limbata	9.2, 19.6, 18.7	(Kazemi, 2015)
				(Salehi et al., 2004)
				(Jassbi et al., 2012; Sonboli et al., 2006)
		S. officinalis	9.1	
		S. sahendica	12.6	
		S. santolinifolia	7.0, 6.6	
6	Myrcene 	*S. bracteata*	7.7	(Amiri, 2007)
		S. hypoleuca	8.3	(Jassbi et al., 20120
7	Hexyl acetate $C_8H_{16}O_2$	*S. reuteriana*	6.7, 7.6	(Jassbi et al., 2012; Mirza & Sefidkon, 1999)
8	ρ-Cymene 	*S. santolinofolia*	5.1	(Jassbi et al., 2012)
9	Limonene 	*S. aegyptiaca*	46.4	(Jassbi et al., 2012)
		S. bracteata	7.2	(Amiri, 2007)
		S. eremophila	6.2	(Jassbi et al., 2017)
		S. multicaulis	8.3	(Ahmadi & Mirza, 1999)
		S. reuterana	5.2	(Batooli et al., 2013)
		S. rhytidea	14.9	(Habibi et al., 2008)
		S. santolinifolia	6.0, 7.7, 5.3	(Jassbi et al., 2012; Sonboli et al., 2006; Bahadori et al., 2016)
				(Zare & Jassbi, 2014)
		S. sharifii	7.0	

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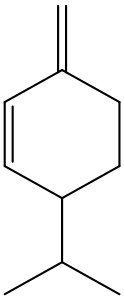
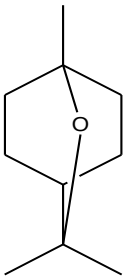
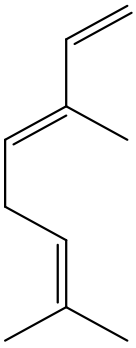
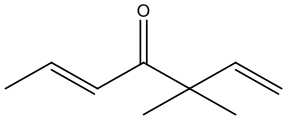
10	β-Phellandrene	*S. bazmanica*	15.3	(Akhgar et al., 2011)
		S. rhytidea	22.7	(Rustaiyan et al., 2005)
				
11	1,8-Cineol	*S. aristata*	8.3	(Emadipoor et al., 2016)
		S. brachyantha	10.2	(Jamzad et al., 2012)
		S. hydrangea	12.7, 15.2	(Sonboli et al., 2006; Ebrahimi & Ranjbar, 2016) (Jamzad et al., 2011) (Attaran et al., 2016; Monfared & Ghorbanli, 2010)
		*S. indica*	13.4	
		S. leriifolia	20.2, 20.0	(Sajjadi & Shahpiri, 2004; Rajabi et al., 2014) (Kazemizadeh et al., 2010)
		S. limbata	11.0, 9.3	(Rajabi et al., 2014) (Sonboli et al., 2006; Yamini et al., 2008; Mirza, 2003; Asadipour et al., 2013
		S. macrochlamys	18.9	(Ahmadi & Mirza, 1999)
		S. macrosiphon	5.3	(Kazemi, 2015; Alizadeh & Shaabani, 2012)
		S. mirzayanii	21.2, 8.0, 12.1, 8.7, 9.4	(Shakeri et al., 2018) (Norouzi-Arasi et al., 2005)
		S. multicaulis		
		S. officinalis	8.3	
			15.0, 7.9	
		S. pachystachys		
		S. suffruticosa	13.5	
			18.6	
12	(E)-β-Ocimene	*S. reuterana*	32.3	(Mirza & Sefidkon, 1999)
		S. sclareoides	11.8	(Sepahvand et al., 2014)
		*S. spinosa*	30.9, 12.3	(Salehi et al., 2014; Bahernik & Mirza, 2005)
13	Artemisia ketone	*S. leriifolia*	62.9	(Monfared & Ghorbanli, 2010)
				

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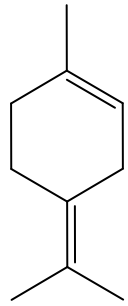
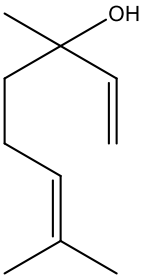
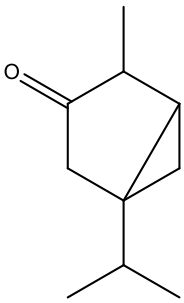
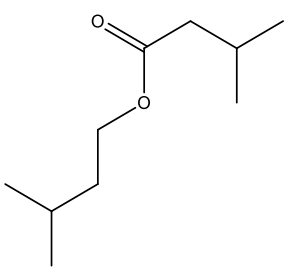
14	α-Terpinolene	*S. spinosa*	32.7	(Salehi et al., 2014)
				
15	Linalool	*S. aethiopis*	51.6	(Rajabi et al., 2014)
		S. eremophilla	12.7-46.2	(Rajabi et al., 2014)
		S. limbata	17.5	(Mirza et al., 2005)
		*S. macrosiphon*	21.8, 34.0, 54.8, 16.1-27.8,	(Jassbi et al., 2012; Rajabi et al., 2014; Sefidkon et al., 2013; Kariminik et al., 2019) (Sonboli et al., 2006; Yamini et al., 2008; Mirza et al., 2003; Asadipour et al., 2013; Javidnia et al., 2002)
		S. mirzayanii	8.9, 9.0, 19.0, 11.8, 5.2	Rajabi et al., 2014) (Rajabi et al., 2014; Salimpour et al., 2011) ((Sepahvand et al., 2014)
		S. nemorosa		
		S. sclarea	5.0-8.1 7.4, 12.2-21.4	
		S. sclareoides	27.6	
16	α-Thujone	*S. officinalis*	5.7, 41.5	(Kazemi , 2015; Alizadeh & Shaabani, 2012)
				
17	Isopentyl isovalerate	*S. spinose*	9.5	(Bahernik & Mirza, 2005)
				

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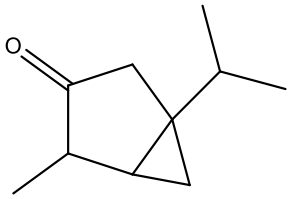
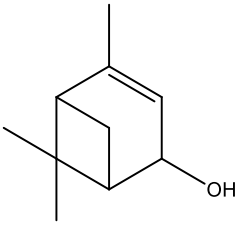
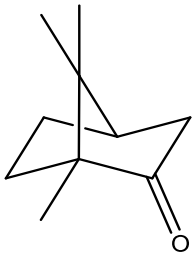
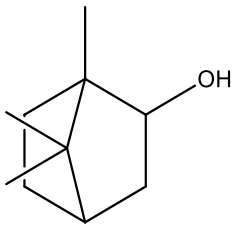
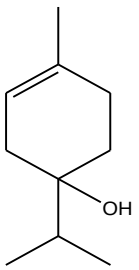
18	β-Thujone	*S. officinalis*	6.8	(Alizadeh & Shaabani, 2012)
				
19	Verbenol	*S. macrosiphon*	7.3	(Rajabi et al., 20140
				
20	Camphor	*S. hydrangea*	12.1	(Ebrahimi & Ranjbar, 2016) (Monfared & Ghorbanli, 2010)
		*S. leriifolia*	18.5	(Kazemi, 2015) (Shakeri et al., 2018)
		S. officinalis	6.0	(Norouzi-Asadi et al., 2005)
		S. pachystachys	31.0	
		S. suffruticosa	48.5	
21	Borneol	*S. compressa*	9.3	(Mirza & Bahernik et al., 2007) (Jassbi et al., 2012)
		*S. eremophila*	22.9	(Sonboli et al., 2006)
		S. hydrangea	5.2	(Kazemi, 2015; Alizadeh & Shaabani, 2012)
		S. officinalis	12.7, 8.3	(Jassbi et al., 2012)
		S. santolinifolia	5.2	
22	4-Terpineol	*S. limbata*	8.9	(Morteza-Semnani et al., 2014) (Sajjadi & Ghannadi, 2005)
		*S. rhytidea*	5.5	

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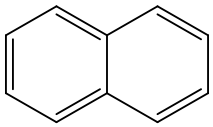
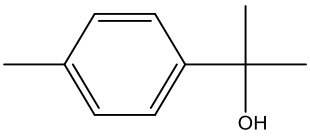
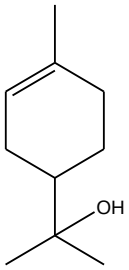
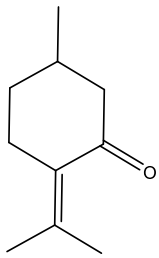
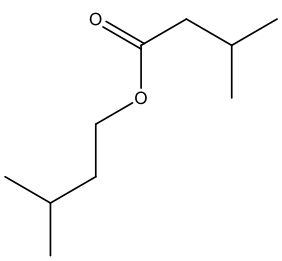
23	Naphthalene 	*S. hydrangea*	10.8	(Ebrahimi & Ranjbar, 2016)
24	ρ-Cymene-8-ol 	*S. rhytidea*	11.9	(Sajjadi & Ghannadi, 2005)
25	α-Terpineol 	*S. mirzayanii* *S. sclarea*	6.3 5.6	(Asadipour et al., 2013) (Rajabi et al., 2014)
26	Decanal $C_{10}H_{20}O$	*S. palaestina*	7.0	(Salehi et al., 2005)
27	Pulegone 	*S. rhytidea*	6.4	(Sajjadi & Ghannadi, 2005)
28	Hexyl isovalerate $C_{11}H_{22}O_2$	*S. macrosiphon* *S. reuteriana*	5.8, 9.4 12.1	(Jassbi et al., 2012; Mirza et al., 2005) (Jassbi et al., 2012)
29	Geraniol 	*S. compressa*	10.5	(Mirza & Bahernik, 2007)

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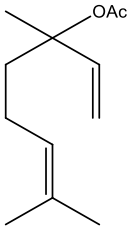
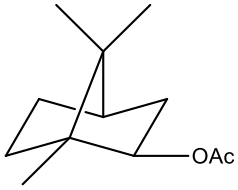
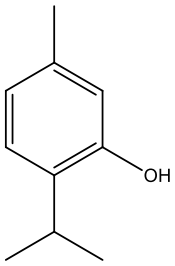
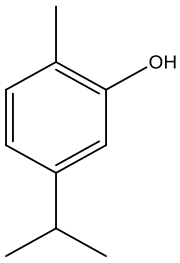
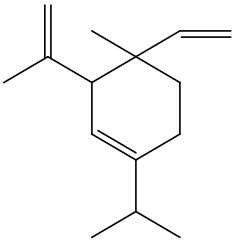
30	Linalyl acetate 	*S. limbata*	16.1	(Mirza et al., 2005)
		S. mirzayanii	5.4, 7.6, 12.9, 11.8	(Sonboli et al., 2006; Yamini et al., 2008; Asadipour et al., 2013) (Rajabi et al., 2014)
		S. reuterana		(Rajabi et al., 2014; Salimpour et al., 2011)
		S. sclarea	5.0	(Rajabi et al., 2014)
			6.8, 13.1-52.6	
		S. virgata	5.2	
31	Hexyl valerate $C_{11}H_{22}O_2$	*S. macrosiphon*	5.0	(Salimpour et al., 2011)
32	Bornyl acetate 	*S. chloroleuca*	5.9	(Jassbi et al., 2012)
		S. eremophila	5.4	(Jassbi et al., 2012)
		S. limbata	12.5	(Rajabi et al., 2014)
		S. macrosiphon	7.8	(Rajabi et al., 20140
		S. multicaulis	18.1	(Ahmadi & Mirza, 1999)
		S. officinalis	5.0	(Kazemi, 2015)
		S. syriaca	10.5	(Lari Yazdi et al., 2005)
		S. urumiensis	7.7	(Khalilzadeh et al., 2011)
33	Thymol 	*S. macilenta*	5.2	(Sonboli et al., 2005)
34	Carvacrol 	*S. aristata*	22.7	(Emadipoor et al., 2016)
35	δ-elemene 	*S. limbata*	5.1	(Sajjadi & Shahpiri, 2004)
		S. syriaca	7.9	(Lari Yazdi et al., 2005)

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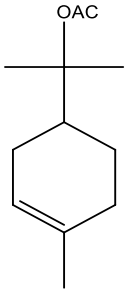
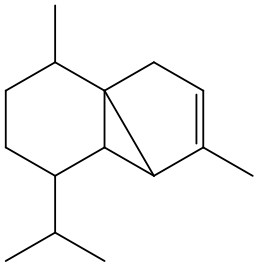
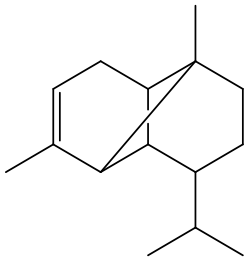
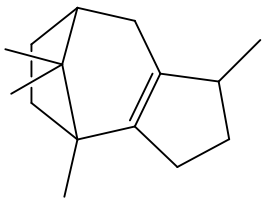
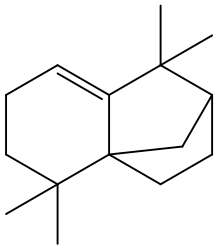
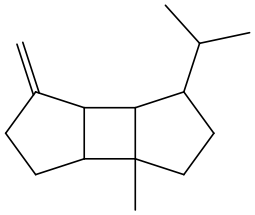
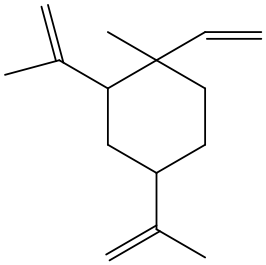
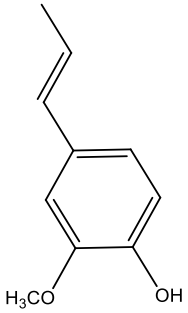
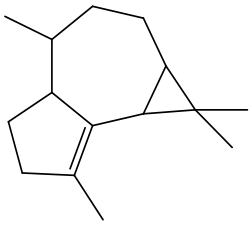
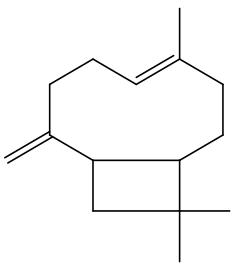
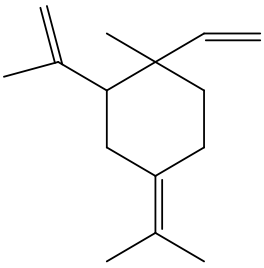
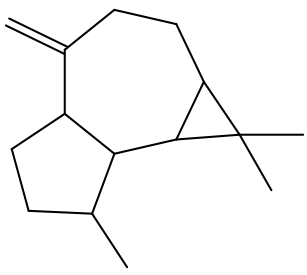
36	α-Terpinyl acetate 	*S. mirzayanii*	22.6, 11.5, 11.0, 9.5, 5.2	(Sonboli et al., 2005; Mirza et al., 2003; Haghighat et al., 2012; Javidnia et al., 2002)
37	α-Cubebene 	*S. atropatana*	13.0	(Salimpour et al., 2011)
38	α-Copaene 	*S. aethiopis*	16,6, 24.3	(Rajabi et al., 2014; Salimpour et al., 2011)
				(Rajabi et al., 2014)
		S. macrosiphon	9.1	(Sajjadi & Ghannadi, 2005)
		S. rhytidea	5.3	(Batooli et al., 2013)
		S. sclarea	6.8	(Salehi et al., 2013)
		S. staminea	7.0	(Khalilzadeh et al., 2011; Salehi et al., 2005)
		S. xanthocheila	11.9, 14.1	
39	β-Patchoulene 	*S. spinosa*	12.8	(Salehi et al., 2014)
40	Hexyl hexanoate $C_{12}H_{24}O_2$	*S. macrosiphon*	6.0	(Jassbi et al., 2012)
41	Isolongifolene 	*S. oligophylla*	9.9	(Salimpour et al., 2011)
		S. reuterana	6.5	(Salimpour et al., 2011)

42	β-Bourbonene	*S. macrosiphon*	6.0	(Rajabi et al., 2014)
				
43	β-Elemene	*S. aethiopis*	9.4	(Rajabi et al., 2014)
		S. macrosiphon	5.4	(Salimpour et al., 2011)
		*S. reuteriana*	11.7, 6.3	(Jassbi et al., 2012; Salimpour et al., 2011)
44	*cis*-Isoeugenol	*S. aethiopis*	9.2	(Salimpour et al., 2011)
				
45	α-Gurjunene	*S. nemorosa*	14.1	(Mirza & Sefidkon, 1999)
		S. verticillata	5.7	(Rajabi et al., 2014)
				
46	β-Caryophyllene	*S. aegyptiaca*	8.3	(Jassbi et al., 2012)
		S. aethiopis	12.0, 9.0-18.9	(Rajabi et al., 2014; Salimpour et al., 2011) (Emadipooor et al., 2016)
		*S. aristata*	6.1	(Jassbi et al., 2012; Salimpour et al., 2011, Mirza & Ahmadi, 2000)
		S. atropatana	14.5, 6.1, 16.3	(Sefidkon et al., 2007) (Jassbi et al., 2012; Khalilzadeh et al., 2011)
		S. bracteata	41.6	(Ebrahimi et al., 2014)
		S. chloroleuca	5.2, 6.7	(Rajabi et al., 2014) (Tavassoli et al., 2009)
		S. chorassanica	37.0	(Mirza & Bahernik, 2010)

		S. eremophilla	5.6-14.7	(Sonboli et al., 2006)
		S. glutinosa	20.9	(Jassbi et al., 2012; Sonboli et al., 2016; Nickavar et al., 2005)
		S. grossheimii	22.4	(Sajjadi & Shahpiri, 2004)
		S. hydrangea	25.1	(Kazemizadeh et al., 2010)
		S. hypoleuca	13.8, 21.7, 14.6	(Rajabi et al., 2014)
		S. limbata	5.3	(Ahmadi & Mirza, 1999)
		S. macrochlamys	32.7	(Rajabi et al., 2014; Mirza & Sefidkon, 1999)
		S. macrosiphon	6.3-16.3	(Rustaiyan et al., 2005; Salehi et al., 2005)
		S. multicaulis	16.5	(Jassbi et al., 2012; Rajabi et al., 2014; Lari Yazdi et al., 2005)
		S. nemorosa	14.6, 18.8-60.6	(Rajabi et al., 2014; Batooli et al., 2013; Salimpour et al., 2011)
		S. palaestina	6.1, 36.4	(Sepahvand et al., 2014)
		S. reuteriana	6.9, 13.1, 9.7	(Zare & Jassbi, 2014; Asgarpanah et al., 2017)
		S. sclarea	7.6, 17.3, 8.5	(Bahernik & Mirza, 2005)
		S. sclareoides	16.6	(Goldansaz et al., 2017)
		S. sharifii	12.8, 12.3	(Rajabi et al., 2014)
		S. spinosa	10.2	(Rajabi et al., 2014; Sefidkon & Mirza, 1999)
		S. tebesana	7.7	(Khalilzadeh et al., 2011; Salehi et al., 2005)
		S. verticillata	17.0-41.0	
		S. virgata	46.6, 30.0	
		S. xanthocheila	6.7, 14.8	
47	γ-Elemene 	*S. hypoleuca*	7.7	(Nickavar et al., 2005)
		S. macilenta	6.1	(Sonboli et al., 2005)
		S. officinalis	6.2	(Kazemi, 2015)
48	Aromadendrene 	*S. indica*	10.1	(Bahernik & Mirza, 2010)

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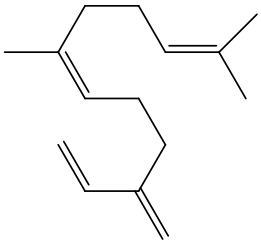
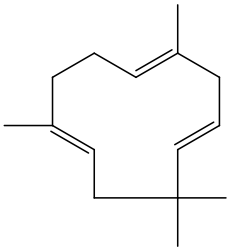
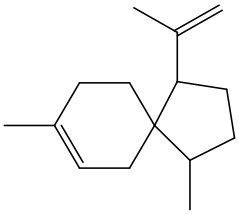
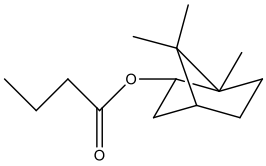
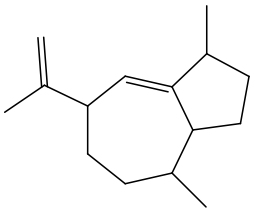
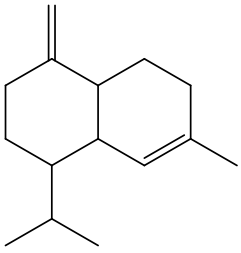
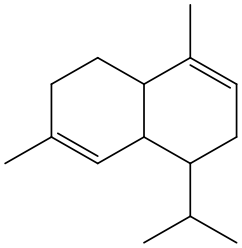
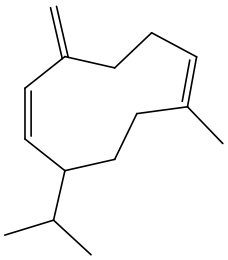
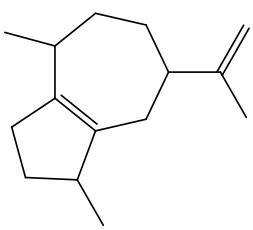
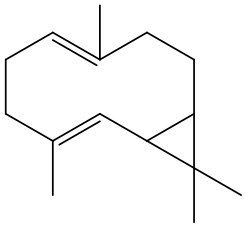
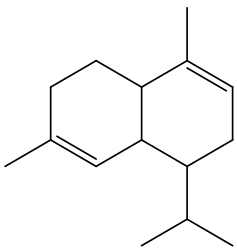
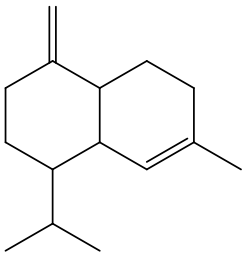
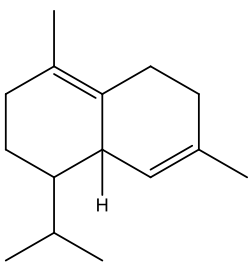
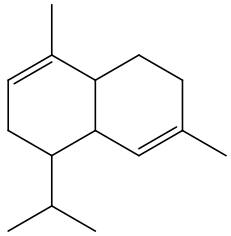
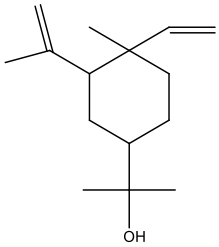
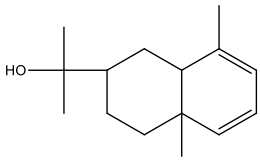
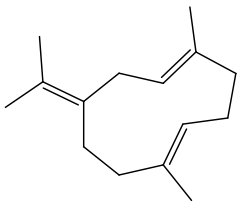
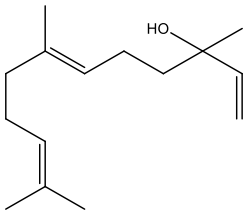
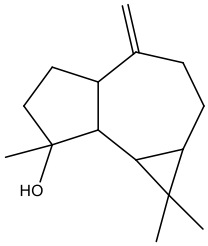
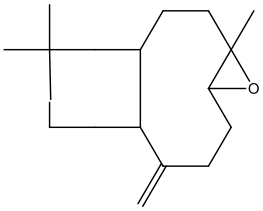
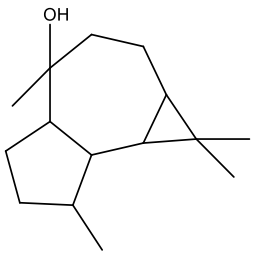
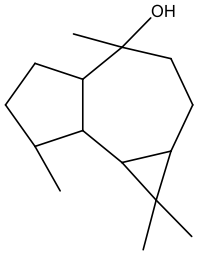
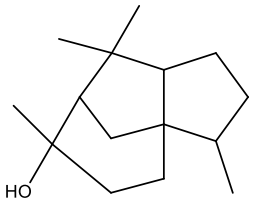
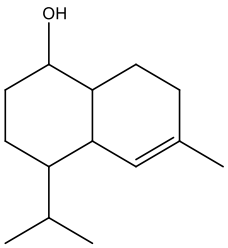
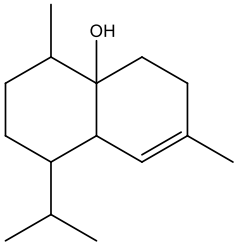
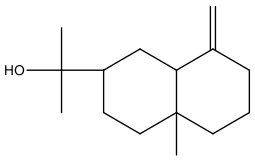
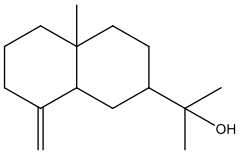
49	Z-β-Farnesene 	*S. aethiopis* *S. nemorosa*	8.6 6.0	(Rajabi et al., 2014) (Mirza & Sefidkon, 1999)
50	. α-Humulene 	*S. glutinosa* *S. sclareopsis* *S. verticillata*	9.4 18.4 5.4-14.0	(Tavassoli et al., 2009) (Hemmati et al., 2018) (Rajabi et al., 2014)
51	Acoradiene 	*S. oligophylla*	5.6	(Salimpour et al., 2011)
52	Isobornyl butanoate 	*S. palaestina*	5.8	(Salehi et al., 2005)
53	γ-Gurjunene 	*S. virgata*	6.5	(Rajabi et al., 2014)
54	γ-Muurolene 	*S. brachyantha* *S. bracteata*	7.5 36.3	(Jamzad et al., 2012) (Sefidkon et al., 2007)

55	α-Amorphene 	*S. hydrangea*	9.3	(Ebrahimi & Ranjbar, 2016)
56	Germacrene D 	*S. aethiopis*	61.5, 25.2	(Rajabi et al., 2014; Salimpour et al., 2011)
		S. atropatana	19.7, 5.1	(Jassbi et al., 2012; Salimpour et al., 2011) (Jassbi et al., 2012; Khalilzadeh et al., 2011)
		S. chloroleuca	15.7, 15.7	(Tavassoli et al., 2009) (Mirza & Bahernik, 2010)
		S. glutinosa	18.0	(Jassbi et al., 2012) (Bahrnik & Mirza, 2010)
		S. grossheimii	45.4	(Mirza et al., 2005)
		S. hypoleuca	8.8	(Rajabi et al., 2014)
		S. indica	10.4	(Rajabi et al., 2014; Mirza & Sefidkon, 1999)
		S. limbata	25.7	(Salehi et al., 2005)
		S. macrosiphon	10.5-8.1	(Jassbi et al., 2012; Mirza & Sefidkon, 1999;
		S. nemorosa	5.6, 5.5-6.3	Batooli et al., 2013; Salimpour et al., 2011) (Salehi et al., 2004)
		S. palaestina	14.0	(Rajabi et al., 2012; Batooli et al., 2013;
		S. reuteriana	14.1, 11.2, 11.2, 21.2	Salimpour et al., 2011) (Sepahvand et al., 2014) (Zare & Jassbi, 2014; Asgarpanah et al., 2017)
		S. sahendica		(Salehi et al., 2013)
		S. sclarea	8.3	(Sefidkon & Mirza, 1999)
			12.7, 20.8, 9.5-17.7	(Khalilzadeh et al., 2011) (Rajabi et al., 2014)
		S. sclareoides		(Sefidkon & Mirza, 1999)
		S. sharifii	10.0	(Khalilzadeh et al., 2011; Salehi et al., 2005)
		S. staminea	9.5, 30.3	
		S. syriaca		
		S. urumiensis	36.3	
		S. verticillate	29.2	
		S. virgata	5.2	
		S. xanthocheila	6.4-13.0	
			5.7	
			44.0, 17.6	
57	*cis*-β-Guaiene 	*S. macrosiphon*	8.6	(Rajabi et al., 2014)

58	Bicyclogermacrene 	*S. aethiopis*	5.8	(Rajabi et al., 2014)
		S. bracteata	9.9	(Sefidkon et al., 2007)
		S. chloroleuca	18.5, 17.0	(Jassbi et al., 2012; Khalilzadeh et al., 2011) (Mirza & Baher, 2010)
		S. grossheimii	7.1	(Jassbi et al., 2012; Sonboli et al., 2016; Nickavar et al., 2005) (Sajjadi &Shahpiri, 2004) (Haghighat et al., 2012) (Rajabi et al., 2014)
		S. hypoleuca	19.8, 37.3, 15.3	
		S. limbata	21.1	(Batooli et al., 2013)
		S. mirzayanii	8.0	(Salehi et al., 2004)
		S. nemorosa	7.7	(Batooli et al., 2013)
		S. reuterana	8.2	(Zare & Jassbi, 2014; Asgarpanah et al., 2017)
		S. sahendica	15.8	(Rajabi et al., 2014)
		S. sclarea	8.8	
		S. sharifii	5.6, 15.7	
		S. verticillata	13.0-21.0	
59	α-Muurolene 	*S. sclareopsis*	8.4	(Hemmati et al., 2018)
60	γ-cadinene 	*S. mirzayanii*	5.2, 5.8	(Sonboli et al., 2006; Javidnia et al., 2002) (Rajabi et al., 2014)
		S. virgata	6.2	
61	δ-Cadinene 	*S. aethiopis*	8.2	(Salimpour et al., 2011)
		S. hydrangea	8.3	(Ebrahimi & Ranjbar, 2016) (Asadipour et al., 2013; Haghighat et al., 2012)
		S. mirzayanii	5.7, 7.5	(Rajabi et al., 2014)
		S. virgata	16.0	

62	α-Cadinene	*S. syriaca*	10.3	(Lari Yazdi, 2005)
		*S. virgata*	32.0	(Rajabi et al., 2014)
63	Elemol	*S. brachyantha*	5.9	(Jamzad et al., 2012)
				
64	Occidentalol	*S. oligophylla*	24.0	(Salimpour et al., 2011)
				
65	Germacrene B	*S. atropatana*	10.0	(Mirza & Ahmadi, 2000)
		S. nemorosa	21.3	(Mirza & Sefidkon, 1999)
		*S syriaca*	34.8	(Sefidkon & Mirza, 1999)
		S. virgata	13.9	(Sefidkon & Mirza, 1999)
66	Nerolidol	*S. tebesana*	12.1	(Goldansaz et al., 2017)
				
67	Hexyl octanoate	*S. atropatana*	12.2	(Mirza & Ahmadi, 2000)
	$C_{14}H_{28}O_2$	*S. macrosiphon*	8.9	(Sefidkon et al., 2013)
68	Spathulenol	*S. brachyantha*	6.2	(Jamzad et al., 2012)
		S. chloroleuca	5.1	(Khalilzadeh et al., 2011)
		S. chorassanica	39.5	(Ebrahimi et al., 2014)
		S. eremophilla	22.0-35.4	(Rajabi et al., 2014)

		*S. hypoleuca*	12.4, 12.5	(Jassbi et al., 2012, Nickavar et al., 2005)
		S. limbata	8.1, 8.2, 13.2	(Sajjadi & Shahpiri, 2004; Rajabi et al., 2012; Morteza-Semnani et al., 2014)
				(Rajabi et al., 2012; Salimpour et al., 2011)
				(Javidnia et al., 2002)
		S. macrosiphon	5.8, 17.1	(Rajabi et al., 2012)
				(Rajabi et al., 2012; Lari Yazdi et al., 2005)
		S. mirzayanii	10.4	(Sajjadi & Ghannadi, 2005)
		S. nemorosa	6.8-26.0	(Rajabi et al., 2012; Batooli et al., 2013; Salimpour et al., 2011)
		S. reuterana	12.4, 17.0	(Zare & Jassbi, 2014)
		S. rhytidea	7.3	(Lari Yazdi et al., 2005)
		S. sclarea	6.9, 10.0, 5.0	(Khalilzadeh et al., 2011)
				(Rajabi et al., 2014)
				(Sefidkon & Mirza, 1999)
		S. sharifii	6.9	(Khalilzadeh et al., 2011)
		S. syriaca	18.6	
		S. urumiensis	14.6	
		S. verticillata	11.0-17.0	
		S. virgata	6.4	
		S. xanthocheila	7.0	
69	Caryophyllene oxide	*S. aethiopis*	10.3	(Rajabi et al., 2014)
		S. atropatana	17.6, 19.3	(Jassbi et al., 2012; Salimpour et al., 2011)
				(Jamzad et al., 2012)
		S. brachyantha	6.7	(Ebrahimi et al., 2014)
		S. chorassanica	22.7	(Mirza & Bahernik, 2007)
		S. compressa	15.7	(Rajabi et al., 2014)
		S. eremophilla	9.5-17.8	(Sonboli et al., 2006)
		S. hydrangea	11.5	(Rajabi et al., 2014)
		S. macrosiphon	11.3-26.9,	(Rajabi et al., 2014; Mirza & sefidkon, 1999)
		S. nemorosa	6.8, 19-27	(Rajabi et al., 2014)
				(Rajabi et al., 2014; Batooli et al., 2013)]
		S. reuterana	38.0	(Hemmati et al., 2018)
		S. sclarea	10.4, 6.3-8.5	(Zare & Jassbi, 2014)
				(Goldansaz et al., 2017)]
		S. sclareopsis	7.7	(Rajabi et al., 20140
		S. sharifii	5.5	(Rajabi et al., 2014; Sefidkon & Mirza, 1999)
		S. tebesana	6.8	(Khalilzadeh et al., 2011)
		S. verticillata	7.0-10.0	
		S. virgata	13.2, 10.0	
		S. xanthocheila	15.5	
70	Globulol	*S. indica*	24.1	(Bahernik & Mirza, 2010)
		S. mirzayanii	5.4	(Haghighat et al., 2012)
				

71	Viridiflorol 	*S. hypoleuca* *S. officinalis* *S. sclareopsis*	17.5 5.9 23.5	(Nickavar et al., 2005) (Alizadeh & Shaabani, 2012) (Hemmati et al., 2018)
72	Cedrol 	*S. mirzayanii*	16.3	(Haghighat et al., 2012)
73	*tau*-Cadinol 	*S. compressa*	36.0	(Mirza & Bahernik, 2007)
74	Cubenol 	*S. leriifolia* *S. palaestina*	9.4 9.8	(Monfared & Ghorbanli, 2010) (Salehi et al., 2005)
75	β-Eudesmol 	*S. eremophilla* *S. macrosiphon* *S. reuterana* *S. santolinifolia*	7.2 9.7-16.1 7.6 20.0	(Rajabi et al., 2014) (Rajabi et al., 2014) (Rajabi et al., 2014) (Bahadori et al., 2016)
76	α-Eudesmol 	*S. macrosiphon*	5.3	(Rajabi et al., 2014)

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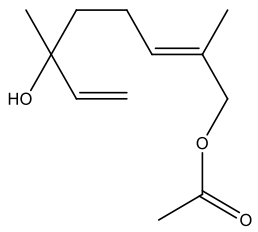
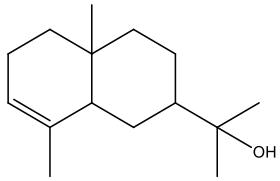
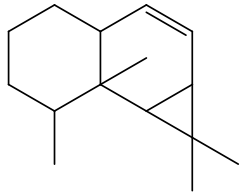
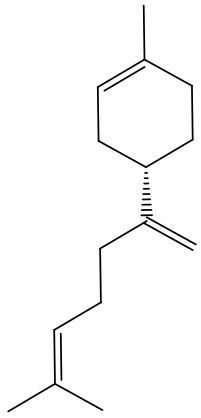
77	8-Acetoxy linalool 	*S. mirzayanii*	11.0	(Yamini et al., 2008)
78	7-epi-α-eudesmol 	*S. tebesana*	17.5	(Goldansaz et al., 2017)
79	Aristolene 	*S. macrosiphon*	5.7	(Salimpour et al., 2011)
80	β-Bisabolene 	*S. palaestina*	11.9	(Salehi et al., 2005)
81	Octadecane $C_{18}H_{38}$	*S. sclareopsis*	20.6	(Hemmati et al., 2018)
82	Hexahydrofarnesyl acetone $C_{18}H_{36}O$	*S. staminata*	11.2	(Salehi et al., 2013)

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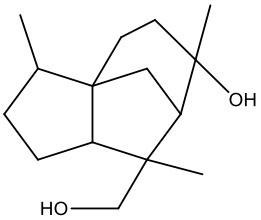
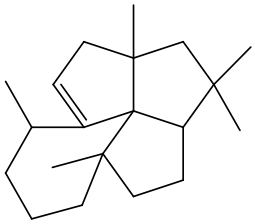
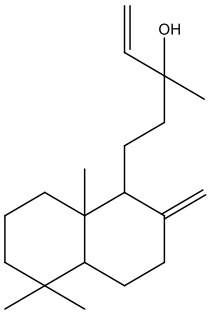
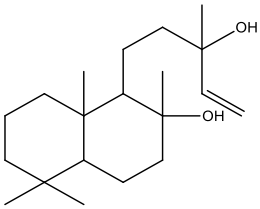
83	8,13-Cedranediol 	*S. reuterana*	9.9	(Batooli et al., 2013)
84	*epi*-Laurenene 	*S. reuterana*	9.5	(Batooli et al., 2013)
85	Manool 	*S. macrosiphon* *S. persepolitana*	27.3 37.3	(Kariminik et al., 2019) (Habibi et al., 2008)
86	Sclareol 	*S. atropatana* *S. macrosiphon* *S. sclarea*	13.3 15.8, 8.6 5.3	(Mirza & Ahmadi, 2000) (Sefidkon et al., 2013; Salimpour et al., 2011) (Salimpour et al., 2011)

As shown in Table 2, 86 compounds were identified in the essential oil of Persian *Salvia* species in sizeable amounts (>5%). The essential oil composition of these species is dominated by mostly the presence of monoterpene hydrocarbons, sesquiterpene hydrocarbons, oxygenated monoterpenes and oxygenated sesquiterpenes.

β-Caryophyllene as the most abundant component in many *Salvia* species essential oil, has been reported in *S. aegyptiaca, S. aethiopis, S. aristata, S. atropatana, S. bracteata, S. chloroleuca, S. chorassanica, S. eremophilla, S. glutinosa, S. grossheimii, S. hydrangea, S. hypoleuca, S. limbata, S. macrochlamys, S. macrosiphon, S. multicaulis, S. nemorosa, S. palaestina, S. reuteriana, S. sclarea, S. sclareoides, S. sharifii, S. spinosa, S. tebesana, S. verticillata, S. virgata, S. xanthocheila* of which the oils of *S. bracteata, S. nemorosa* and *S. virgata* contain more than 40 % β-caryophyllene. Germacrene D is the other abundant component reported in the most *Salvia* species such as *S. aethiopis, S. atropatana, S. chloroleuca, S. glutinosa, S. grossheimii, S. hypoleuca, S. indica, S. limbata, S. macrosiphon, S. nemorosa, S. palaestina, S. reuteriana, S. sahendica, S. sclarea, S. sclareoides, S. sharifii, S. staminea, S. syriaca, S. urumiensis, S. verticillata, S. virgata,*

S. xanthocheila of which the S*. aethiopsis, S. grossheimii* and *S. xanthocheila* produce essential oils contained more than 40 % of germacrene D.

α-Pinene as the dominant component of the first category of the Iranian sage presents in 18 *Salvia* species including *S. aegyptiaca, S. aristata, S. bazmanica, S. bracteata, S. chloroleuca, S. eremophila, S. hydrangea, S. hypoleuca, S. indica, S. leriifolia, S. limbata, S. macilenta, S. multicaulis, S. pachystachys, S. sahendica, S. santolinifolia, S. tebesana* and *S. urumiensis* among which the *S. macilenta* and *S. santolinifolia* possess the essential oil with more than 60 % of α-pinene.

Conclusion

Data in the Table 2 represents a complex composition of the essential oils, especially for the same species. Indeed, the composition of essential oils depends on climatic and ecological conditions, plant organ and vegetative cycle stage. Thus, it is of utmost importance to characterize the essential oils composition as well as the influence of the referred parameters on its quality, in order to obtain essential oils of constant composition. This could only be possible if essential oils are extracted under the same conditions from the same organ of the plant which has been growing on the same soil, under the same climate and has been picked in the same season. It was also concluded that the most of the studied Iranian *Salvia* species were considered as sesquiterpene chemotype and β-caryophyllene followed by germacrene D, spathulenol and caryophyllene oxide were the most common and abundant in Persian *Salvia* species.

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CONFLICTS OF INTEREST

The author does not have conflicts of interest to declare.

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RESEARCH ARTICLE

Chemical composition and lipoxygenase inhibitory activity of *Alseodaphne peduncularis* Meisn. essential oil

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Abstract

This study examined the chemical composition of the essential oil extracted from the bark of *Alseodaphne peduncularis* Meisn. (Lauraceae) by hydrodistillation with 0.2% yield. The essential oil was chemically characterised by gas chromatography-flame ionization detector and gas chromatography-mass spectrometry, and 31 constituents comprising 94.3% of the total oil content were identified. These constituents included β-caryophyllene (24.5%), viridiflorol (12.3%), δ-cadinene (9.8%), and bicyclogermacrene (4.2%), respectively. The tested essential oil showed moderate *in vitro* lipoxygenase activity with an IC_{50} = 60.2 µg/mL. To the best of our knowledge, there is no report and study on the bark part of this species.

Keywords: Lauraceae, *Alseodaphne peduncularis*, hydrodistillation, GC-MS, lipoxygenase

Introduction

The tree species *Alseodaphne peduncularis* Meisn. (Lauraceae), locally known as *medang*, occurs widely in mixed dipterocarp and sub-montane forests up to 1000 m above sea level in the Peninsular Malaysia, Sumatra, and Borneo (Turner, 1995; Thakur et al., 2012). Although the tree is a common wood source for fuel, furniture, house, and boat-building, little is known about its medicinal values. Recently, the chemical composition and biological activity of the essential oil extracted from the leaves of *A. peduncularis* were reported in our study (Salleh et al., 2016a). The leaf oil was weak in the DPPH radical scavenging (IC_{50}: 253.2 µg/mL) with relatively low phenolic content (32.5 mg GA/g) and modest inhibitory effect against acetylcholinesterase (I: 45.2%) and butyrylcholinesterase (IC_{50}: 48.6%). In the continuation of our studies on essential oils from this species *A. peduncularis*, herein we report the chemical constitution of essential oil extracted from its bark and the lipoxygenase inhibitory effect of the essential oil.

Materials and Methods

Plant material and isolation of essential oil

In this study, the essential oil was extracted from the fresh bark of *A. peduncularis*, which was sampled from the secondary forest at Behrang, Perak in September 2019. The tree was taxonomically identified by Shamsul Khamis from Universiti Kebangsaan Malaysia (UKM). Meanwhile, voucher specimens of the plant (SK128/19) were stowed at UKMB Herbarium. A Clevenger apparatus was used to extract the essential oil from the fresh bark (200 g) mixed with water via hydrodistillation for 4 h. The essential oil was desiccated using the dry magnesium sulfate and kept at 4 – 6 °C until analysis.

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Analysis of essential oil

Hydrocarbons were detected via a gas chromatography-flame ionization detector (Agilent Technologies 7890B, USA) equipped with an HP-5MS capillary column of 30 m in length, 0.25 µm thick, and an inner diameter of 0.25 mm. Analytes were moved through the column by helium gas at a flow rate of 0.7 mL/min. Temperatures were adjusted to 250 °C and 280 °C for the injector and detector, respectively, while the oven was set at 50 °C, but slowly increased to 280 °C at 5 °C/min and eventually kept isothermally for 15 min. Samples were diluted with diethyl ether at 1: 100 (v/v) and analyzed in triplicates, in which 1.0 µL of the diluted sample was manually interpolated at a split ratio of 50: 1. The average of triplicates was calculated to generate the percentage of peak area. Meanwhile, a gas chromatography-mass spectrometer (GC/MS; 5890A for GC, 5898A for MS, Hewlett Packard, USA) was used to detect various substances in the test sample. Analytes were moved by helium through the HP-5 column fixed to the GC at 1 mL/min. The injector was kept at 250 °C, and the oven set to gradually increase from 50 to 280 °C (5 min hold) at 10 °C/min and eventually kept isothermally for 15 min. An electron ionization energy of 70 eV was employed in the GC/MS detection at a scan rate of 0.5 s (cycle time: 0.2 s), encompassing a mass range of 50 - 400 atomic mass unit (Salleh et al., 2012; Salleh et al., 2016b).

Identification of constituents

For the identification of chemical constituents in GC/MS, samples were co-injected with the standards (major components) with the corresponding retention indices and mass spectra following the study of Adams (2007). Constituents of essential oil were semi-quantified through the normalization of peak areas by taking into account the same reactionary component for all the detected volatile constituents. The average of three GC analyses yielded the relative percentage (%) value.

Lipoxygenase inhibitory activity

The LOX inhibition was largely based on the technique of Ellman (Salleh et al., 2016c), in which 5 µL of essential oil was buffered with 1.74 mL borate (0.2 M, pH 9.2) and added with 5 µL (50,000 U/mL) of 5-LOX enzyme. The reaction began upon adding 250 µL linoleic acid (5 mg linoleic acid mixed with 15 µL ethanol and 15 mL borate in brisk shaking). The absorbance at 234 nm was assayed for 5 min in a UV-visible spectrophotometer (Genesys 10Se, Thermo Scientific, USA). The dimethyl sulfoxide (5 µL) served as a negative control, while the quercetin (Sigma-Aldrich, St. Louis, MO, USA), a positive control, was prepared in the same strength as the essential oil. The percentage inhibition (I%), which is equivalent to the concentration of drug required for 50% inhibition (IC_{50}) in µg/mL , was computed by the equation below:

$$I\% = [A_{\text{initial activity}} - (A_{\text{inhibitor}} / A_{\text{initial activity}})] \times 100$$

where $A_{\text{initial activity}}$ is the absorbance of the control, and $A_{\text{inhibitor}}$ is the absorbance of the test sample. Averaging the absorbance values of the triplicates and quercetin yielded the LOX inhibitory activity.

Results and Discussion

The bark of *A. peduncularis* yielded 0.2% yellow oil based on the fresh weight. Table 1 shows, in order of elution, 31 constituents identified in the essential oil, comprising 94.3% of the total chemical content. Sesquiterpenes were the main hydrocarbon groups found in the 16 oil components identified, constituting 59.2% of the total oil content. Besides, oxygenated sesquiterpenes, oxygenated monoterpenes, and monoterpene hydrocarbons contributed 25.7%, 5.7%, and 3.7%, respectively to the overall oil content.

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Table 1. Chemical composition of *Alseodaphne peduncularis* essential oil

No	RRI[a]	RRI[b]	Components	Percentage[c]	Identifications[d]
1	935	935	α-Pinene	2.0 ± 0.1	RI, MS
2	945	946	Camphene	1.2 ± 0.1	RI, MS
3	967	965	Sabinene	0.5 ± 0.2	RI, MS
4	1082	1082	Linalool	1.2 ± 0.1	RI, MS
5	1175	1175	Terpinen-4-ol	2.3 ± 0.2	RI, MS
6	1185	1189	α-Terpineol	2.2 ± 0.1	RI, MS
7	1352	1350	α-Cubebene	1.2 ± 0.2	RI, MS
8	1374	1374	α-Copaene	0.2 ± 0.2	RI, MS
9	1385	1386	β-Cubebene	0.5 ± 0.1	RI, MS
10	1409	1405	α-Cedrene	0.2 ± 0.2	RI, MS
11	1425	1420	β-Caryophyllene	24.5 ± 0.2	RI, MS, Std
12	1455	1453	α-Humulene	0.5 ± 0.2	RI, MS
13	1458	1458	Aromadendrene	2.5 ± 0.1	RI, MS
14	1478	1480	Germacrene D	5.2 ± 0.2	RI, MS, Std
15	1485	1482	α-Amorphene	3.5 ± 0.2	RI, MS
16	1495	1495	Cadina-1,4-diene	1.2 ± 0.1	RI, MS
17	1500	1501	Bicyclogermacrene	4.2 ± 0.2	RI, MS, Std
18	1502	1500	α-Muurolene	2.0 ± 0.1	RI, MS
19	1529	1530	δ-Cadinene	9.8 ± 0.2	RI, MS, Std
20	1535	1537	α-Cadinene	1.4 ± 0.1	RI, MS
21	1542	1545	Germacrene B	2.1 ± 0.2	RI, MS
22	1545	1543	γ-Cadinene	0.2 ± 0.2	RI, MS
23	1546	1545	Elemol	1.8 ± 0.2	RI, MS
24	1570	1570	Globulol	2.0 ± 0.1	RI, MS
25	1575	1575	Spathulenol	2.2 ± 0.2	RI, MS
26	1592	1595	Viridiflorol	12.3 ± 0.2	RI, MS, Std
27	1602	1602	Guaiol	1.0 ± 0.1	RI, MS
28	1635	1635	t-Muurolol	0.2 ± 0.2	RI, MS
29	1652	1650	α-Cadinol	2.5 ± 0.1	RI, MS
30	1654	1652	α-Eudesmol	3.2 ± 0.2	RI, MS
31	1682	1685	α-Bisabolol	0.5 ± 0.1	RI, MS
			Monoterpene hydrocarbons	3.7	
			Oxygenated monoterpenes	5.7	
			Sesquiterpene hydrocarbons	59.2	
			Oxygenated sesquiterpenes	25.7	
			Total identified	**94.3**	

[a]Linear retention index, experimentally determined using homologous series of C_6-C_{30} alkanes. [b]Linear retention index taken from Adams (2007). [c]Relative percentage values are means of three determinations ±SD. [d]Identification methods: Std, based on comparison with authentic compounds; MS, based on comparison with Wiley, Adams, FFNSC2, and NIST08 MS databases; RI, based on comparison of calculated RI with those reported in Adams, FFNSC2 and NIST08.

The major constituents of the essential oil consisted of β-caryophyllene (24.5%), viridiflorol (12.3%), δ-cadinene (9.8%), and bicyclogermacrene (4.2%). Other notable constituents included α-amorphene (3.5%), α-eudesmol (3.2%), aromadendrene (2.5%), α-cadinol (2.5%), terpinen-4-ol (2.3%), α-terpineol (2.2%), spathulenol (2.2%), germacrene B (2.1%), α-pinene (2.0%), α-muurolene (2.0%), and globulol (2.0%). Both β-

caryophyllene and δ-cadinene occurred in a substantial amount in the bark oil when compared to the leaf oil of the same plant species (Salleh et al., 2016a), and the majority of the bark oil constituents also occurred in the leaf oil. However, 12 constituents were not detected in the bark oil; they were α-cedrene, α-humulene, aromadendrene, germacrene D, α-amorphene, cadina-1,4-diene, bicyclogermacrene, α-muurolene, elemol, viridiflorol, guaiol, and α-cadinol. Such differences were probably due to different genotypes and chemotypes of the plant, which may affect the content of essential oil (Salleh et al., 2014; Marjo et al., 2001).

Arachidonate 5-lipoxygenase is the key enzyme in leukotriene biosynthesis and catalyzes the initial steps in the conversion of arachidonic acid to biologically active leukotrienes. Leukotrienes are considered potent mediators of inflammatory and allergic reactions and regarding their pro-inflammatory properties, the inhibition of 5-lipoxygenase pathway is considered to be interesting in the treatment of a variety of inflammatory diseases. Besides 5-lipoxygenase inhibitors, drugs able to block the 5-lipoxygenase as well as the cyclooxygenase metabolic pathway are also of therapeutic value. The LOX inhibitory effect of essential oil extracted from the bark was modest at inhibition of 60.2%, compared to that of quercetin with inhibition of 92.5%. Since sesquiterpene hydrocarbons of essential oils extracted from various plant species (e.g., *Syzygium aromaticum*, *Cannabis sativa*, *Rosmarinus officinalis,* and *Tagetes minuta*) were generally reported to show anti-inflammatory effect (Gertsch et al., 2008; Ghiasvand et al., 2011), thus, the high amount of β-caryophyllene and δ-cadinene in the essential oil of *A. peduncularis* might at least partially contribute to the anti-inflammatory potential.

This study provides valuable and useful information and indications for further exploring the potential nutraceutical and pharmaceutical applications of the genus Lauraceae. The next step will be to evaluate the *in vivo* of the essential oil in order to valorize this species with a special ecological character.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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RESEARCH ARTICLE

Evaluation of chemical profiles and biological properties of *Gliricidia sepium* (Jacq.) Walp. volatile oils from Nigeria

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Abstract

Volatile oils obtained by hydrodistillation from *Gliricidia sepium* (Jacq.) Walp. leaf and stem were examined for their chemical composition and biological activity. The oils were analyzed by gas chromatographic-mass spectrometric (GC-MS) techniques. Toxicity, antimicrobial and antioxidant activities were evaluated via brine shrimp lethality assay, agar-diffusion, and DPPH radical-scavenging methods, respectively. A total of 43 and 44 constituents were identified in the leaf and stem oils, correspondingly. The major components recognized in the leaf oil were (*E*)-hexadecatrienal (16.9%) and pentadecanal (16.0%) while humulene epoxide II (17.5%) and caryophyllene oxide (10.6%) dominated the stem oil. There was no significant activity against the bacteria but moderate inhibition zones (IZ) between 1.8±0.3 and 8.1±0.1 mm were observed against the fungi. The stem oil showed better antifungal activity than the leaf oil but not as active as the standard drug ketoconazole which inhibited the test fungi with IZ range of 10.4±0.4-21.0±1.4 mm at 200 µg. Both oils were toxic to brine shrimp (*Artemia salina*) giving LC_{50} of 79.7 µg/mL (leaf) and 38.7 µg/mL (stem). The leaf and stem oils had IC_{50} of 84.3 µg/mL and 142.2µg/mL, respectively, in the DPPH radical-scavenging assay, indicating moderate antioxidant activity relative to positive controls, butylated hydroxylanisole (IC_{50}=45.1 µg/mL) and α-tocopherol (IC_{50} =81.6 µg/mL). The results suggest that *G. sepium* volatile oil may find potential use as a natural antioxidant and antifungal agent.

Keywords: *Gliricidia sepium*; volatile oils; chemical compositions; biological activities

Introduction

Investigations of chemical and biological properties of medicinal plants have been a great help to pharmacists in dealing with the global health challenges mostly caused by microbial infections and oxidative deterioration. *Gliricidia sepium* (Jacq.) Walp. (Fabaceae), commonly known as quick stick, is a multipurpose plant that is easily propagated and drought tolerant. It provides wood fuel, animal feed, green manure, shade, plants support and used in folk medicine as fumigant, mosquito repellent, rat control, antiviral, dysentery cure, CNS depressant and wound dressing (Asolkar et al., 1992; Csurhes & Edward, 1998; Gupta, 1995; Kumar & Simon, 2016).

The leaf, which contains sufficient amounts of crude protein and minerals with its ability to thrive during dry season make the plant a good food supplement for livestock (Aye & Adegun, 2013). Reported pharmacological activities include nematicidal (Nazli et al., 2008), larvicidal (Jiby et al., 2015), antibacterial (Akharaiyi et al., 2012; Sukumar & Apama, 2014) and anti-inflammatory (Kumar et al., 2014).

Previously isolated compounds include gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, gentisic acid, β-resorcyclic acid, vanillic acid, syringic acid, *p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid, ferulic acid, sinapinic acid (*Z* and *E*), coumarin, myricetin, protocatechuic acid, stigmasterol glucoside and 7,2'' -dihydroxy-6,4''-dimethoxyisoflavone from the heartwood and leaves extracts (Ramamoorthy & Paliwal, 1993; Herath & deSilva, 2001).

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The purpose of this work is to investigate the chemical compositions and the biological properties of the volatile oils hydrodistilled from the leaves and stem of *Gliricidia sepium* from Nigeria. Biological activities, i.e., antimicrobial activity, free radical-scavenging potential, brine shrimp toxicity, and the chemical composition have not been reported before.

Materials and Methods

Sample preparation

Gliricidia sepium was collected at Saunders road, University of Ibadan (7° 23' 0.2" N/3° 54' 28.8" E) and authenticated at Forestry Research Institute of Nigeria where a voucher specimen was deposited (FHI 112496). The plant parts (leaves and stem) were air dried and pulverized. The pulverized samples were separately hydrodistilled for 3 h using an all-glass Clevenger-type apparatus, according to the British Pharmacopeia specification (1980). The oils were dried over anhydrous sodium sulfate and kept in sealed glass vials under refrigeration at 4 °C prior to analysis and bioassays.

Gas chromatography/mass spectrometry (GC-MS) analysis

The volatile oils were analysed by gas chromatography-mass spectrometry technique using a Shimadzu GC-MS-QP2010 equipped with ZB-5 fused silica capillary column (Phenomenex, Torrance, CA, USA) with a (5% phenyl)-polymethylsiloxane stationary phase and a film thickness of 0.25 µm. The MS detector was operated in the electron impact (EI) mode of 70 eV, scan range 40-400 atomic mass units at the rate of 3.0 scan/s. The carrier gas was helium with a column head pressure of 552 kPa and flow rate of 1.37 mL/min. The injector initial temperature was 50 °C and increased at the rate of 2 °C/min to 260 °C. A 5% w/v solution of the sample in CH_2Cl_2 was prepared and 0.1 µL was injected with a splitting mode (30:1). Identification of constituents in the volatile oils was based on comparison of their retention indices relative to the homologous series of *n*-alkanes, and comparison of their mass spectra fragmentation patterns with the ones in the literature (Adams, 2017) and those of reference compounds stored in the home database library (Satyal, 2015).

Antimicrobial assays

The antimicrobial activity of the essential oils was determined using agar-diffusion method. The oils were tested against two bacteria standard strains (*Staphylococcus aureus* ATCC 6571 and *Escherichia coli* ATCC 25925) and three clinical fungi isolates (*Candida albicans*, *Aspergillus niger* and *Fusarium solani*) from University College. Bacteria strains were maintained on Mueller-Hinton Agar and fungi on Sabouraud Dextrose Agar (SDA). Each medium was prepared following the manufacturer's instructions. Diluted overnight cultures (10^{-2} CFU/ mL) were inoculated each into sterile agar. Wells of uniform diameter were created in the seeded agar plates using 8 mm cork borer. Volatile oils of varied concentrations were allowed to diffuse into the seeded agar for 1 h before incubating for 24 h at 37 °C and 48 h at 25-32 °C for bacteria and fungi respectively after which the inhibition zones were observed and recorded. Gentamycin (10 µL/mL) was used as positive control for bacteria and ketoconazole (200 µg) for fungi.

DPPH free radical-scavenging assay

DPPH free radical-scavenging assay is one of the standard technique used to study antioxidant property of plant volatile oils (Kedare & Singh, 2011). Free radical-scavenging potential of the *G. sepium* leaf and stem oils was evaluated using the method explained by Saleh *et al.,* (2010) with some modifications. A volume of 1.5 mL of different concentrations of the oil samples (5 mg/mL, 25 mg/mL and 100 mg/mL) were separately mixed with 1.5 mL of 0.2 mM DPPH (dissolved in methanol) and incubated in the dark for 20 min at room

temperature. The absorbance at 517 nm was recorded as $A_{(sample)}$ using CE 2021, 2000 series double beam UV-Vis spectrophotometer. A blank experiment was also carried out using the same procedure but without the volatile oils and the absorbance was recorded as $A_{(blank)}$. Each experiment was carried out in triplicate and the free radical-scavenging activity of the oils was calculated as percentage inhibition using the formula;

$$\% \text{ inhibition} = A_{(blank)} - A_{(sample)} / A_{(blank)} \times 100$$

The free radical-scavenging activity of butylated hydroxylanisole (BHA) and α-tocopherol were also evaluated as positive controls for comparison. IC_{50} was calculated through Microsoft EXCEL by plotting the graph of percentage inhibition against concentration as described by Kumawat et al., (2012).

Brine shrimp lethality test

The toxic effect of the volatile oils against brine shrimps of *Artemia Salina* (Artemiidae) was evaluated at different concentrations. Ten shrimp nauplii were introduced into 5 mL of 1000 ppm, 100 ppm and 10 ppm solution of essential oils (dissolved in DMSO) and sea water. The experiment was performed in triplicate making a total of 30 shrimps per dilution. Blank experiment was also carried out by introducing 10 shrimps into 5 mL seawater with DMSO to serve as negative control in other to eliminate other factors contributing to the total number of dead nauplii. The number of surviving shrimps were counted and recorded after 24 hours. The median lethal concentration (LC_{50}) was analyzed at 95% confidence intervals using a probit regression analysis (Finney, 1971). High toxicity is attributed to LC_{50} values less than 100 ppm while values between 100-500 ppm is moderately toxic, 500-1000 ppm is less toxic and greater than 1000 ppm is non-toxic (Meyer et al., 1982).

Results and Discussion

Identification of *Gliricidia sepium* volatile oils constituents

The GC-MS results of the essential oil obtained from the leaf and stem of *Gliricidia sepium* are listed in Table 1 in order of their elution from a ZB-5 fused silica capillary column. A total of 43 compounds were identified in the leaf oil amounting for 97.6% of the whole volatile oil. The oil comprised mainly non-terpenes which made up 73.0% of the total oil. The most abundant constituents were *E*-hexadecatrienal (16.9%), pentadecanal (16.0%) and methylcyclohexane (8.0%). The leaf oil characterized via GC-FID and GC-MS from Costa Rica (Chaverri and Cicció, 2015) presented 96 compounds with pentadecanal (18.7%), (*Z*)-phytol (7.8%) and nonanal (5.1%) as most abundant constituents, which were also detected in the present study in quantity 16.0%, 0.9% and 1.5%, respectively.

In contrast, Kaniampady et al. (2007) gave account of propylene glycol (25.1%), coumarin (18.2%), (*Z*)-3-hexanol (17.7%), β-farnesene (14.2%) and 2*E*-hexanol (6.5%) as the major components in *G. sepium* leaf oil amid the sixteen constituents identified by GC.

Likewise, safrole (12.3%) and 2''-hydroxy-acetophenone (12.1%) were found to be main constituents among the eighty volatile compounds from Colombia leaf oil analysed by GC-FID and GC-MS (Quijano-Célis et al., 2015).

The stem oil contained 44 compounds including 64.7% sesquiterpenes, 1.2% monoterpenes and 29.3% non-terpenes equivalent to 95.2% of the whole essential oil. The main constituents were determined as (9*Z*)-docosenamide (18.0%), humulene epoxide II (17.5%), caryophyllene oxide (10.6%) and α-cadinol (5.0%). The only monoterpene hydrocarbon present was α-pinene (0.4%). Alongside were oxygenated monoterpenes (*E*)-myrtanol (0.5%) and geranyl acetone (0.3%). In contrast, Jose and Reddy (2010) reported methyl-3-(*E*)-

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pentenyl ether (11.6%), 3-methyl-2-butanol (10.7%), 1-(1-ethoxyethoxy)-2-hexene (9.7%), 2-decanol (9.0%), coumarin (8.1%) and hexadecanoic acid (5.2%) as main components among the 19 compounds identified from the stem bark oil. Although, the leaf and stem oils were extracted from the same plant, there was significant difference in their chemical compositions. All the dominant constituents in the leaf oil were absent in the stem oil and vice versa except methylcyclohexane (8.0%, 0.2%), phytone (4.5%, 0.5%) and (*Z*)-hexadecatrienal (4.9%, 0.9%) that were present in the leaf and stem volatile oils, respectively.

Table 1. Chemical Compositions of *G. sepium* Volatile oils

Constituents	**RI**	**Percentage composition (%)**	
		Leaf	**Stem**
Z-1,2-Dimethylcyclopentane	716	1.3	-
Methylcyclohexane	718	8.0	0.2
2-Methyl-2-pentanol	723	0.7	-
Ethylcyclopentane	726	0.5	-
3-Methyl-2-pentanol	745	0.4	-
2-Methylheptane	754	0.3	-
Toluene	760	2.7	0.5
3-Methylheptane	763	0.2	-
Z-1,3-Dimethylcyclohexane	777	0.6	-
E-1,3-Dimethylcyclohexane	780	0.3	-
2-Hexanone	786	0.6	-
1-Methylcyclopentanol	792	0.7	-
Octane	798	0.5	-
Hexanal	800	0.8	0.5
Z-1,4-Dimethylcyclohexane	807	0.4	-
Ethylcyclohexane	832	0.3	-
Ethylbenzene	856S	1.0	-
1-Hexanol	864	0.4	-
o-Xylene	866	1.0	-
p-Xylene	867	0.4	-
Nonane	903	-	0.4
α-Pinene	930	-	0.4
1-Octen-3-ol	977	0.8	-
2-Pentylfuran	987	-	0.8
2-Ethylhexanol	1025	-	0.4
Limonene	1027	0.8	-
Linalool	1098	1.0	-
Nonanal	1103	1.5	0.7
Decanal	1204	0.5	-
E-*p*-Menthan-2-one	1208	2.0	-
E-Myrtanol	1260	-	0.5
β-Bourbonene	1381	-	1.4
β-Elemene	1386	-	0.3
β-Caryophyllene	1416	-	1.8
Geranyl acetone	1444	0.7	0.3

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α-Humulene	1452	-	3.9
Z-Muurola-4(14),5-diene	1471	-	0.4
(*E*)- *β*-Ionone	1475	0.5	-
Germacrene D	1478	-	0.3
β-Selinene	1486	-	0.7
α-Selinene	1492	-	0.7
α-Muurolene	1495	-	0.3
Tridecanal	1508	0.5	-
δ-Cadinene	1514	-	0.6
(3*Z*)-Hexenyl benzoate	1568	0.5	-
Dendrolasin	1570	0.7	-
Spathulenol	1573	-	1.9
Hexyl benzoate	1576	0.3	-
Caryophyllene oxide	1579	-	10.6
Salvial-4(14)-en-1-one	1589	-	1.9
allo-Cedrol	1592	-	0.6
Humulene epoxide II	1606	-	17.5
Tetradecanal	1609	-	0.3
Pentadecanal	1611	16.0	-
Muurola-4,10(14)-dien-1*α*-ol	1622	-	0.4
Caryophylla-4(12),8(13)-dien-5α-ol	1629	-	3.8
Caryophylla-4(12),8(13)-dien-5β-ol	1633	-	1.4
τ-Cadinol	1638	-	0.7
τ-Muurolol	1640	-	1.7
δ-Cadinol	1643	-	1.1
α-Cadinol	1652	-	5.0
Selin-11-en-4α-ol	1655	-	2.8
14-Hydroxy-9-*epi*-(*E*)-Caryophyllene	1667	-	1.5
Cadalene	1669	-	0.5
Germacra-4(15),5,10(14)-trien-1α-ol	1686	-	1.9
Pentadecanal	1711	-	4.1
Hexadecanal	1813	0.5	-
Phytone	1837	4.5	0.5
(*Z*)-Hexadecatrienal	1882	4.9	0.9
(*E*)-Hexadecatrienal	1887	16.9	-
Heptadecanal	1915	0.9	-
Palmitic acid	1953	0.8	-
Phytol	2102	0.9	-
(9Z)-Octadecenamide	2349	-	0.6
1-Docosanol	2484	0.6	0.5
Heptacosane	2700	1.0	-
(9Z)-Docosenamide	2754	-	18.0
Monoterpene hydrocarbons		0.8	0.4
Oxygenated monoterpenes		3.7	0.8
Sesquiterpene hydrocarbons		0.7	10.9
Oxygenated sesquiterpenes		-	52.8

Diterpene	0.9	-
Apocarotenoids	0.5	-
Non-terpene derivatives	91.0	29.4
Total compounds identified	43	44
Percentage identified	**97.6**	**95.2**

RI- Retention indices determined with respect to a series of *n*-alkanes on a ZB-5 column.

Antimicrobial activity

The result of the antimicrobial assay is given in Table 2. The activity of the volatile oil was compared with gentamycin (10 µL/mL) and ketoconazole (200 µg) used as positive control in the antibacterial and antifungal assays respectively. DMSO used as solvent was used as negative control while the inhibition zones diameter values were reported as 1 decimal place mean±SD. There was no activity against the *E. coli* and *S. aureus* but moderate zones of inhibition were observed on the fungi. Only the stem oil gave significant antifungal activity with inhibition zones varying from 2.1±0.1 to 8.1±0.1 mm but not as active as the referenced antifungal drug (Figure 1). On the other hand, Jose and Reddy (2010) reported significant activity of leaf and flower essential oils of *G. sepium* against *E. coli* and *S. aureus* using agar-diffusion method. Similarly, good activity was documented for leaf extracts and (Chevian & Thambi, 2019; Kumar & Simon, 2016; Sukumar & Aparna, 2014; Akharaiyi et al., 2012). Variation in chemical compositions due to factors like geographical location, plant maturity, season harvested and extraction procedure might have contributed to their different activity.

Previous researches showed that most essential oils with good antimicrobial activities were made up of high percentage of monoterpenes and sesquiterpenes as well as their related alcohols, phenols and other oxygenated compounds (Griffin et al., 1999) due to the hydrophilic character of their functional groups and lipophilic nature of their hydrocarbon chains (Daferera et al., 2000; Kalemba and Kunicka 2003). The above information probably explains antimicrobial activity presented by *G. sepium* stem oil, since it was dominated by terpenes, i.e., 10.9% sesquiterpene hydrocarbons and 52.8% oxygenated sesquiterpenes. Spathulenol, *α*-selinene and *δ*-cadinene found in the stem oil but absent in the leaf oil have been reported to possess antifungal and antibacterial activity (Cakir et al., 2005; Cheng et al., 2005). Besides, synergistic effects of the oil constituents can also contribute to their activity.

Table 2. Zones of Inhibition (mm) observed in antimicrobial assay of *G. sepium* volatile oils

Essential oils	**Conc (µL/mL)**	**Inhibition zones diameter (mm)**				
		E. coli (ATCC 25925)	*S. aureus* (ATCC 6571)	*C. albicans* (clinical)	*A. niger* (clinical)	*F. solani* (clinical)
Leaf	100	0.0	1.8±.0.3	0.0	0.0	6.0±0.1
	10	0.0	0.0	0.0	0.0	3.9±0.1
	1	0.0	0.0	0.0	0.0	2.0±0.1
Stem	100	0.0	0.0	8.1±0.1	1.9±0.1	6.1±0.1
	10	0.0	0.0	4.3±0.4	0.0	3.8±0.4
	1	0.0	0.0	2.1±0.1	0.0	0.0
Positive control		9.0±1.4	11.5±0.7	21.0±1.4	10.5±0.7	10.3±0.4
Negative control		0.0	0.0	0.0	0.0	0.0

Antioxidant activity

DPPH radical-scavenging assay is one of the standard technique commonly used to evaluate the antioxidant potential of essential oils or other phytochemicals extracted from plant. The DPPH has a characteristic absorption at 517 nm. It reduces when exposed to an antioxidant agent that can scavenge its free radical either by donating an electron or a hydrogen atom. At the concentrations observed (5, 25 and 100 µg/ mL), the percentage inhibition of the DPPH• by the leaf and stem volatile oils of *Gliricidia sepium* were between 40-55 %. The leaf and stem oils demonstrated a concentration dependent scavenging ability and the IC_{50} values obtained from the graph of percentage inhibition against the concentration were 84.26 and 142.15 µg/ mL respectively (Table 3), indicating lower scavenging ability than BHT (IC_{50} = 45.11 µg/ mL) that was used as positive control while only the leaf volatile oil showed higher scavenging activity than α-tocopherol (IC_{50}= 81.58 µg/ mL). The lower the IC_{50} value the higher the free radical-scavenging activity. In the previous studies, the leaf extracts especially the ethanoic fractions showed high antioxidant property (Ang et al., 2019; Akharaiyi et al., 2012).

Table 3. IC_{50} values obtained from DPPH radical-scavenging activity of the volatile oils

Tested Samples	Leaf oil	Stem oil	α-Tocopherol	BHA
IC_{50} (µg/mL)	84.26	142.15	81.58	45.11

IC_{50} was obtained by plotting the values of % inhibition against concentration using Microsoft EXCEL

Toxicity

The data from the toxicity study of plant volatile oils is important in order to ascertain their safety when used by human as a source of drug. The brine shrimp lethality assay result was given in Table 4. The stem oil has higher toxicity (LC_{50}= 38.7081 µg/mL) than the leaf oil (LC_{50}= 79.6717 µg/mL). β-elemene (0.3 %), β-caryophyllene (1.8 %) and α-humulene (3.9 %) present in the stem oil have shown high toxicity although not against brine shrimps but tumour cells (Hanusova et al., 2017; Jiang et al., 2017). These compounds are not the major constituents but their presence might have added up to the toxic effect of the stem oil in addition to the synergistic effect of other components of the oil. Several studies have shown good correlation between lethal concentrations (LC_{50}) obtained from the brine shrimps lethality assay using *Artemia salina* and the acute oral toxicity assay using mice (Arlsanyolu and Erdemgil, 2006). This implies the volatile oils from the leaf and stem of *G. sepium* may possess antitumor potential. The toxicity of *G. sepium* leaf extract against brine shrimps have been reported (Ang et al., 2019).

Table 4. The Brine shrimp lethality assay result

Tested samples	LC_{50}	LC limit	UC limit	CL
Leaf oil	79.6717	128.0301	203.9742	0.2275
Stem oil	38.7081	20.5002	66.9387	0.1200

CL-Confidence limit, LC-Lower confidence, UC-Upper confidence, LC_{50}- Conc at which there was 50 % mortality

Conclusion

Chemical compositions, antimicrobial, antioxidant and toxicity of the volatile oils from the leaf and stem of *Gliricidia sepium* from Nigeria are reported for the first time. There were differences in the biological activities of the studied essential oils and the ones from the literatures due to variation in their geographical locations, extraction procedure and chemical compositions. The leaf and stem oils showed poor antibacterial

activity but moderate antifungal activity. The leaf oil is largely non-terpenes: dominated by (*E*)-hexadecatriene (16.9 %) and pentadecanal (16.0 %) while the stem oil composed principally terpenes mainly humulene epoxide II (17.5 %) and caryophyllene oxide (10.6 %). The marked difference between the chemical compositions of the two essential oils was because they were extracted from different plant organs. Therefore, the antifungal activity exerted by the stem oil may be as a result of the synergic effect of several sesquiterpenes present in the oil but absent in the leaf oil. The leaf oil showed better antioxidant activity than the stem oil while both oils were toxic to *Artemia salina* larvae. The results suggest that the stem essential oil from *G. sepium* may find potential use as a natural antiseptic agent. The lower LC_{50} values of the oils signify they are biologically active. Thus several other bioassays will be helpful to search for more activities of these volatile oils whether they possess the same activities like the extract or support their traditional use.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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RESEARCH ARTICLE

Essential oil of *Rosmarinus officinalis* L. from West Highlands of Algeria: Chemical characterization and *in vitro* antifungal activity against *Fusarium oxysporum* f. sp. *albedinis*

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Abstract

Rosmarinus officinalis is a well-studied species; however, *R. officinalis* essential oil (EO) from West highlands of Algeria was not investigated chemically and biologically. In this context, chemical composition of *R. officinalis* EOs obtained from leaves and stem were determined by GC/MS analysis and their antifungal activity against *Fusarium oxysporum* f. sp. *albedinis* (Foa) were evaluated. The GC/MS analysis indicated that monoterpenes were the dominant class of compounds in both leaves and stem (oxygenated 84.9%, 52.4%) and hydrocarbons (12.2%, 8.4 %), respectively. Among them, 1,8-cineole was the main component (leaves: 54.4%, stem: 29.7%), which classifies these EOs as 1,8-cineole chemotype. The *in vitro* antifungal activity of Eos was evaluated through micro-atmosphere and direct contact methods. Best inhibitory activity against Foa was determined after 7-day incubation using direct contact method by relative growth reduction (RGR= 0.398; RGR=0.383) with EOs from leaves and stem, respectively.

Keywords: Essential oil, *Rosmarinus officinalis*, antifungal, fusariosis, GC-MS analysis

Introduction

The worldwide interest in the use of medicinal plants has been growing, and its beneficial effects being rediscovered for the development of new drugs (Andrade, et al., 2018). Essential oils (EOs) are known to have various bioactivities (antibacterial, antifungal etc.). Consequently, studies on their biological activities have become important (Shaaban, et al., 2012).

Rosmarinus officinalis species (syn.: *Salvia rosmarinus* Schleid, *R. angustifolius* Mill., *R. communis* Noronha) from Lamiaceae family, commonly known as 'Lazir' is an evergreen plant typical of the Mediterranean region. It is widely used in the Algerian ethnopharmacopoeia as a spice and for the treatment of various diseases such as digestive troubles, gallbladder disorders, cephalic pains, headaches, migraines, colic, diarrhoea, cough and broncho-pulmonary infections (Cheriti, et al., 1995; Cheriti, 2000).

Despite the huge number of studies on *R. officinalis*, the most focused on phytochemistry then on biological activities. The studied biological activities were interested principally to human health (antimicrobial, insecticidal, antioxidant, aromatherapy) (Andrade, et al., 2018; Durak, et al., 2016; Mekonnen, et al., 2016; Isikber, et al., 2006); less was concerned by *Fusarium oxysporum* (phytopathogen) (Mekonnen and Manahile, 2017; Ozkan and Chalchat, 2008). *R. officinalis* EOs from Algeria were found to be rich in 1,8-cineole and camphor. In addition, these EOs presented many biological activities such as antibacterial effect against human pathogenic bacteria (Boutekedjiret, et al., 1998; Djeddi et, al., 2007; Boutabia, et al., 2016).

Fusarium oxysporum f. sp. *albedinis* (Foa) is the causal agent of lethal disease of date palm called Bayoud. We have studied Bayoud disease for many years (Boulenouar, et al., 2009; Boulenouar, et al., 2011; Boulenouar, et al., 2012; Boulenouar, et al., 2014; Belhi, et al., 2020; Ghazi, et al., 2020); but till now there

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is no efficient treatment. The aim of this study is the investigation of antifungal effect of *R. officinalis* EO on phytopathogen fungus Foa. To the best of our knowledge, this is the first time that EO of *R. officinalis* from Algerian West highlands was investigated on phytochemical level and as potential antifungal agent against Foa.

Materials and Methods

Plant material and essential oil extraction

Aerial parts of *R. officinalis* were collected from West highlands (El-Bayadh, Algeria, Latitude: 33° 40′ 49″ N; Longitude: 1° 01′ 13″ E; Altitude: 1313m) during March 2018. The species was identified, and a voucher specimen is kept at Phytochemistry and Organic Synthesis Laboratory under N° CA99/11. The leaves and stem were separated, washed, dried in shade, then grinded until obtaining a fine homogenous powder.

The *R. officinalis* essential oil was obtained from dry plant material (100 g) by hydrodistillation using a Clevenger apparatus for 3 h, in accordance with the 3rd Edition of the European Pharmacopoeia cited by Bruneton (1999). The process was repeated five times to get a sufficient amount of EO for antifungal tests and chemical analysis. The obtained oil was dried over anhydrous sodium sulphate and stored in hermetically closed small vials at 4°C until use.

GC-MS analysis

Gas chromatographic (GC) analysis was performed on a Perkin Elmer Clarus 680 gas chromatograph equipped with an FID and fitted with a fused-silica Rtx-5MS capillary column (30 m x 0.25 mm, ID 0.25 µm film thickness). The analytical conditions were: Carrier gas was He (1.0 mL/min), injector and detector temperature were 280°C. The temperature program used was 4 min isothermal at 70 °C, increased to 180 °C at a rate of 4 °C/min, then increased to 240 °C at a rate of 10 °C/min and ending with a 10 min at 300°C. Samples were injected by splitting and the split ratio 1:5. The relative amounts of the individual components found in the oil are based on the GC peak areas obtained (FID response).

The GC/MS analysis was performed on Perkin Elmer Clarus 680 gas chromatograph, interfaced with Clarus SQ 8T mass spectrometer, operating at electron impact of 70 eV with an ion source temperature at 250°C, scan mass range of 30-300 m/z at a sampling rate of 0.5 scan/s. A fused-silica Rtx-5MS capillary column (30 m x 0.25 mm, ID 0.25 µm film thickness was used under the same conditions as those used for gas chromatography analysis as described above. The EOs component identification from the GC/MS spectra was confirmed by comparison of mass spectral fragmentation patterns with the computer library (NIST MS Library), and verified by comparison of their retention indices, determined relatively to the retention times of a *n*-alkanes homologous series (C4–C40) of the identified compounds with literature (Adams, 2007; Babushok, et al., 2011; Benabed, et al., 2017; Boukhobza, et al., 2020).

Antifungal test

Fungal strain

The fungal strain used in this study is *Fusarium oxysporum* f. sp. *albedinis*. It was obtained from the Technical Institute for Development of Saharan Agronomy (TIDSA), Adrar, Algeria. It was identified and a voucher specimen was stored at Phytochemistry and Organic Synthesis Laboratory under N° Foa-POSL/2011/01. Preparation of Foa culture was realized as described by Boulenouar et al. (2012).

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Procedure

Micro-atmosphere method. A 10mm diameter mycelial disc taken from 7 days culture of Foa was deposited upside-down in the center of PDA medium. Four volumes of EO (10, 20, 30, 40µL) were spread on the lids of the Petri dishes (85mm diameter). PDA inoculated with Foa and without EO was used as a negative control. The observation of the results was carried out after incubation for 7 days and 10 days at 25±1 °C. The tests were performed with three repetitions (n=3) (Stupar et al., 2014).

Direct contact method. Four volumes of the EO (10, 20, 30, 40µL) were spread on PDA medium (85mm diameter Petri dishes). A 10mm diameter mycelial disc taken from 7 days Foa culture was placed upside-down in the center of the PDA medium. The negative control was performed with PDA medium inoculated with Foa and without EO. The observation of the results was carried out after incubation for 7 days and 10 days at 25±1 °C. The tests were performed with three repetitions (*n*= 3) (Ozkan and Chalchat, 2008).

Evaluation of antifungal activity

Antifungal activity was evaluated using: growth rate (GR), percentage of growth inhibition (Inhib%) and relative growth reduction (RGR). GR represents the speed of mycelium growth as millimeter per day (mm/day) (Kibar and Piksen, 2011). Inhib% was calculated using the following formula: Inhib% = [(DC-DT)/DC] × 100. Where DC and DT are the average diameters (mm) of fungal growth from control and treatment samples, respectively (Ozkan and Chalchat, 2008). RGR (%.mm^{-1}.μL^{-1}) evaluation takes in consideration the size of inoculums and quantity of analysed substance in the antifungal activity. It was calculated using the following formula: RGR = Inhib% / (In × V). Where: "Inhib%" is the percentage of growth inhibition (%) calculated as cited above, "In" is the diameter (mm) of mycelial inoculum from fungi culture, "V" is the volume (µL) of EO used in treatment. No substance has been reported effective on *Fusarium oxysporum* f. sp. *albedinis* to be used as positive control. The negative control was test passed all protocol without using essential oils.

Experimental design and data analysis

The experimental design used in this study was factorial experiment. All experiments were conducted in triplicate. The significance of activities had been analysed with ANOVA test. Correlation between different factors was tested. The probability "*P* value" less than 0.05 was considered significant (α= 5%).

Results and Discussion

Chemical composition of *R. officinalis* EO

The green yellowish EO yield from leaves was significantly higher (2.17±0.02 %) than from stem (0.98±0.01 %), which is in accordance with the limits cited by Jawad et al. (2018). The extraction yield can be affected by many factors (seasonal and geographic conditions, distillation technique, harvest period…) (Rao, et al., 2014; Singh, et al., 2014; Kumar, et al., 2016). A total of 21 and 19 compounds representing 98.8% and 68.3% of the total EO were identified in leaves and stem, respectively. The chemical composition of the EOs were presented in Table 1. The components were listed in order of their elution on the Rtx-5MS capillary column.

The oxygenated monoterpenes were the dominant class of compounds in both leaves and stem (84.9%, 52.4%), respectively. The 1,8-cineole (54.4%, 29.7%) was the main constituent, followed by camphor (10.1%, 7.9%), α-terpineol (7.6%, 5.6%) and borneol (5.5%, 4.0%), respectively.

The EOs of *R. officinalis* were found to contain six monoterpenes hydrocarbons (leaves: 12.2%, stem: 8.4 %), of which α-pinene (leaves: 5.5%, stem: 4.0%) was the most represented component. In addition, EOs from both parts contains lower amounts of sesquiterpene (leaves: 1.4%, stem: 7.4%). When compared with leaves EO, higher amount of caryophyllene oxide (5.4%) was measured in the stem EO. It's probably due to change of cytological, biochemical and physiological activities within organs (Zaouali, et al., 2013), and the importance of leaves as a centre of volatile compounds production in the plant (Boix, et al., 2011). In accordance with the richness of leaves in terms of volatile compounds, in this study, yield of leaves EO (98.8%) was detected higher than that of stem EO (68.3%) (Boix, et al., 2011).

Table 1. Chemical composition of the *R. officinalis* essential oil

N°	Compounds	RI[a]	RI[b]	Content (%)	
				Leaves	Stem
1	α-Pinene	931	932	5.5	4.0
2	Camphene	946	946	2.6	1.8
3	β-Pinene	976	974	3.1	2.0
4	α-Terpinene	1016	1014	0.3	tr[c]
5	1,8-Cineole	1030	1026	54.4	29.7
6	γ-Terpinene	1059	1054	0.5	0.4
7	Sabinene hydrate	1068	1065	0.1	-
8	α-Terpinolene	1089	1086	0.2	0.2
9	Linalool	1103	1095	4.0	2.8
10	Camphor	1144	1141	10.1	7.9
11	Borneol	1170	1165	5.5	4.0
12	Terpinen-4-ol	1180	1174	1.6	1.3
13	α-Terpineol	1193	1186	7.6	5.6
14	Verbenone	1212	1204	0.5	0.4
15	Bornyl acetate	1289	1284	1.2	0.8
16	Carvacrol	1306	1298	0.1	0.1
17	Methyl eugenol	1405	1403	0.1	-
18	β-Caryophyllene	1421	1417	0.8	1.9
19	(*E*)-Geranylacetone	1453	1451	0.1	0.1
20	α-Humulene	1455	1454	0.2	0.1
21	Caryophyllene oxide	1589	1583	0.5	5.4
			Monoterpene hydrocarbons	12.2	8.4
			Oxygenated monoterpenes	84.9	52.4
			Sesquiterpene hydrocarbons	1.0	2.0
			Oxygenated sesquiterpenes	0.5	5.4
			Others	0.2	0.2
			Total	**98.8**	**68.3**

[a]Retention indices on Rtx-5MS column. [b]Retention Indices obtained from literature (Adams 2007, Babushok et al. 2011). [c]Trace amount < 0.01

The qualitative composition of EO from both parts was similar, but a marked quantitative difference was observed, which may be attributed to the growth phase of the plant, as it's well known that chemical variability may be related with different vegetative phases of the plant (Barra, 2009). Many studies have

been carried out on the chemical composition of different samples of *R. officinalis* from different Mediterranean geographical regions revealing that chemical composition and percentage vary depending upon the plant parts, vegetative phases, extraction methods and environmental and growing conditions (e.g. seasonal and geographical variations, soil composition) (Carvalho, et al., 2005; Figueiredo, et al., 2008; Zaouali, et al., 2013; Andrade, et al., 2018).

According to previous studies, 1,8-cineole ranging from 57.7 to 11.0%, camphor from 36.7 to 7.9% and α-pinene from 24.7 to 5,7%, are the most represented components in all samples of *R. officinalis* EO from Mediterranean region : Algeria (Fellah, et al., 2018), Egypt (Fadel & El-Massry, 2000), France (Chalchat, et al., 1993), Italy (Napoli, et al., 2010; Serralutzu, et al., 2020), Lebanon (Diab, et al., 2002), Morocco (Chalchat, et al., 1993; Rahmouni, et al., 2019), Portugal (Mata, et al., 2007), Spain (Chalchat, et al., 1993; Salido, et al., 2003), Tunisia (Hcini, et al., 2013; Zaouali, et al., 2013) and Turkey (Celiktas, et al., 2007; Ozcan & Chalchat. 2008).

The EO extracted from *R. officinalis* collected from El-Bayadh (Algerian West highlands) was characterized by high content of 1,8-cineole, which classifies it as 1,8-cineole chemotype according to the classification of Napoli et al. (2010). It has been reported that altitude affects the chemical composition of EOs in many plant species (Barra, 1990). Concerning *R. officinalis* EOs, Sabbahi et al. (2020) has demonstrated that only the major constituent (1,8-cineole) has a significant relationship with altitude. However, the effect of endogenous and exogenous factors on secondary metabolites biosynthesis –at the same time- makes the evaluation of altitude effect difficult.

Evaluation of antifungal activity

Among plant extracts, essential oils are the most difficult to be analyzed for antimicrobial activity, because of their limited yield, less-stability, less-solubility in media and their complex composition (Lahlou, 2004). Antimicrobial activities reported in the literature have been evaluated with diverse sets of methodologies, degrees of sensitivity, amount of test-compounds and microbial strains, often difficult to compare (Valgas, et al., 2007). Many laboratories have modified antimicrobial evaluation methods for specific samples, such as essential oils and non–polar extracts and these modifications became impossible to directly compare results (Scorzoni, et al., 2007).

It's well known that inoculum size and antimicrobial quantity influence the efficacy of antimicrobials (Cerero, et al., 2010; Xie, et al., 2017). The effect of inoculum density is observed to be strain dependent (Bedenic, et al., 2001). It has been concluded that the extent of antifungal effect varied depending on the levels of EO used in the experiment (Ozkan & Chalchat, 2008). Thus, the use of RGR principle in relation to Inhib%, mycelia inoculum and EO volume gives more opportunity to compare EO effect on filamentous fungi.

The principle of radial growth calculation is based on the diameter of control growth covering the Petri dish, in our case after 10 days. However, we preferred to evaluate the effect after 7-day incubation as well, to calculate the progression of antifungal effect through two periods of time.

Antifungal activity of *R. officinalis* EO on Foa

The date palm fusariosis caused by Foa presents a serious problem for desertic regions, especially in Algeria and Morocco. The development of efficient treatment is more than necessary to protect the oases.

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Table 2. Effect of essential oils from *Rosmarinus officinalis* L. on *Fusarium oxysporum* f. sp. *albedinis* expressed as GR, Inhib% and RGR using the micro-atmosphere and direct contact methods.

Method	EO[a] part	Incubation (days)	EO volume (µL)	GR[b] (mm/day)	Inhib %[c]	RGR[d] (%.mm^{-1}.µL^{-1})
Micro-atmosphere	Leaves	7	10	8.57±0.17	6.61±1.80	0.066
			20	7.95±0.05	13.36±0.52	0.067
			30	7.19±0.13	21.66±1.37	0.072
			40	6.90±0.13	24.77±1.37	0.062
		10	10	7.90±0.06	6.51±0.68	0.065
			20	7.63±0.09	9.66±1.04	0.048
			30	7.30±0.12	13.61±1.37	0.045
			40	7.20±0.12	14.79±1.37	0.037
	Stem	7	10	8.62±0.13	6.09±1.37	0.061
			20	8.05±0.05	12.32±0.52	0.062
			30	8.00±0.08	12.84±0.90	0.043
			40	7.28±0.08	20.62±0.90	0.052
		10	10	7.80±0.06	7.69±0.69	0.077
			20	7.70±0.06	8.88±0.69	0.044
			30	7.60±0.06	10.06±0.69	0.034
			40	7.37±0.09	12.82±1.04	0.032
Direct contact	Leaves	7	10	5.52±0.17	39.82±1.87	0.398
			20	4.76±0.17	48.12±1.87	0.241
			30	3.28±0.38	64.20±4.11	0.214
			40	3.19±0.13	65.24±1.38	0.163
		10	10	6.30±0.06	25.44±0.68	0.254
			20	5.57±0.12	34.12±1.42	0.169
			30	4.93±0.20	41.62±2.40	0.139
			40	4.37±0.18	48.32±2.09	0.121
	Stem	7	10	5.67±0.17	38.26±1.87	0.383
			20	3.95±0.42	56.95±4.61	0.285
			30	4.14±0.16	54.87±1.80	0.183
			40	3.19±0.37	65.24±4.05	0.163
		10	10	6.40±0.06	24.26±0.68	0.243
			20	5.63±0.12	33.33±1.42	0.167
			30	4.87±0.24	42.41±2.84	0.141
			40	4.00±0.36	52.66±4.27	0.132

[a]Essential oil, [b]Growth rate, [c]Percentage of growth inhibition (Inhib % values were calculated referred to radial growth of negative control. The radial growth of negative control was: 64.25±0.75mm (7 days of incubation); 84.50±0.29mm (10 days of incubation), [d]Relative growth reduction.

Best inhibitory activity of Foa was observed for 10µL of EOs after 7 days incubation using direct contact method (leaves: RGR=0.398; stem: RGR=0.383). The comparison of Inhib% values between the two periods

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shows a significant decrease from 7 to 10 days ($p<0.05$). This effect may be explained by the development of resistance mechanism through production of metabolites or enzymes by the fungus to detoxify the antifungal compounds in EO. Ozcan and Chalchat (2008) showed that EO from *R. officinalis* leaves inhibits *F. oxysporum* after 7 days but no significant inhibition was observed after 10 days. When we link this result with the EO quantity, the increase of EO volume in contact with Foa gave more brake to Foa growth. Farooq et al. (2002) present this phenomenon of detoxification by plant pathogenic fungus. (Table 2)

The antifungal activity of our essential oil might be related to their monoterpenes components which constitute dominant class of compounds in both leaves and stem, with oxygenated monoterpenes (84.9%, 52.4%) and monoterpenes hydrocarbons (12.2%, 8.4%) respectively. Besides a variety of biological activities of monoterpenes, EOs containing high amounts of oxygenated monoterpenes have also been reported to be important antifungal agents (Burt, 2004; Farooq, et al., 2002; Dias, et al., 2017; Danielli, et al., 2019). In addition, it is possible that antifungal activity of *R. officinalis* EO is due to cell membrane disruption by lipophilic compounds (Cowan, 1999).

Inhibition activity can be related to presence of aromatic ring and OH group (present in the minor compounds such as carvacrol and methyl eugenol) that is known to be reactive forming hydrogen bonds with enzymes causing their inhibition (Velluti, et al., 2003). Thus, antifungal activity may be related to these minor constituents. The synergism between EO constituents is among probable antifungal effect observed in this study.

Lee et al. (2007) demonstrated that *F. oxysporum* is inhibited by commercial EOs with Inhib% values 57 to 76% using micro-atmosphere principle. However, the inoculum size was presented as plugs without specification of diameter. Therefore, it is not possible to compare our results with this study.

Analysis of variance in micro-atmosphere method revealed that effects of all factors on GR and RGR are significant ($p<0.05$). For GR values, the highest antifungal effect was observed for 7 days of incubation, leaves and EO volume equal to 40µL (6.90±0.13mm/day). For RGR values, maximum inhibitory activity was observed with stem EO 10µL (RGR=0.077).

The link between EO amount used and antifungal activity can be explained through two principles. First, for GR and Inhib%, the increase in volume permit the increase in EO components responsible for antifungal effect, so 40µL is more efficient than 10µL. Second, for RGR, the increase of volume is not related directly to antifungal effect but other parameter is engaged which is fungus quantity (culture diameter). On the other hand, decrease of effect between 7 and 10 days reflects a detoxification phenomenon as explained previously.

The highest effects were observed for direct contact technique. This may be due to the fact that micro-atmosphere technique permits to only highly volatile compounds to act on Foa (Stupar, et al., 2014). Thus, the antifungal effect using this method is underestimated. However, the micro-atmosphere method has a positive point related to low contamination risk because there is no direct contact between EO and medium.

The results presented in (Table 2) showed a strong correlation among couples RGR/method (r=0.80), Inhib%/method (r=0.86), GR/method (r=-0.88) and Inhib%/GR (r=-0.99). A moderate correlation was observed among couples RGR/Inhib% (r=0.62) and RGR/GR (r=-0.60). No correlation was observed among others.

The effect of part used (leaves, stem) was more significant in direct contact method than micro-atmosphere one ($p<0.05$). This may be due to the specificity of direct contact method which facilitates the contact between EOs components and Foa.

If we use only Inhib% values, the best effect was observed for 40 µL EO from stem and leaves using direct contact method after 7-day incubation (65.24% for both). However, using RGR values, the efficient effect was observed for 10µL EO from leaves and stem using direct contact method after 7-day incubation (0.398 and 0.383, respectively). Among the problems related to antifungal effect evaluation is the high assay dosage that may lead to overestimation (Scorzoni, et al., 2007). Thus, the evaluation of antifungal effect without referring to dose used is not sufficient to talk about efficacy. The RGR values reflect the effectiveness of 1µL of EO in the presence of 1mm of mycelial inoculums. Therefore, RGR is more suitable to reflect the effect of this EO on Foa. (Table 2)

Its common knowledge that *R. officinalis* is rich in natural products with interesting biological activities. This is the first report on *R. officinalis* EO from Algerian West highlands as source of potential antifungal compounds against Foa.

The GC/MS analysis has demonstrated the richness of this EO in monoterpenes in both leaves and stem. Eucalyptol (1,8-Cineole) was the main component which classifies this EO as 1,8-cineole chemotype.

R. officinalis EOs has presented an important antifungal effect against Foa in first stage. Best inhibitory activity against Foa was determined after 7-day incubation using direct contact method (RGR= 0.398; RGR=383) with EOs from leaves and stem, respectively. Antifungal effect is probably due to the major compounds in the EO (oxygenated and hydrocarbons monoterpenes) or to synergism between constituents. However, it seems that Foa has developed a kind of resistance mechanism. This resistance may be related to detoxification phenomenon. RGR evaluation allows focusing on the efficient dose to avoid insignificant higher doses and work on other parameters to increase effect.

To the best of our knowledge, this is the first report of antifungal activity of *R. officinalis* EO (collected from West highlands of Algeria) on the causal agent of Bayoud disease. The obtained results shed the light on the possibility to use *R. officinalis* EO as source of treatment against Foa by proceeding with further advanced studies.

Conclusion

Upcoming new insights may focus on major compounds in this EO, especially 1,8-cineole, to develop efficient treatment. Further *in vivo* experiments are needed to be performed.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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RESEARCH ARTICLE

Zerumbone induces growth inhibition of Burkitt's lymphoma cell line via apoptosis

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Abstract

Zerumbone (ZER), a natural compound has been extracted from *Zingiber zerumbet* with known pharmacological activities. The aim was to determine the anti-human Burkitt's lymphoma (Raji) cell effect of ZER. The 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide (MTT) assay was used to determine cytotoxic effect while the Annexin-V-fluorescein isothiocyanate/propidium iodide-PI flow cytometric assays was used to determine apoptotic effect of ZER on the human Burkitt's lymphoma (Raji) cell (ATCC CCL-86) cell line. The expressions of Bax, Bcl-2, and c-Myc genes were determined via real-time PCR. ZER suppressed the proliferation of Raji cells with a 48 h IC_{50} value of 5.1 µg/mL. Treated Raji cells also underwent late apoptosis especially after treatment with 100 µg/mL ZER. The apoptotic effect of ZER is associated with increase in Bax and decrease in Bcl-2 and c-Myc gene expressions. These results suggest that ZER inhibited the proliferation of Raji cells through the modification of apoptosis-related gene expressions. Therefore, ZER has potential as a candidate for the treatment of Burkitt's lymphoma.

Keywords: Cytotoxicity, cell proliferation, Burkitt lymphoma.

Introduction

Burkitt's lymphoma is among most common cancers worldwide (Al-Attar et al., 1979; Achmad et al., 2019). Like all cancers, conventional treatment for lymphomas with immunotherapy and chemotherapy often lack efficacy due to the development of cancer resistance to the compounds. Therefore, there is need for the discovery of more effective and safer anticancer drugs than the conventional therapeutics. Currently, the most attractive candidates as anticancer compounds are products from medicinal plants (Albaayit et al., 2021a; Ling et al., 2016).

Anticancer compounds primarily targets the cellular mechanisms regulating proliferation of cancer cells, particularly the apoptotic pathways (Albaayit et al., 2021b; Sharifi et al., 2014). The driving force behind these pathways is the Bax proteins, which are associated with the mitochondrial control of cell proliferation (Glab et al., 2017). The members of the Bcl-2 family are among Bax proteins that function either as pro- or anti-apoptotic molecules (Albaayit et al., 2020a; Sharifi et al., 2014). The Bcl-2 protein, an anti-apoptotic molecule is among the most popular targets for anticancer therapeutics, and in lymphoma this is no exception. Lymphomas, for example Burkitt's lymphoma, are also governed by the expression of the oncogene, c-Myc, that is responsible for the regulation and expression of various apoptosis-related genes. Thus, the c-Myc gene can be modulated to make cancer cells more susceptible to apoptosis (Benassi et al. 2012; Cerquetti et al., 2015; Nguyen et al., 2017).

Zingiber zerumbet (L.) Smith is a perennial herb found in South East Asian countries and its rhizomes had been used traditionally in these countries for different purposes like food flavouring, appetizer, and herbal

medicines (Albaayit et al., 2020b; Attyah & Ismail, 2012). Till date, many manuscripts related to this plant has been published and discussed its ethnomedicinal uses like treatment of diarrhoea, toothache, fever, inflammation, fever, constipation, indigestion, severe sprains, pain relief, antirheumatic, antispasmodic, antiplatelet aggregation, and diuretic agents (Jantan et al. 2008; Bhuiyan et al. 2009; Zakaria et al. 2010; Yob et al. 2011,). Due to its high medicinal value, several researchers had isolated its compounds and described the phytochemical contents of rhizomes of *Z. zerumbet*. Till date many compounds have been identified from different extracts of its rhizomes such as humulene, monoterpenes, zerumbone, humulene monoxide, humulene dioxide, linalool, α-pinene, α-terpineol, β-pinene, camphor, borneol, humulene epoxide-I, humulenol-I, zerumbone oxide, zerumbone epoxide, diferuloylmethane, acetylated rhamnopyranoside, (*Z*)-nerolidol, kaempferol derivatives, *p*-hydroxybenzaldehyde, vanillin, saponins, terpenoids, and zederone (Varier et al. 1945; Balakrishnan et al. 1956; Dev et al. 1960; Ramaswami et al. 1962; Dũng et al.1995). Among all bioactive compounds identified from different extracts of rhizomes of *Z. zerumbet*, zerumbone has got special attention and extensively studied.

Zerumbone (ZER) is a crystalline sesquiterpene isolated from the essential oil of the rhizomes of *Z. zerumbet* (L.) Smith. ZER is unique in structure, containing a cross-conjugated ketone in an 11-membered ring (Albaayit and Maharjan, 2018). Among the pharmacological properties of ZER are the hepatoprotective, anti-inflammatory, antidiabetic, immune-modulatory, analgesic, antiadipogenetic, antioxidant, antifungal, and antimycobacterial effects (Sakinah et al., 2007; Haque et al., 2017; Albaayit et al., 2021c). ZER has also been shown to possess cytotoxic properties toward several cancer cell lines including the hepatic (HepG2), breast (MCF-7), cervical (HeLa), colon (COLO205), and ovarian (Caov-3) cells (Girisa et al. 2019). Murakami et al.1999 had shown the ZER inhibitory activity on human Burkitt lymphoma (Raji) cell line, however till date, its molecular mechanistic study on Raji cell line was not found, therefore this study was carried out to investigate the *in vitro* apoptotic effect of zerumbone on Burkitt lymphoma (Raji cell lines) through MTT assay, flow cytometry, and PCR techniques.

Materials and Methods

Material

Zerumbone crystals were prepared and characterized by using the method described by Mohamad et al. (2014). The crystals were diluted with 0.1% dimethyl sulfoxide (DMSO) to obtain the stock solution.

Cytotoxicity of ZER against Raji cell lines

Raji cell suspension at a concentration of 5×10^3 cells/well in a 96-well plate were treated with ZER at concentrations ranges from 3.1 to 100 µg/mL, while the control was treated with 0.1% dimethyl sulfoxide (DMSO) for 48 h at 37°C and 5% CO_2. 20 µL of MTT solution (5 mg/ml) was added and the plate incubated for 4 h in the dark. Formed purple formazan crystals were dissolved with 200 µL DMSO.

The absorbance of the samples was then measured at 570 nm using an ELISA plate reader (Tecan, California, USA). The half cancer cell growth inhibition (IC_{50}) value was obtained from the graph of inhibition percentage versus concentration (Albaayit et al. 2019). The following equation was used to calculate percentage growth inhibition for each concentration of ZER:

% growth inhibition = 100-(Average reading of test compound/Average reading of control) x100

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Apoptosis

Annexin-V/propidium iodide assay

Apoptosis in Raji lymphoma cell line were also determined by using the Annexin/PI staining technique (Thermo Fisher Scientific). Briefly, 1 ml of 1 × 10^6 Raji cells was seeded into each well of a 6-well plate and the plate incubated for 24 h at 37°C. ZER at either 5.1 or 100 µg/mL was added to the respective wells and the plate incubated for 48 h. After the treatments, the cells were harvested by trypsinization, washed thrice with phosphate buffered saline (PBS), and stained with 5 µL fluorescein isothiocyanate (FITC)-conjugated annexin-V and 5 µL PI for 15 min in the dark at room temperature. The percentage of cells undergoing apoptosis and necrosis were determined by flow cytometrically (FACSCalibur™, Becton Dickinson) (Albaayit, 2021).

Quantitative real-time polymerase chain reaction

RT-qPCR was used to determine expression of apoptosis-related genes in the treated Raji cells. 1 × 10^6 Raji cells/well/0.5mL suspension cells seeded into each well of a 6-well plate. The cells were treated with ZER at 5.1 (IC_{50}) and 100 µg/mL for 48 h. Total RNA was isolated from harvested cells using the TRIzol® Reagent (Bio basic BS410A, Canada). Complementary DNA (cDNA) was synthesized using the cDNA synthesis kit (Thermo Fisher Scientific K0221). 1 µL of cDNA was amplified using real-time PCR and primers listed in Table 1. The amplification of genes was ran with SYBR Green Master Mix (Thermo Fisher Scientific). LightCycler® 480 Gene Scanning Software (Agilent Technologies Stratagene Mx 3000P, Santa Clara, USA) was used to generate the results and calculate the amount of transcripts relative to the control. GAPDH was used as the housekeeping gene control (Hidayat et al. 2016).

Table1: Genes and primer sequences

Gene	Forward primer sequence	Reverse primer sequence
BCL2-associated X (*BAX)*	AAGAAGCTGAGCGATGTC	GGCCCCAGTTGAAGTTGC
B cell lymphoma -2 (BCL2)	GGCATTCAGTGACCTGACATC	AGTCATGCCCGTCAGGAAC
C-Myc	CACAGCAAACCTCCTCACAG	GGTGCATTTTCGGTTGTTGC
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	CCAGAACATCATCCCTGCCT	CCAGAACATCATCCCTGCCT

Statistical analysis

Differences among means were determined by one-way analysis of variance and Tukey's *post-hoc* test using SPSS (Version 19.0; IBM Corporation, Armonk, NY, USA) at $P<0.05$. The results are expressed as mean ± SD. All experiments were performed in triplicates.

Results and Discussion

Burkitt's lymphoma is among the most aggressive cancers. Currently, the mainstay of treatment for lymphoma is chemotherapy, which has many side effects. For that reason, phytochemicals with minimal side effects has been sought as potential anticancer compounds. Among natural compounds shown to have potential in the treatment of Burkitt's lymphomas are β-elemene (Tonglin and Gao, 2018), shikonin (Ni et al., 2018), and resveratrol (Jara et al., 2018).

ZER, the most prominent phytochemical in *Zingiber zerumbet*, was shown to have potent anticancer properties by acting on multiple pathways and suppresses the growth of cancers (Samad et al. 2015; Girisa

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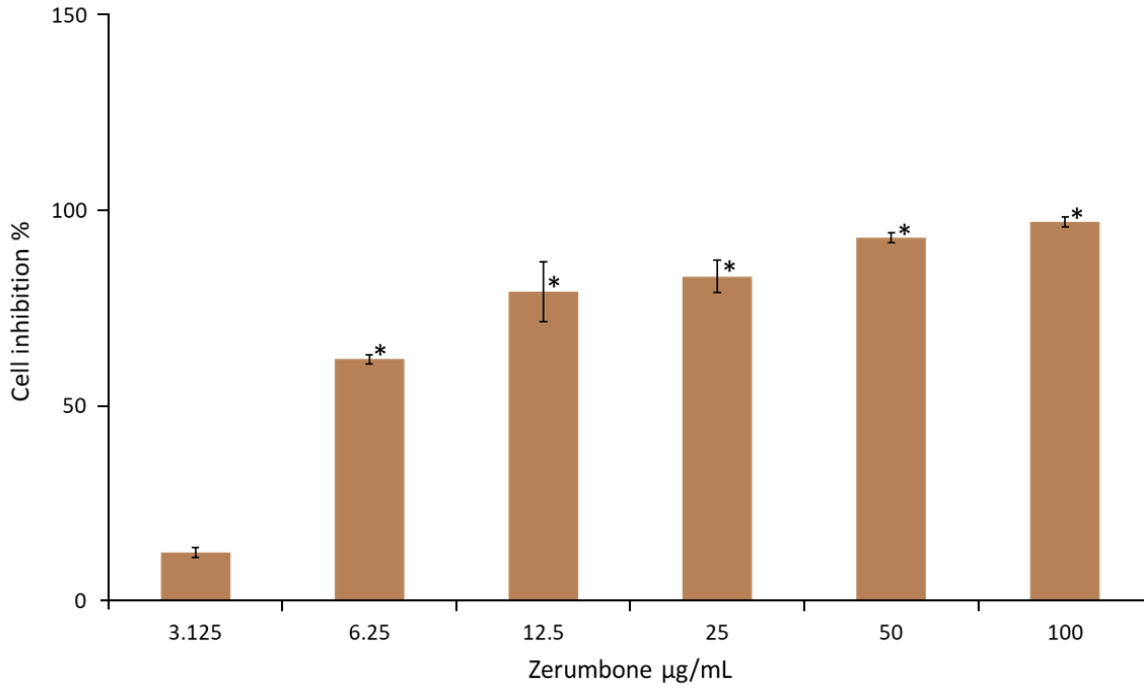
et al. 2019). In different cancer cell lines, ZER downregulated CD1d, IL-1β, TGF- β1, CD44, MMP-3, NF-κB, TNF-α, IL-8, β-catenin, BCl-2, VEGF, MMP-9, PI3K/AKT/mTOR, JAK2/STAT3 genes and upregulated p53, Caspase-3, Bax, Caspase 8, Caspase 9, IL-6, DR4, DR5 genes (Girisa et al. 2019). However, it is still unknown how ZER affects Raji cells. By the MTT colorimetric assay, ZER, in fact, significantly inhibited the proliferation of Raji cells as shown in figure 1. The lowest mean IC50 of 5.1 µg/mL against Raji cells, and hence was chosen for subsequent studies. It is believed that the effect of ZER on Raji cells is attributed to its β-unsaturated carbonyl group (Sadhu et al. 2007). ZER, especially at high doses, inhibited Raji cell proliferation via the induction of late apoptosis. Flow cytometric analysis clearly showed that ZER induced apoptosis in Raji cells (Figure 2). Zerumbone, especially at 100 µg/mL induced the majority of Raji cells to undergo apoptosis.

The Bcl-2 family of proteins, especially the Bax and Bcl-2 proteins are popular targets for anticancer drug candidates (Pfeffer and Singh, 2018). ZER caused significant upregulation in BAX by ~1.3 and 1.8-fold while downregulation in the Bcl-2 gene of the Raji cells by ~1.7 and 2.7-fold at 5.1 and 100 µg/ml, respectively. This suggests that ZER has potential as anti-Burkitt's lymphoma agent (Figure 3).

c-Myc is proto-oncogene protein that both activates and represses target gene via several mechanisms (Dang et al., 2006). Cancer cells have high c-Myc expression (McMahon, 2014) and this is presumably associated with its role as an activator of pro-proliferative genes. The c-Myc gene was significantly downregulated in Raji cells with ZER treatment, further showing that this compound is anti-proliferative toward Raji cells (Figure 3).

Figure 1. Effect of zerumbone on the Raji cell inhibition



After 48 h exposures determined via MTT assay. Values are mean ± standard deviation. *means significantly different (P <0.05) from control (0% inhibition) means.

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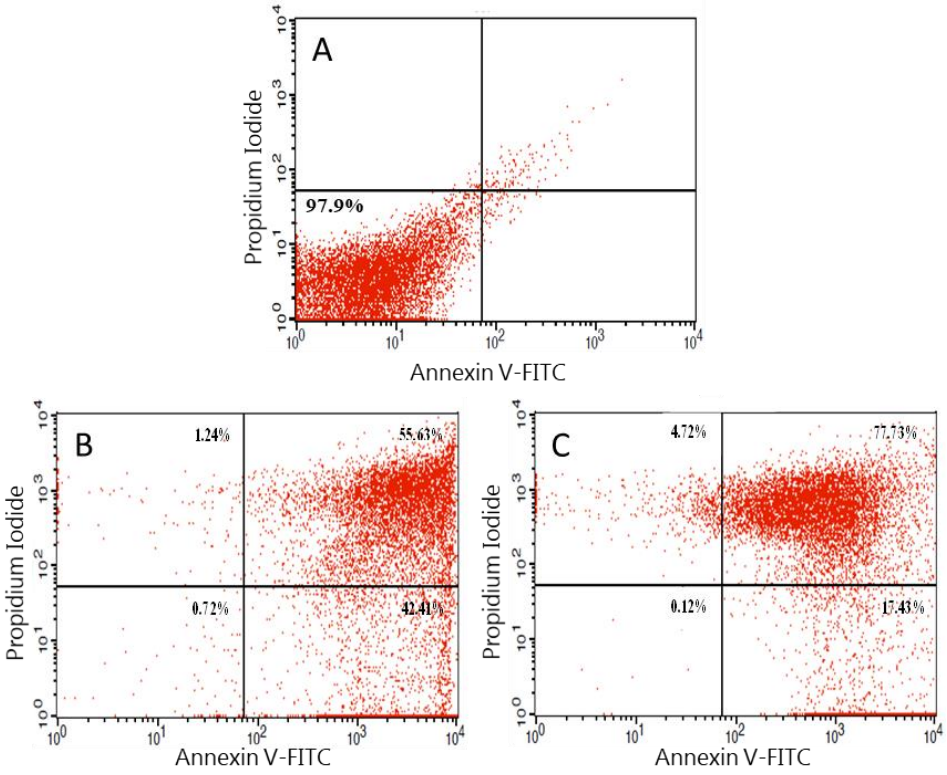
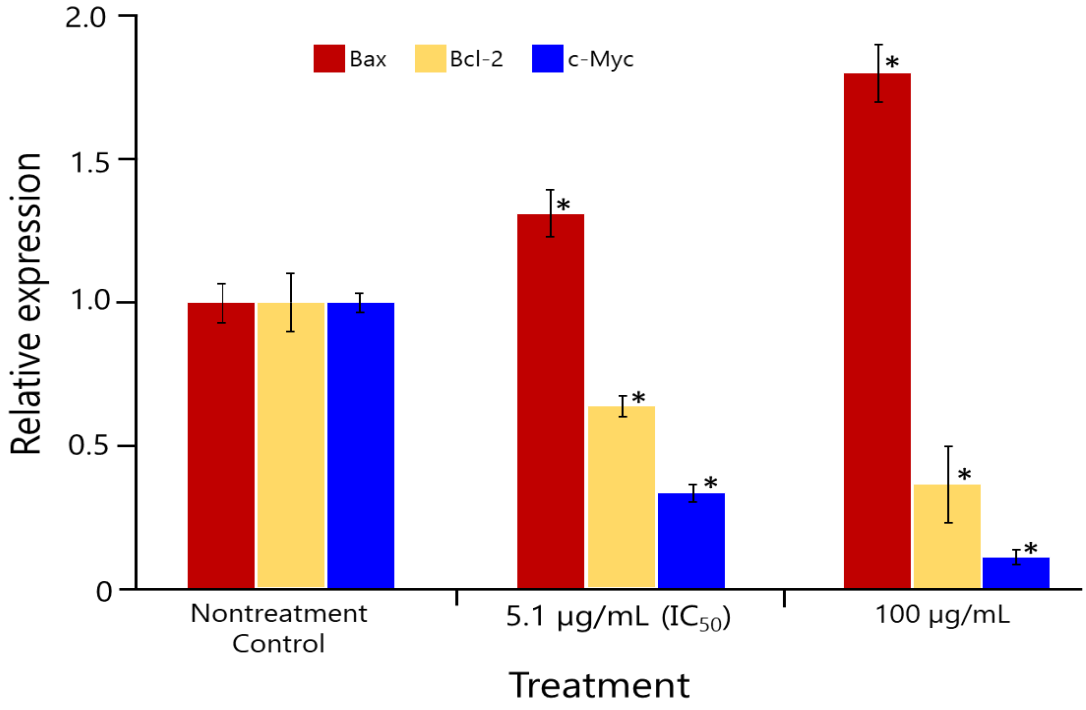
Figure 2. Effect of Zerumbone on apoptosis of Raji cells



After 48-hour treatment. (A) Nontreatment control, (B) 5.1 and (C) 100 µg/mL zerumbone for 48 h. Zerumbone, especially at high dose, induced late apoptosis in Raji cells.

Figure 3. Effects of Zerumbone on Bax, BcL-2 and c-Myc gene expressions



After 48 hour treatment. Zerumbone increased Bax and decreased Bcl-2 and c-Myc gene expressions in Raji cells.

Conclusion

Till date, many *in* vitro and *in* vivo studies had demonstrated the potent anti-tumour effect of zerumbone on different cancer cell lines. However, no significant antiproliferative effect of zerumbone on human Burkitt lymphoma (Raji) cell line had been described. This current study has shown the anti-tumour effect of zerumbone on Raji cell lines by up-regulating the pro-apoptotic Bax, and downregulating the anti-apoptotic Bcl-2 and c-Myc genes. To further confirm the potential candidate of zerumbone in the treatment of Burkitt lymphoma, more molecular studies have to be done regarding its effects on other apoptotic and anti-apoptotic genes, protein expression, cell cycle, and immunocytochemistry.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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RESEARCH ARTICLE

Antimicrobial and antioxidant activities and volatile constituents of *Eurhynchium angustirete* (Broth.) T. J. Kop. and *Isothecium alopecuroides* (Lam. ex Dubois) Isov. from Turkey

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Abstract

The aim of the present study was to determine the chemical analysis, antimicrobial and antioxidant effects of the *Eurhynchium angustirete* (Broth.) and *Isothecium alopecuroides* (Lam. Ex Dubois) essential oil obtained by distillation method (Clevenger apparatus hydrodistillation). Obtained volatile oils were identified by gas chromatography-mass spectrometry (GC/MS) which revealed the presence of twenty-two components analyzed in the essential oil of *E. angustirete* representing 95.7% of the total identified compounds and twenty compounds were determined in the essential oil of *I. alopecuroides* representing 94.3% of the total identified compounds. *Trans*-pinocarveol and myrtenal as major constituents were 23.0% and 14.1% in *E. angustirete* and the main components were: biformene (9.9%), α-pinene (9.1%), bornyl acetate (8.4%) in *I. alopecuroides.* The essential oils from *E. angustirete* and *I. alopecuroides* showed higher metal-ion chelating activities which were 65.93±0.39% and 31.01±2.75, respectively. There was no significant difference results DPPH activity of essential oils 16.8±6.98 and 22.8%±8.91, respectively. The antimicrobial activity was investigated by microdiution method. The isolated essential oils were showed effective antimicrobial activities against *Escherichia coli* ATCC25922*, Enterococcus faecalis* ATCC29212*, Staphylococcus aureus* ATCC25923*, Pseudomonas aeruginosa* ATCC2785 and *Streptococcus mutans.* Antimicrobial effect values were found ranging of 90.76-95.38%. According to antimicrobial effect value against to microorganism, essential oil of *E. angustirete* was higher than *I. alopecuroides.*

Keywords: Mosses, essential oil, GC/MS, antimicrobial activity, antioxidant activity.

Introduction

Bryophyta (mosses, 14000 species) belongs to Bryophytes have about 23000 species in the worldwide comprise the second largest group of plants after angiosperms (Asakawa et al., 2013; Asakawa and Ludwiczuk, 2017). Bryophytes have spread all over the world except the sea. (Vollar et al., 2018; Önder and Özenoğlu, 2019). Bryophytes play a valuable role in maintaining ecosystems because they have the dense cuticle layer which holds the water and have partitions in their structure which protect the insect into. The mosses are represented by approximately 25000 taxa around the world (Smith, 2004). In previous studies, although there are many studies are about usually biomonitoring/bioindicator of waters and air pollution in addition to its use for determination of heavy metal accumulation for mosses, photochemical structures and biological activity of mosses studies have been limited (Yushin et al., 2020; Matos et al. 2021; Limpens et al., 2017)

Mosses as *Rhodobryum, Bryum* and *Philonotis* genus have been used as traditional medicine to reduce the risk of infection from burns, wounds and cuts in countries such as North American Indians and China (Glime, J.M. 2007; Harborne, J.B. 1998). Recently, it has attracted great attention from scientists for pharmacology and drug discovery due to mosses can be a source of metabolites. Previously, there are several studies about antibacterial, antifungal, antiviral, antioxidant, insecticidal, cytotoxic and antitumor activity against some cell cancer extracts of many species Bryophyta (Cheng et al., 2013; Onbaşlı and Yuvalı, 2021). Terpenoid compounds, which are intensely determined in essential oils extracted from plants, have

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many biological activities. Among these compounds, monoterpenoids containing two isoprene units were identified as compounds such as *β*-cyclocitral, *β*-ionone, geosmin and *α*-pinene which were most detected in the essential oils of mosses. Moreover, sesqui- and diterpenoids including respectively three and four isoprene units isolated from some mosses represented cytotoxic activity against cancer cells such as momilactones A and B from the moss *Hypnum plumaeforme* (Hypnacea) (Chen et al., 2018). Lately mosses have begun to attract great interest from the field of chemistry research in search of a source of new and/or bioactive compounds in pharmacology and for drug discovery.

In Turkey, there are three species of *Isothecium* genus belong Lembophyllaceae family, and two species of *Eurhynchium* genus belong Brachytheciaceae family. In one of the previous studies about *Isothecium alopecuroides* species, it was observed that *I. alopecuroides* showed the highest effect in terms of the number of microorganisms it affected (Altuner, 2008). Other studies about this species generally were biomonitoring, metal accumulation and the epiphytic bryophyte flora (Alataş et al., 2015; Sun et al., 2007; Sert et al., 2011). Also, *Eurhynchium angustirete* was chosen as another moss species in this study, because there are a few reports on the antimicrobial activities of essential oil of *Eurhynchium angustirete* grown in different parts of the world including Turkey (Tosun et al., 2015; Nikolajeva et al., 2014).

In this study, the essential oils of *Eurhynchium angustirete* (Broth.) (*E. angustirete)* and *Isothecium alopecuroides* (Lam. Ex Dubois*) (I. alopecuroides)* were extracted by hydrodistillation (HD) using a Clevenger type apparatus and the variation in the essential oils were characterized by gas chromatography mass spectrometry (GC/MS). Moreover, antimicrobial activity of essential oils was identified by microdilution methods against to *Escherichia coli* ATCC25922, *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853 and *Streptococcus mutans* (clinical isolate). The antioxidant activity of essential oils was also examined by DPPH scavenging assay and metal-ion chelating assay. There are no previous reports on the chemical compositions, antimicrobial and antioxidant activity of the *Isothecium alopecuroides* volatile oils but in a previous study, chemical profile and antimicrobial activity of the essential oil of *Eurhynchium angustirete* has differences in terms of volatile compounds and microorganism, which can be explained by the environmentally, locality and the subspecies of the plant used (Altuner and Çetin 2018; Tosun et al. 2015).

Materials and Methods

Plant material

E. angustirete was collected on 5th June 2018 from Erikbeli plateau with coordinate position (Lat. 40°45'00.80"N/39°13'39.18"E), Tonya, Trabzon, at an altitude of 1440 m. *I. alopecuroides* was collected from rocks and stony places in the Erikbeli plateau with coordinate position (Lat. 40°43'12.39"N/39°38'41.21"E) at 848 meters in the centre of Tonya district of Trabzon, Turkey in May 2018, The voucher specimens were identified by Professor Batan, and deposited under number KTUB 1610, KTUB 1609 respectively, at the Herbarium of Department of Biology, Karadeniz Technical University, Trabzon, Turkey.

Isolation of essential oil

The collected fresh moss samples were cleaned to ward off insects if any and other soil particles. The fresh aerial parts of *E. angustirete* and *I. alopecuroides* were divided into smaller pieces. A sample weighing 30 g of each moss samples were subjected to hydro-distillation using a Clevenger-type apparatus for 4 h (yields: 0.05%(w/w), 0.08%(w/w), respectively). The obtained oils were dissolved in HPLC grade *n*-hexane (0.5 mL)

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and dried over anhydrous sodium sulphate and stored at 4-6 °C in a sealed brown vial. One µL of the essential oils was directly injected separately into GC and GC/MS instrument. The oils were dried over anhydrous sodium sulfate and preserved in a sealed vial at 4 °C until further analysis.

GC/MS condition

The GC/MS analysis was performed using Shimadzu 2010 Plus gas chromatograph coupled to a Shimadzu QP2010 Ultra mass selective detector. The separation was performed by means of a Restek Rxi-5MS capillary column, 60 m length, 0.25 mm i.d. and a 0.25 µm phase thickness. The split mode was used. The oven program was as follows: Initial temperature was 60 °C for 2 min, which was increased to 240 °C at 3 °C min^{-1}, 250 °C was maintained for 4 min. Helium (99.999 %) was used as carrier gas with a constant flow-rate of 1 mL min^{-1}. Detection was carried out in electronic impact mode (EI); ionization voltage was fixed to 70 eV. Scan mode (40-450 m/z) was used for mass acquisition.

The volatile compounds were identified by comparison with their retention index (RIs)(relative to C_7-C_{30} n-alkane standard) for identification (Adams, 2004). The mass spectral were compared value of the two libraries (FFNSC1.2 and W9N11) (Çelik, 2020).

Antioxidant activity

Measurement of metal-ion chelating capacity

The chelation of ferrous ions by the essential oils of each species was determined as the previous method (Decker and Welch, 1990). Different concentrations of essential oil (25, 50, 100, 200, 400, and 800 µg/mL) were added to the reaction mixture as described by Decker and Welch. The absorbance of the reaction mixture was measured at 562 nm. EDTA and Trolox were used as standards in the same concentration of essential oils. The percentage of chelating capacity of the test sample was calculated as follows:

$$\text{Chelating capacity\%} = [(A_1 - A_2)/A_1 \times 100]$$

where A_1 is the absorbance of control and A_2 is the absorbance in the presence of essential oil or EDTA.

DPPH• scavenging activity assay

The antioxidant activity of each species' essential oil was first determined by measuring the DPPH• scavenging activity. The essential oil at various concentrations (25, 50, 100, 200, 400, and 800 µg/mL) was added to the reaction mixture including DPPH•. When DPPH• reacts with an antioxidant in the essential oil that can donate hydrogen, it gets reduced form and the resulting decrease in absorbance at 517 nm was recorded using a UV-Vis spectrophotometer (Jasco, V-630, Tokyo, Japan) (B. Williams et al., 1995). In this study, Trolox and BHT were used as antioxidant standards. DPPH scavenging activity of the test sample was calculated as follows:

$$\text{DPPH}^{\bullet}\ \% = 100 - (A_0 - A_1/A_0 \times 100)$$

Antimicrobial activity

Growth inhibitory activity of the essential oils were tested against *Escherichia coli* ATCC25922, *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853 and *Streptococcus mutans* (clinical isolate). Antimicrobial activity test was carried out according to the CLSI laboratory standard for broth microdilution assays (CLSI, 2012). For this purpose, antibiotic stock solutions were prepared for the antibiotic test and serial dilutions were made in separate tubes from here. An overnight culture on a nonselective medium such as bloody agar was used in the experiment. A standard

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inoculum of 0.5 McFarland units (108 colony forming units/µL) was prepared in MRS broth. It has diluted 1:30 (5x106 CFU/mL) and 50 µL (2.5x105 CFU/mL) inoculated into each well excluding the control well. Therefore, bacteria inoculum of approximately 2.5x105 CFU/mL was adjusted in a final volume of 100 µL in each well. MRS was used in bacterial suspension and antibiotic dilutions were prepared by DMSO. A well of each plate was used as growth control and a well of each plate was used as a sterilization control and included only broth. The same antibiotics stock solutions and dilutions of the same antibiotics were analyzed on appropriate ATCC strains in another plate for quality control of the experiment. The plates were incubated at 35-37 °C for 24 hours in a normal atmosphere. Ampicillin (10.000 µg/mL), fluconazole (5.000 µg/mL), and streptomycin (10.000 µg/mL) were used as standard antibacterial and antifungal. When MIC values of quality control strains were appropriate and the results of the growth control, the sterilization control, the inoculum purify control, and the inoculum density control was valid, the plates were read by a microplate reader in 600 nm. The results obtained are calculated as % inhibition.

Statistical analysis

The obtained essential oils were tested for antioxidant and antimicrobial activities. A statistical package (SPSS version 20.0) was used for data analysis. The statistical analysis was performed using a one-way ANOVA (p<0.05). Each biological activity test was done twice in duplicate and the results are expressed as mean ± standart deviation (SD).

Results and Discussion

The essential oil compositions of *E. angustirete* and *I. alopecuroides* are listed in Table 1. As shown, twenty-two components, representing almost 95.7% of the essential oil of *E. angustirete*, were characterized. The major components were: pinocarveol (23.0%), myrtenal (14.1%) and verbenone (7.3%). Üçüncü et al (2010) reported that *Tortula muralis* growing in the Gümüşhane region in Turkey contained pinocarveol (0.6%), myrtenal (0.7%) and verbenol (0.8%). On the other hand, it was reported that *Pseudoscleropodium purum* growing in Artvin, Turkey contained pinocarveol (0.6%) and myrtenal (0.8%) (Tosun et al., 2015).

Thirty-one components were identified for *I. alopecuroides* (Table 1), representing almost 94.3% of total oils. The main components were determined as biformene (9.9%), *α*-pinene (9.1%), bornyl acetate (8.4%), respectively. Biformene, a labdane-type diterpene was reported in *Bazzania francana* which is a moss-like plant (Metoyer et al., 2018). It is known that the labdane diterpenes have been shown to possess cardiovascular effects, anti-fungal activity, and anti-inflammatory and cytotoxic effects (Singh et al., 1999; Demetzos et al., 2001; Lahlou et al., 2007). Also, 1-Heptatriacontanol (1.6%) was detected in essential oil of *I. alopecuroides*. 1-Heptatriacontanol an alcoholic compound exhibit anti-hypercholesterolemic, antioxidant, anti-inflammatory, anticancer and antimicrobial effect (Baskaran et al., 2015; Kalaimagal, 2019).

Table 1. Chemical composition of essential oils of mosses

Retention time[a]	Compounds	A %	B %	$RI^{[a]}_{LİT}$	$RI^{[b]}_{EXP}$
	Monoterpenes				
6.66	Tricyclene	-	1.2	927	931
6.90	*α*-pinene	4.3	9.1	932	935
7.36	Camphene	-	4.1	956	961
8.07	Sabinene	-	3.0	975	977
8.30	*β*-pinene	6.5	5.0	979	982

9.78	Limonene	-	1.2	1030	1035
10.03	*Z*-*β*-Ocimene	-	1.7	1037	1042
10.25	*E*-*β*-Ocimene	3.1	7.4	1050	1050
11.94	Terpinolene	0.6	-	1089	1090
12.59	Linalool	0.8	-	1091	1095
	Oxyganeted monoterpenes				
14.69	*trans*-Pinocarveol	23.0	-	1135	1140
14.71	*trans*-Limonene oxide	1.4	1.8	1142	1141
18.24	*trans*-Carveol	1.5	-	1217	1220
	Oxygenated monoterpenes related				
15.48	Citronellal	0.9	-	1153	1155
15.72	Pinocarvone	3.6	1.3	1165	1162
15.87	Borneol	2.4	2.0	1166	1167
16.37	Terpinen-4-ol	5.9	1.1	1177	1178
16.96	*α*-Terpineol	2.2	1.1	1186	1192
17.17	Myrtenal	14.1	1.7	1196	1195
17.75	Verbenone	7.3	3.0	1207	1205
21.08	Bornyl acetate	1.3	8.4	1289	1291
26.63	*α*-Ionone	-	0.9	1430	1432
28.47	*β*-Ionone	0.4	1.5	1487	1492
	Sesquiterpenes				
24.80	*α*-Copaene	0.7	1.9	1375	1372
26.35	Trans-caryophyllene	-	1.2	1418	1420
27.10	*Z*-*β*-Farnesene	0.5	-	1443	1445
27.48	*α*-Humulene	-	1.0	1452	1456
	Oxyganeted sesquiterpenes				
31.22	Caryophyllene oxide	1.9	5.4	1582	1585
32.83	*α*-Cadinol	-	1.3	1652	1650
	Oxygenated sesquiterpenoids related				
36.06	Hexahydrofarnesyl acetone	-	5.2	1844	1848
	Diterpenes				
41.53	Biformene	-	9.9	2026	2030
	Carboxyllic acids				
37.23	Pentadecanoic acid	-	2.6	1871	1875
39.10	*cis*-13-eicosenoic acid	-	1.5	1915	1918
	Others				
12.55	2-Nonanone	-	2.3	1090	1093
13.28	Nonanal		2.5	1100	1101
14.90	(2*E*,6*Z*)-Nonadienal	13.3	2.4	1153	1148
42.37	1-Heptatriacontanol	-	1.6	2303	2300
	Monoterpenes	15.3	32.7		
	Oxygenated Monoterpenes	25.9	1.8		
	Oxygenated Monoterpenes related	38.1	21		
	Sesquiterpenes	1.2	4.1		
	Oxygenated Sesquiterpenes	1.9	6.7		
	Oxygenated Sesquiterpenes related	-	5.2		
	Diterpene	-	9.9		
	Carboxyllic acid	-	4.1		
	Others	13.3	8.8		
	Total	**95.7**	**94.3**		

[a]Percentages obtained by FID peak-area normalization. [b]RI calculated from retention times relative to that of *n*-alkanes (C_7-C_{30}) on the non-polar Rxi-5MS column. A: *E. angustirete*, B: *I. alopecuroides.*

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Antioxidant activity of essential oils

Metals such as iron and copper prevent redox reactions that can occur in the biological system. Excessive accumulation of these metals causes many diseases such as DNA damage, lipid peroxidation and protein modification (Jomova and Valko, 2011). The chelation of redox-active metals prevents oxidative damage by preventing a redox reaction from occurring. In this study, maximum metal ion chelating activity was observed in *E. angustirete* and *I. alopecuroides* as the value of 31.01±2.75 and 65.93±0.39, respectively (Table 2).

The DPPH• method was performed by converting free radical DPPH to non-radical DPPH-H in the presence of a hydrogen-donating antioxidant and measuring the concentration of this compound at 517 nm. Both species represented similar activity to reduce DPPH• (Table 2). DPPH• activity was determined as value of 16.88±6.98 and 22.84±8.91, respectively. ± values indicate the standard deviation. As a result of Anova test, no statistical significance was observed between samples in metal chelate data and DPPH• data (p<0.953).

Table 2. DPPH• and metal ion-chelating activities of essential oils obtained from *E. angustirete and I. alopecuroides* (Inhibition% ± SD)

Samples	% DPPH radical scavenging[a]	% Metal ion-chelating[b]
E. angustirete	16.88±6.98	31.01±2.75
I. alopecuroides	22.84±8.91	65.93±0.39
BHT	94.69±0.04	-
Trolox	90.57± 0.06	-
EDTA		97.06± 0.01

[a]Values are given as mean±S.D. (n= 3), and there is no significantly different at p<0.05. [b]Values are given as mean±S.D. (n= 3), and considered to be significantly different at p<0.05. BHT, Trolox and EDTA antioxidant standards

Figure 1. Metal chelating activity of essential oil

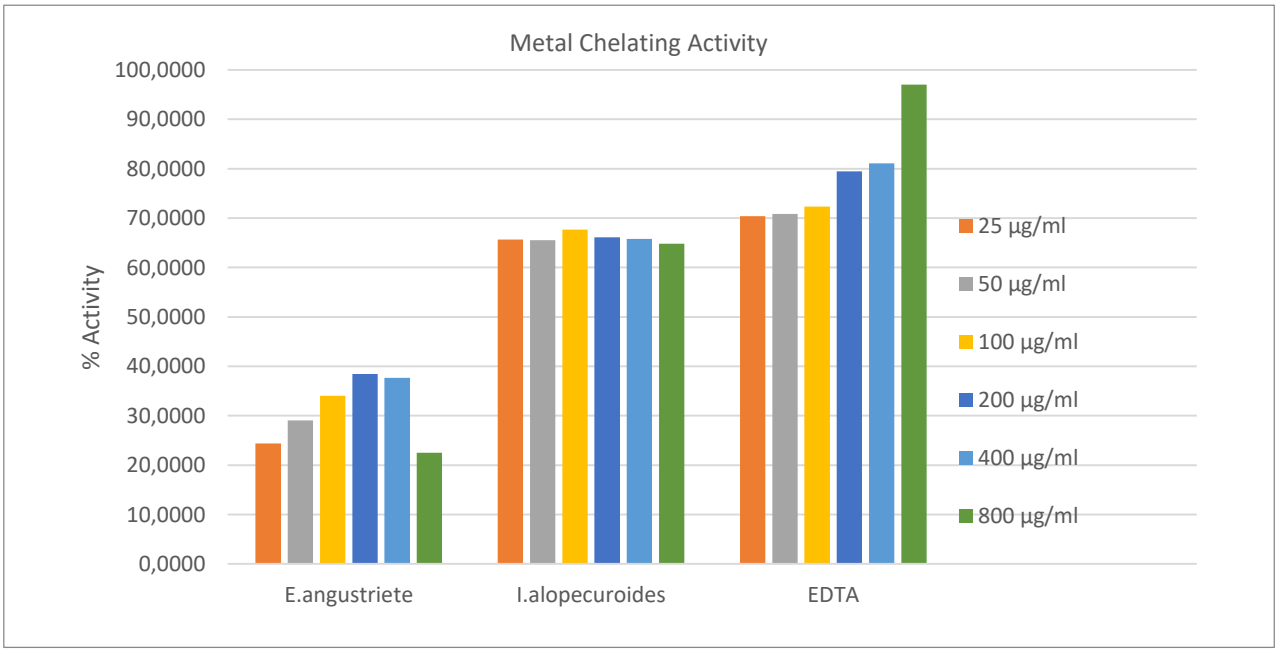

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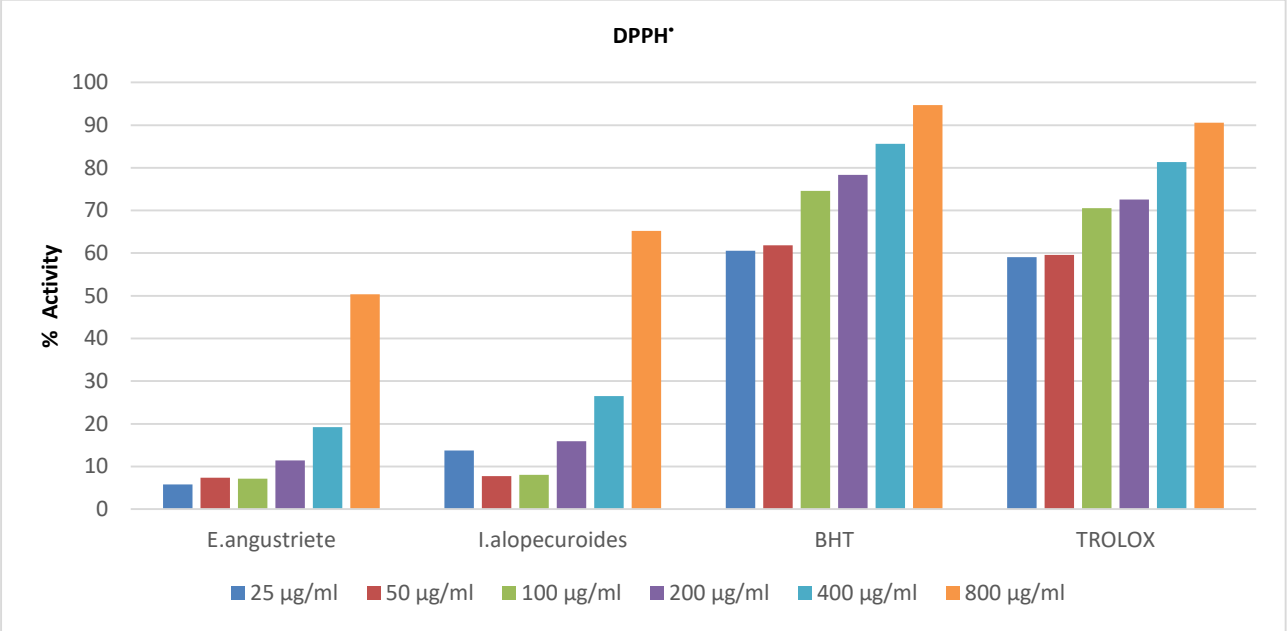
Figure 2. DPPH• activity of essential oil



Antimicrobial activity of essential oils

The essential oils showed moderate antibacterial activity against 3 gram-positive (*S. aureus*, *E. faecalis* and *S. mutans*) and 2 gram-negative bacteria (*E. coli* and *P. aeruginosa*). The % inhibition values of essential oils from five species against determined in a broth microdilution assay, as shown in Table 3. According to the results, essential oils from each species showed a potent inhibitory effect on the growth of microorganisms without significant differences between groups.

Table 3. Minimal inhibitory concentrations (µg/mL) of essential oils against.

Microorganisms	Sample	% İnhibition[a]
E. coli ATCC 25922	*E. angustirete*	95.36±0.14
	I. alopecuroides	94.26±0.03
E. faecalis ATCC 29212	*E. angustirete*	94.23±0.15
	I. alopecuroides	94.15±0.04
S. mutans	*E. angustirete*	95.43±0.06
	I. alopecuroides	94.25±0.05
P. aeruginosa ATCC 27853	*E. angustirete*	95.38±0.08
	I. alopecuroides	95.21±0.03
S. aureus ATCC 25923	*E. angustirete*	91.19±0.10
	I. alopecuroides	90.76±0.03

[a]Values are given as mean±S.D. (n= 3) and considered to be significantly different at $p<0.05$.

Conclusion

The distillation method was developed in Spain and France at the beginning of the 1300s to obtain essential oil, and in the 1550s, new techniques were started to be applied in order to meet the needs of different branches such as pharmacology (Rangahau, 2001). Today, it is applied in modern methods using advanced technology as well as classical distillation methods. These methods include classical methods such as water distillation and solvent extraction and pressing, as well as modern methods such as microwave extraction

and solid-phase microextraction (Kılıç, 2008). In this study essential oils were obtained from these moss species using the hydro-distillation method. Essential oils are very complex natural mixtures that contain many ingredients in different concentrations.

In this study, it was observed that the essential oil of *I. alopecuroides* represented highly inhibitory effects on microorganism. According to antioxidant activity results, it's DPPH activity and metal chelating capacity values are higher than essential oil of *E. angustirete* (22.84±8.91 and 65.93±0.39, respectively). Biformene exhibits antioxidant properties (Öztürk et al., 2009). Also, according to the antimicrobial activity tests findings, there was no significant difference within moss materials as shown in Table 3 in the inhibition effect against microorganisms.

In a study by Altuner and Çetin (2018), antimicrobial activity chloroform, benzene, diethyl ether, ethanol, methanol, ethyl acetate, sdH2O and 0.5 M Tris-HCl buffer (pH: 8.0) of *I. alopecuroides* extracts were tested against *Bacillus subtilis* ATCC 6633*, Candida albicans* ATCC 95071*, Escherichia coli* ATCC 11230*, Escherichia coli* O157:H7*, Listeria monocytogenes* ATCC 7644*, Salmonella enteretidis* ATCC 13076*, Shigella flexneri* (clinical isolate), *Staphylococcus aureus* ATCC 25923 and *Yersinia enterocolitica* O3 by disk diffusion, minimum inhibitory concentration test and minimum bactericidal/fungicial concentration tests. According to this study, benzene extract of a sample collected from Aydın was only active against *B. subtilis*, but the sample collected from Muğla was active against *B. subtilis, S. aureus, E. coli* and *C. albicans* with its different extracts at different ranges. The essential oil of *I. alopecuroides* was determined as a good antimicrobial activity against all microorganisms especially *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922.

In another study, methanol extract of *I. alopecuroides*, was shown to have an effective antimicrobial effect on *P. larve* isolates (Sevim et al., 2017). Previous studies confirm the high antimicrobial activity values that this species showed in our study.

Also, Altuner (2008) investigated the effect on the antimicrobial activity of moss variety as the third variable in Ph.D thesis study, he found that 32 of 113 moss extracts were bactericidal, 27 were fungicidal, 25 were fungistatic, and 24 were bacteriostatic. Among the studied 20 moss species, he observed that *I. alopecuroides* showed the highest antimicrobial effect. The obtained moss extracts had different effect on microorganisms. The microorganisms least affected by moss extracts were *Listeria monocytogenes* and *E. coli*.

The essential oil of *I. alopecuroides* contained biformene-diterpene as a major component (Table 1). Diterpenes compounds such as pimaradienes, *ortho*- or *para*-quinone and pyhtol have many biological effects for example anticancer, antioxidant, and anti-inflammatory (Bisio et al., 2019; Costa et al., 2014, Reveglia et al., 2018).

Oxygenated monoterpenes consist of a 10 carbon backbone (2 isoprene units) structure. These compounds divided into three classes as aliphatic (myrcene, linalool), monocylic (limonene, carvone, menthol) and bicyclic (thujene, Δ^3-carene) oxygenated monoterpenes. They are found in many plants and show many biological effects such as antibacterial, antiviral, antimicrobial and anticandidal effects (İşcan, 2017; Hosseinkhani et al., 2015; Taylor et al., 2020). *trans*-pinocarveol (23.0%,-) and limonene oxide (1.4%, 1.8%) as oxygenated monoterpenes and *α*-pinene (4.3%, 9.1%), myrtenal (14.1%, 1.7%) monoterpene compounds are major components of essential oils of *I. alopecuroides* (i) and *E. angustirete* (ii), respectively. In our study, the high antimicrobial effect of the two species containing these oxygenated monoterpenes supports the literature. In addition, the metal ion chelating capacity of the essential oils of *E. angustirete* (31.01%)

and *I. alopecuroides* (65.93%), and were high supporting the antioxidant properties of oxygenated monoterpenes in the literature.

Nikolajeva et al. (2012) studied the antimicrobial activity of aqueous and ethanolic extracts of 11 Bryophyta species including *Eurhynchium angustirete* (Broth.) T. J. Kop. and 9 Marchantiophyta species collected in Latvia were tested against *Staphylococcus aureus* MSCL 334, *Escherichia coli* MSCL 332 and *Bacillus cereus* MSCL 330. The most comprehensive researches were conducted on the effect of water and ethanol extracts of bryophytes on the growth of *Staphylococcus aureus*. Minimal inhibitory concentration of ethanolic extracts in the indicated interval of concentration of *Eurhynchium angustirete* was found 10-30 against *Staphylococcus aureus*. In this study, antimicrobial activity of the essential oil of *Eurhynchium angustirete* was found against *S. aureus* lower than that against *P. aeruginosa, E. coli, E. faecalis* and *S. mutans.*

In another study, Tosun et al. characterized the essential oil content of *E. angustirete* grown Artvin, Turkey, and studied its antimicrobial properties (Tosun et al*.,* 2015). According to the results of the chemical composition, hydrocarbons (81.6%) and monoterpenes (18.3%) were the major groups in the essential oil and moderate antimicrobial activity against the bacteria *Y. pseudotuberculosis*, *P. aeruginosa*, *S. aureus*, *E. faecalis*, *B. cereus* and *M. smegmatis* were observed. In our study, the essential oil of this species contained less monoterpene (15.30%) and more oxygenated monoterpenes (25.9%) and represented highly antimicrobial activity against *P. aeruginosa, S. aureus and E. faecalis.*

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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RESEARCH ARTICLE

Chemical composition and antibacterial activity of *Clinopodium nepeta* subsp. *glandulosum* (Req.) Govaerts essential oil

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Abstract

Clinopodium nepeta subsp. *glandulosum* (Req.) Govaerts is a natural perennial grass belonging to the Lamiaceae family, growing spontaneously on the Mediterranean coast. It is locally known as "kedi fesleğeni" in Turkey. This aromatic plant is used as a mint-like spice in food preparations and in the composition of some recipes during religious feasts. In folk medicine, it is used as stimulant, antiseptic and antispasmodic. Aerial parts of *C. nepeta* subsp. *glandulosum* were collected from Bilecik in July 2017, and was air dried. The essential oil was isolated by hydrodistillation using a Clevenger-type apparatus and the composition of the essential oil was simultaneously analysed by GC-FID and GC-MS. Piperitenone oxide (47.8%), limonene (18.6%) and piperitone oxide II (13.6%) were found as major components. Antibacterial activity of the essential oil was tested against *Escherichia coli* NRRLB-3008, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, *Bacillus cereus* NRRL-B3711 and *Streptococcus sanguinis* ATCC 10556 were used by broth microdilution method. Ciprofloxacin was used as control and Minimal Inhibitory Concentrations (MIC) were determined. The highest activity was found against *S. typhimurium* (1250 µg/mL). The essential oil is more effective against *B. cereus* (2500 µg/mL) and *S. sanguinis* (2500 µg/mL). The lowest activities were determined against *E. coli* (5000 µg/mL) and *P. aeruginosa* (10000 µg/mL).

Keywords: Lamiaceae, *Clinopodium nepeta* subsp. *glandulosum*, essential oil, chemical composition, antibacterial activity

Introduction

The genus *Clinopodium* (Lamiaceae) is represented by 39 taxa (Govaerts, 1999, Conforti et al., 2012). *Clinopodium* species are used in folk medicine like mints, mainly as stimulant, digestive, tonic, antiseptic (Baytop, 1999). *Clinopodium nepeta* subsp. *glandulosum (Req.)* Govaerts (syn. *Calamintha nepeta* subsp. *glandulosa*) is a perennial aromatic plant widespread in the Mediterranean region. *C. nepeta* occurs in Southern, Western and Southern Central Europe from South-eastern England to Crimea, Caucasus and Turkey, and is naturalised in North America (Conforti et al., 2012). This aromatical plant is effective as an antiseptic, antispasmodic and tonic. The essential oil of plant has antimicrobial, antifungal, antispasmodic, antioxidant and antitumor activities (Brankovic et al., 2009; Debbabi et al., 2020; Dzhambazov et al., 2002; Sarac & Uğur, 2009; Tepe et al., 2007).

Microorganisms develop resistance to antibiotics due to the unconscious and inappropriate use of antibiotics resulting in the difficult treatment of infectious diseases. Especially, strains of *Streptococcus sanguinis, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella typhimurium* are among the resistant pathogens to various antibiotics. *P. aeruginosa* is an ubiquitously distributed opportunistic Gram-negative pathogen that inhabits soil fresh water, marine environments, colonizing plant, animal, and human hosts. It is an opportunistic pathogen that is naturally resistant to many antibiotics. It is one of the causes of hospital infections (Goldberg and Pier, 2000). *P. aeruginosa* is responsible for ventilator-associated pneumonia, contact lens keratitis, otitis externa, cystic fibrosis (Wolska & Szweda, 2009). *Salmonella* species are the most common causes of foodborne illness worldwide and *S. typhimurium* can cause infection in humans. It is a Gram-negative, facultative anaerobe bacterium and the leading cause of gastroenteritis

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(Patricia et al.,1986). *E. coli* is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans (Jang et al., 2017). *S. sanguinis* is an indigenous Gram-positive bacterium that has been recognized for a long time as a key player in colonization of the human oral cavity. *S. sanguinis* is considered a benign, or even a beneficial, bacterium with regard to dental caries (Kreth et al., 2005). *B. cereus*, Gram-positive, an ubiquitous organism, commonly found in soil, hay, live trees, and other plant material, raw and processed foods, post-operative wound infections, local infections with necrosis, intravenous drug abuse, alcoholism, osteomyelitis and trauma caused by *B. cereus* (Kotiranta et al. 2000). Diseases caused by multidrug resistant microorganisms can be treated by plant sources, especially with the antimicrobial activity of essential oils.

In this study, essential oil obtained from aerial parts of *C. nepeta* subsp. *glandulosum* was evaluated for its *in vitro* antibacterial properties against food and human pathogenic standard bacterial strains. Besides, the volatile components of the essential oil were also investigated.

Materials and Methods

Materials

Aerial parts of *C. nepeta* subsp. *glandulosum* were collected from Bilecik in July 2017, herbarium samples were prepared. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy in Ankara University with the herbarium number of AEF 27033 and was air dried. The essential oil was isolated by hydrodistillation for 3 h using a Clevenger-type apparatus. Antibacterial activities of the *C. nepeta* subsp. *glandulosum* essential oil against *Escherichia coli* NRRL B-3008 (Agricultural Research Service Culture Collection), *Bacillus cereus* NRRL B-3711, *Pseudomonas aeruginosa* ATCC 27853 (American Type Culture Collection), *Salmonella typhimurium* ATCC 13311, and *Streptococcus sanguinis* ATCC 10556 strains were screened. Standard antibiotic ciprofloxacin and resazurin were acquired from Sigma-Aldrich.

GC-FID and GC-MS analyses

Gas Chromatography-Flame Ionization Detection (GC-FID) and Gas Chromatography-Mass Spectrometry (GC-MS) analyses processes were performed with reference to Demirci et al. (2008).

Identification of the components

Identification of the essential oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder Software 4.0) and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils.

Antibacterial activity

The antibacterial activity of the essential oil was evaluated by broth microdilution assay according to a modified Clinical and Laboratory Standards Institute (CLSI) method (CLSI, 2006). *Escherichia coli* NRRL B-3008, *Bacillus cereus* NRRL-B3711, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, and *Streptococcus sanguinis* ATCC 10556 were used as test microorganisms. Minimal Inhibitory Concentrations (MIC) of the samples were determined and given in Table 2, where ciprofloxacin was used as a positive control in the experiments. The tests were carried out in 96-well micro plates. The sample (100 µL per well) was diluted two-fold, with a final concentration range of 5000 to 9.76 µg/mL, respectively. Standard antibacterial agent ciprofloxacin (64 to 0.125 µg/mL) was used under the same conditions as positive

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controls. Strains were incubated in Mueller Hinton Broth (MHB) overnight at 37°C for 24h. Cultures, with a final inoculum size of 1 x 10^6 colonies forming units (CFU/mL) were used. Microbial growth was observed by adding 20 µL of resazurin of 0.01% with minor modifications of CLSI standards (Pfaller et al., 2008). A change from blue to pink indicated the reduction of resazurin and, therefore, microbial growth. The minimal inhibitory concentration (MIC) was determined as the lowest drug concentration that prevented the colour change. All experiments were repeated in triplicate, and average results were reported.

Results and Discussion

GC-FID and GC-MS analyses

The greenish-yellow essential oil was analysed by GC-FID and GC-MS, simultaneously to determine its chemical characterization. Piperitenone oxide (47.8%), limonene (18.6%) and piperitone oxide II (13.6%) were found as major components of the essential oil. Other components are given in Table 1.

Table 1. Volatile components of *Clinopodium nepeta* subsp. *glandulosum* essential oil

[a]RRI	Component	%
1018	Methyl-2-methyl butyrate	0.1
1032	*α*-Pinene	1.0
1035	*α*-Thujene	0.1
1063	Ethyl-2-methyl butyrate	[b]tr
1076	Camphene	0.1
1118	*β*-Pinene	1.0
1132	Sabinene	0.6
1174	Myrcene	0.9
1203	Limonene	18.6
1213	1,8-Cineole	0.4
1246	(*Z*)-*β*-Ocimene	tr
1255	*γ*-Terpinene	tr
1280	*p*-Cymene	tr
1393	3-Octanol	1.3
1474	*trans*-Sabinene hydrate	1.0
1494	(*Z*)-3-Hexyl-2-methyl butyrate	0.1
1497	*α*-Copaene	0.1
1535	*β*-Bourbonene	0.3
1532	Camphor	0.2
1553	Linalool	1.0
1556	*cis*-Sabinene hydrate	0.2
1571	*trans*-*p*-Ment-2-en-1-ol	tr
1590	Bornyl acetate	0.2
1612	*β*-Caryophyllene	0.9
1611	Terpinen-4-ol	1.1
1639	*trans*-*p*-Mentha-2,8-dien-1-ol	0.2
1668	(*Z*)-*β*-Farnesene	0.2
1678	*cis*-*p*-Mentha-2,8-dien-1-ol	0.1
1706	*α*-Terpineol	0.6

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1719	Borneol	0.2
1726	Germacrene D	0.6
1733	Piperitone oxide I	0.3
1755	Piperitone oxide II	13.6
1751	Carvone	0.3
1849	Calamenene	0.3
1864	*p*-Cymen-8-ol	0.1
1864	Isopiperitonone	0.6
1880	Benzyl-2-methyl-butyrate	0.2
1949	Piperitenone	0.6
1983	Piperitenone oxide	47.8
2006	8,9-Dehydrothymol	0.2
2016	4aβ, 7α, 7aα Nepatalactone	0.4
2069	4aα, 7α, 7aβ Nepatalactone	2.5
2198	Thymol	tr
	Total	**98.0**

[a]RRI: Relative retention indices calculated against *n*-alkanes, %: calculated from FID data, [b]tr: trace <0.1%

In a previous study of our group, forty-five components representing 91.65% of the oil were identified, piperitenone oxide (43.8%), *trans*-piperitone oxide (25.2%), and limonene (13.0%) were found as the major components *C. nepeta* essential oil (Kirimer et al, 1992). Marongiu et al. (2010) were found pulegone, piperitenone oxide and piperitenone as the main components (64.4–39.9%; 2.5–19.1%; 6.4–7.7%); conversely, the oil distilled from aerial parts of Portuguese *C. nepeta* is predominantly composed of isomenthone (35.8– 51.3%), 1,8-cineole (21.1–21.4%) and *trans*-isopulegone (7.8–6.0%) (Marongiu et al., 2010). Another study reported that, pulegone (40.5-54%) and menthone (23.6-16%) were found as the main components *C. nepeta* essential oil, respectively (Şarer & Pançalı, 1998; Demirci et al. 2011). Kitic et al. (2002) determined that pulegone (37.5%), menthone (17,6%), piperitenone (15.0%) and piperitone were as the main constituents in the oil (Kitic et al., 2002).

Antibacterial activity

The potential *in vitro* antibacterial activity of the essential oil was tested against *Escherichia coli* NRRLB-3008, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, *Bacillus cereus* NRRL-B3711 and *Streptococcus sanguinis* ATCC 10556 were used by broth microdilution method. Ciprofloxacin was used as control and Minimal Inhibitory Concentrations (MIC) were determined. The essential oil is more effective against *B. cereus* (2500 µg/mL), *S. sanguinis* (2500 µg/mL) and *S. typhimurium* (1250 µg/mL). The lowest activities were determined against *E. coli* (5000 µg/mL) and *P. aeruginosa* (10000 µg/mL). The results were given in Table 2.

Table 2. Minimal inhibitory concentration values (MIC) (µg/mL)

Bacteria	***C. nepeta* subsp. *glandulosum* essential oil**	**Ciprofloxacin**
Escherichia coli NRRLB-3008	5000	30
Pseudomonas aeruginosa ATCC 27853	10000	30
Salmonella typhimurium ATCC 13311	1250	30
Bacillus cereus NRRL-B3711	2500	<10
Streptococcus sanguinis ATCC 10556	2500	<10

Numerous studies exist on the *C. nepeta* essential oil antibacterial activity, where varying amounts of the tested pathogens were found to be susceptible to the oil were reported (Arantes et al., 2019; Miladinovic et al., 2012; Kitic et al., 2002). In previous study, MIC values of *C. nepeta* essential oil were determined against *S. aureus* (>2.0 µL/mL), *E. coli* (1.0 µL/mL), *S. typhimurium* (>2.0 µL/mL) and *P. aeruginosa* (1.0 µL/mL) (Arantes et al., 2019). According to other study, essential oil from *C. nepeta* have antimicrobial activity with a range of MIC values from 0.025 to 1.56 µL/mL (Miladinovic et al., 2012). In another study, antimicrobial activity of *C. nepeta* essential oil was determined by disk diffusion method. All tested microorganisms were found to be susceptible to essential oil (Kitic et al., 2002).

Conclusion

In the present study, piperitenone oxide (47.8%), limonene (18.6%) and piperitone oxide II (13.6%) were found as major components of the essential oil. Also, the essential oil of *C. nepeta* subsp*. glandulosum* showed different levels of activities against *E. coli*, *P. aeruginosa*, *S. typhimurium*, *B. cereus* and *S. sanguinis*. It was shown more effective against *B. cereus* (2500 µg/mL), *S. sanguinis* (2500 µg/mL) and *S. typhimurium* (1250 µg/mL). The lowest activities were determined against *E. coli* (5000 µg/mL) and *P. aeruginosa* (10000 µg/mL). More in detail evaluations on biological activity both on *in vitro* and *in vivo* levels are needed to exhaust the potential of essential oil from *C. nepeta*. Further work is ongoing.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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RESEARCH ARTICLE

Characterization of seasonal and chemotypical variability in the essential oil from leaves of *Annona neosalicifolia* H. Rainer (Annonaceae)

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Abstract

The essential oil (EO) from leaves of *Annona neosalicifolia* H. Rainer (Annonaceae) obtained from two chemotypes in the southern Brazilian Atlantic Rainforest was analysed both by GC-FID and GC-MS. Forty compounds were identified, accounting for 95.9 to 99.5% of the total oil in each sample. The VOs were characterized by the predominance of sesquiterpenes in all samples, being main compounds bicyclogermacrene (13.9-41.8%) and beta-caryophyllene (9.3-26.3%); germacrene D was also found in high amounts, but its concentration was more variable. Monoterpene compounds were absent in the summer and fall samples, with higher concentrations in winter samples (6.1-7.4% for α-pinene and 6.4-11.2% for β-pinene). EO from chemotype II was characterized by a high concentration of the sesquiterpene alcohol (*E*)-nerolidol (36.3%), which was absent or in trace amounts in chemotype I. The characterization of these seasonal and individual variations in the chemical composition of the essential oil from *A. neosalicifolia* may have agronomical implications and may be important in the understanding and delimitation of the metabolic plasticity of the highly variable taxon *A. emarginata* as well.

Keywords: Annonaceae, *Annona neosalicifolia*, Essential Oil, Seasonal Variation, (*E*)-nerolidol

Introduction

The Annonaceae family of plants comprises about 120 genera and approximately 2300 mainly forest species around the world, twenty-nine genera comprising ca 260 species in Brazil (Barroso et al., 1978; Maas et al., 2001). Previous reports have identified species from this family as a source for terpenic compounds, specially diterpenes (de Andrade et al., 2004; Moreira et al., 2003), mono- and sesquiterpene rich essential oils (Tairu et al., 1999), isoquinoline alkaloids (Fechine et al., 2002; Jumana et al., 2000; Lima et al., 2004; Moreira et al., 2003), steroids, polyphenols, flavonoids (Moreira et al., 2003), fatty acids and acetogenins (de Andrade et al., 2004; Pontes et al., 2004). The acetogenins uvariamicin-I, solamine, and goniogenin were isolated from the hexane extract from leaves of *Rollinia laurifolia* (Nascimento et al., 2003).

Volatiles were reported from Annonaceae species, especially from *Cananga odorata* (Ylang-Ylang) and edible species in the genus *Annona* (Campos et al., 2014; Felipe G Campos et al., 2019a, Felipe G Campos et al., 2019b; Jirovetz et al., 1998; Pino, 2000; Thang et al., 2013; Wyllie et al., 1987). The most commonly reported volatiles were terpenic in origin: α-pinene, β-pinene, limonene, β-caryophyllene, *p*-cymene, and caryophyllene oxide, which are found in many oils and, therefore, have no special taxonomic value. Other compounds, however, seem to be restricted to some taxa in the family: argentilactone in *Annona haematantha*; the sesquiterpene 1,5-epoxysalvyal-4(14)-ene, with the rare isodaucane nucleus, in *Artabotrys*

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species; and the aromatic compound 2,4,5-trimetoxystyrene in *Pachypodanthium staudii* (Founder et al., 1997; Fournier et al., 1999). Previously containing *ca*. 65 South American, Caribbean, and Mexican species, the genus *Rollinia* was merged into *Annona* (Leboeuf et al., 1980; Rainer, 2006). From the (former) *Rollinia* genus, the fruits of *R. mucosa* have had their volatile chemical constituents described, α-pinene, β-pinene, and β-caryophyllene being the main compounds (Pino, 2000), while in *Rollinia leptopetala cis*-4-thujanol (17.37%), α-terpineol (8.42%), germacrene D (7.72%), bicyclogermacrene (22.47%), and (*E*)-caryophyllene (6.63%) were the main volatiles (Costa et al., 2008).

Annona neosalicifolia H. Rainer (syn. *Rollinia salicifolia* Schltdl., 'ariticunzeiro') is a 7-20-meters tall tree native to Rio Grande do Sul state (RS), Southern Brazil, and Northeast Argentina, on the southern range of the Atlantic Forest. The species produces edible fruits which are consumed *in natura* (Brack et al., 2020) and commercialized in street markets in southern Brazil. In folk medicine, the decoction of stem barks from *A. neosalicifolia* are used in the treatment of conjunctivitis; infusion of leaves are used as 'detoxifying agent'; and seed decoctions are used for killing *Pediculus humanus capitis*. Alkaloids, triterpenes, steroids, anthraquinones, anthocyanins, and essential oils were characterized in a phytochemical screen of leaves from this species (Walker et al., 2003).

Some authors synonymize *A. neosalicifolia* in *A. emarginata* (Maas e tal., 1992), whose variety "terra-fria" is extensively used as a rootstock for the cultivation of the economically promising atemoya fruit (*A. x atemoya*) in southeastern Brazil. The volatile chemistry of that variety was studied and compared with that of another *A. emarginata* variety not suitable as rootsock under the premise that volatiles may be important for the success of the transplant activity (Campos et al., 2014; Campos, Vieira, Baron, et al., 2019; Campos, Vieira, Santos, et al., 2019; Mimi, 2019).

Considering that the chemical composition of essential oils can vary significantly as a function of climatic conditions, vegetative stage, and a specimen genetic constitution, this work aims to describe for the first time the chemical composition of the leaf VOs from two chemotypes of *Annona neosalicifolia* accounting also for seasonal patterns.

Materials and Methods

Plant material

Leaves of *Annona neosalicifolia* H.Rainer were sampled from two chemotypes, in the municipality of São José das Missões, Rio Grande do Sul State, Brazil, in January, March, and July (2005) for chemotype I and July (2005) for chemotype II. Voucher material (SMDB 10.224) was deposited at the Herbarium of the Department of Biology of Universidade Federal de Santa Maria UFSM, Santa Maria–Brazil.

Extraction of the essential oils

The EO from leaves was obtained by hydrodistillation for 4 hours in a Clevenger-type apparatus. The essential oil percentage yield (%) was calculated as volume of oil and the weight of fresh material (w/v). The oil was recovered in ethyl ether and stored under refrigeration after removal of the solvent.

Analysis of the essential oils

The EO from each sample was submitted to GC-FID and GC/MS. GC-FID analysis was performed in a Shimadzu GC17-A system with a DB-5 capillary fused silica column (25m x 0.25mm i.d., 0.25 µm film thickness). Injector and detector temperatures were 220 ºC and 250 ºC, respectively. Column temperature was programmed from 60 ºC to 300 ºC at 3 ºC/min. Helium was used as carrier gas at 80 kPa and 1 mL/min. Split 1:20. Nitrogen,

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synthetic air, and hydrogen (1:1:10) as auxiliary gases. The percentage compositions of the individual components were obtained from electronic integration measurements using flame ionization detection (FID, 250°C). GC/MS analysis was performed the same chromatographic system, with a Shimadzu QP-5000-Quadrupole MS detector, operating with 70 eV ionization energy. Helium as carrier gas, 1 ml/min flow, split. Spectra were recorded between 30 and 400 *m/z*. Compounds were identified by comparison of Retention Index (determined using C9-C22 alkanes) and mass spectra with data from the NIST Mass Spectral Library (National Institute of Standards and Technology) or with spectral data from the literature (Adams, 2007).

Multivariate analysis

Percentage data missing or reported as traces was inputted using the multRepl() function of the R package zCompostions (Palarea-Albaladejo and Martín-Fernández, 2015) and PCA was performed with the option logratio 'CLR' of the function pca() in the R package mixOmics (Rohart et al., 2017).

Results and Discussion

A total of 40 chemical compounds were identified in the EO from the leaves of *A. neosalicifolia*. 95.9 to 99.5% of the total components of each oil were identified and the yield of oil obtained ranged between 0.1 and 0.16% in the different samples as in Table 1. Overall, oils from this species were characterized by the predominance of sesquiterpenes. The hydrocarbon sesquiterpenes β-caryophyllene (9.3-26.3%) and bicyclogermacrene (13.9-41.8%) were among the main compounds in all samples; germacrene D was found in expressive amounts, too, in all samples from the chemotype I. In the second chemotype, the proportion of this compound was approximately five-fold lesser than those obtained in chemotype I (only 4.5%). It is also remarkable the complete absence of monoterpene compounds in the oils obtained in January (Summer) and March (Fall), the beginning and the end of the fruiting period, respectively. However, monoterpene compounds, mainly the hydrocarbons α-pinene (6.7-7.4%) and β-pinene (6.4-11.2%) were found in relatively high concentrations in the samples obtained during Winter (July), when this species was in a sterile period. Oxygenated terpenes were almost absent, except for the second chemotype, where the concentration of the oxygenated aliphatic sesquiterpene (*E*)-nerolidol was 36.3%, contrasting with 0-0.7% in chemotype I. For a long period between Winter and Spring (August-October) the deciduous species did not exhibit any leaves and no Spring samples were obtained for this study.

The most abundant hydrocarbon sesquiterpene in the essential oil from leaves of *A. neosalicifolia*, considering all analyses performed, was the compound bicyclogermacrene; it is a common compound in some *Xylopia*, *Fitzalania*, and *Uvaria* species (Brophy et al., 2004; Maia et al., 2005), but is rarely found as the main compound in oils from other Annonaceae. Another compound present in a high concentration, β-caryophyllene (and its oxidized derivative caryophyllene oxide) seems to be the more widespread sesquiterpene in this family, having no taxonomic value at the tribal and generic levels (Fournier et al., 1997a, 1997b, 1997c). Germacrene D, also found in high amounts, is a relatively common, easily oxidizable hydrocarbon sesquiterpene that can give origin to several other compounds after rearrangement, most commonly selinane and cadinane derivatives (Bülow and König, 2000). Compositional PCA applying centered logratio, used as an exploratory tool, highlighted the importance of pinenes in the winter samples, the preponderance of (*E*)-nerolidol in the chemotype II and TMTT in both the chemotype II and the Fall sample of chemotype I (Figure 1).

In the second chemotype, the oxygenated sesquiterpene (*E*)-nerolidol was observed as the main compound. It is a known feed deterrent (Doskotch et al., 1980), an attractor for some insects, and also produced by flowers for pollination. In some plants, (*E*)-nerolidol is released after herbivore damage, when it acts as an

attractor for carnivores, predators for the herbivore insects that caused the damage, characterizing tritrophic systems (Kappers et al., 2005). The same compound acts as a precursor for the biosynthesis of the homo-monoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT), that is also released after herbivory. In the same way, the homologous diterpene compound geranyl-linalool can originate the homo-sesquiterpene 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), another important signaler in tritrophic systems (Degenhardt and Gershenzon, 2000; Schnee et al., 2002). Two trace compounds were detected with the mass spectrum and retention times similar to those published for DMNT and TMTT. The presence of (E)-nerolidol, DMNT, and TMTT suggests that the specimens could be under biotic stress and, indeed, some *Annona* species can be attacked by gall coccids, which may alter their metabolism in a manner not yet well understood. These alterations can occur in attacked leaves, where gall formation occurs (Soares et al., 2000), but also in the whole plant, preventing herbivore attack to the healthy leaves. In Annonaceae species, the occurrence of (*E*)-nerolidol is not widespread; it has been identified in some genera, such as *Cleistopholis*, *Dennettia*, *Monodora*, *Xylopia*, and *Pachypodanthium*, but in very low concentration only (Boyom et al., 2003; Brophy et al., 2004).

The essential oil from most Annonaceae species is composed of substances of terpenic origin (mono- and sesquiterpenes). Compounds originated in other metabolic routes can occur, sometimes, such as phenylpropanoids and aliphatic derivatives, especially in *Annona* and *Asimina* species. As described for this family, the chemical composition of the essential oil varies in different organs: hydrocarbon monoterpenes in oils from fruits and seeds; hydrocarbon sesquiterpenes in oils from leaves; and oxygenated sesquiterpenes in oils from the stem and root barks (Fournier et al., 1999). In *A. neosalicifolia*, two factors seem to play a significant role in the chemical compositions of VOs. On one hand, the presence or absence of monoterpenes showed a seasonal dependency. On the other hand, each chemotype showed a very different profile on the sesquiterpene fraction, even under the same edaphoclimatic conditions, pointing to a genetically controlled character.

Descriptions of the chemical composition of essential oils sometimes neglect the importance of seasonal variations for characterizing these products; however, an evaluation of those variations allows a better understanding of the metabolic plasticity of a given species in time. This knowledge, allied to the investigation of different populations would allow also the identification of chemotypes, and it should give the investigator more reliability when performing samplings aimed at a specific metabolite or metabolite ensemble.

The taxonomy of *A. neosalicifolia* is disputed at both generic and specific levels. On the generic level, the previously well-defined *Rollinia* was merged into *Annona* (Rainer, 2006). On the specific level, some confusion exists, too. The section *Rolliniella* in the former genus *Rollinia*, used to be composed of eight taxa: *R. ulei* in the Peruvian Amazon Forest, and *R. salicifolia*, *R. rugulosa*, *R. occidentalis, R. glaziovii*, *R. emarginata*, *R. hassleriana*, and *R. intermedia*, reported to occur in the Atlantic Forest between southern and south-eastern Brazil, north-eastern Argentina, and Paraguay (Záchia, 1994). After a brief synonymization under *R. emarginata sesu lato*, due to the impossibility of differentiation based on purely quantitative morphological traits (Maas et al., 1992), four species were delimited again from the new highly variable Atlantic taxon *R. emarginata s.l.*: *R. emarginata sensu stricto*, *R. salicifolia*, *R. rugulosa* and *R. maritima* (Záchia and Irgang, 1996), finally transferred to *Annona* as *A. emarginata*, *A. neosalicifolia, A. rugulosa*, and *A. maritima*, together with *A. neoulei* based on phylogenetics (Rainer, 2006). The concepts *R. occidentalis* and *R. glaziovii* remained merged in *A. rugulosa* while *R. hassleriana* and *R. intermedia* remained merged in *A. emarginata s.s*. The distribution range of *A. neosalicifolia* borders that of *A. maritima* to the east, in the coastal strip of RS, in southern Brazil, and that of *A. emarginata s.s.* to the west, in Argentina, overlapping with that of *A. rugulosa*

in the western regions of Rio Grande do Sul and Santa Catarina states (Záchia and Irgang, 1996). Even though the four species were resurrected from *A. emarginata s.l.* (Záchia and Irgang, 1996) some authors consider them in the sensu lato (Maas et al., 1992; Mendes-Silva et al., 2020) and as a vulnerable taxon in RS (Rio Grande do Sul, 2014).

The description of two chemotypes in *A. neosalicifolia* has possible agricultural, taxonomical, and ecological implications worth being further studied. The agricultural aspect is related to the use of *A. emarginats* s.s. for grafting economically important *Annona* spp. *A. emarginata s.s.* occurs in Argentina, Paraguay, and south-eastern Brazil, where, in São Paulo, several compositions were reported for two varieties, 'mirim' and 'terra-fria'. The variety *A. emarginata* 'terra-fria', highly resistant to stem borers, pests, and diseases (Kavati and Watanabe, 2010) is economically important because it is the most used rootstock for the cultiation of atemoya (*A. x atemoya*), a plant whose fruit has great economic potential due to its well-accepted organoleptic characters. The two varieties of *A. emarginata s.s.* native in São Paulo (where none of *A. maritima*, *A. neosalicifolia*, or *A. rugulosa* were reported), can be differentiated by their volatiles: myrcene ('terra-fria':1-1% x 'mirim':12-14%), linalool (1-2% x 10-16%), (*E*)-caryophyllene (13-27% x 6-10%), and thujene (3-11% x 1-1%) (Mimi, 2019). Other reports referred (*E*)-caryophyllene (29.29%), (*Z*)-caryophyllene (16.86%), γ-muurolene (7.54%), α-pinene (13.86%), and tricyclene (10.04%) as main volatiles in *A. emarginata* var. 'terra-fria' (Campos et al., 2014) and, in the EO from leaves of the rootstock for grafted *A. x atemoya*, β-selinene (12.63%), α-selinene (12.21%), β-elemene (9.66%), spathulenol (7.25%), α-pinene (5.55%), (*E*)-caryophyllene (5.39%) (Campos, Vieira, Baron, et al., 2019b). The volatile chemical profile of the rootstock may influence the success of grafting, probably due to antioxidant, antiradical, and antibiotic activities (Campos, Vieira, Baron, et al., 2019a,b), so it is reasonable to hypothesize that if *A. emarginata s.s.* and *A. neosalicifolia* are actually the same species, the chemical composition of *A. neosalicifolia chemotype II* qualifies it as a new chemotype, different from any reported for *A. emarginata s.s.* and could be tested as an alternative rootstock for grafting *A. x atemoya*.

Both chemotypes of A. *neosalicifolia* were sampled in a 500 m high remnant of the Seasonal Semideciduous or Mixed Ombrophilous Forest, the RS component of the Atlantic Forest, a Biome with a biodiversity 8 times greater than that of the Amazon Forest, but from which less than 13% still exists (Galindo-Leal and Gusmão Câmara, 2003). In RS, the extent of destruction of the Atlantic Forest is even bigger and only 7.5% of the original forest has not fallen to the monocultures such as soybean and corn (Rio Grande do Sul, 2020). Finding industrial and economically important chemotypes of a big deciduous native tree, such as one with VOs composed of almost half or even higher percentages of a highly active compound, (*E*)-nerolidol (Chan et al., 2016), could likely enhance that region's economic chain if adequately exploited.

While *A. cherimola* (cherimoya), *A. muricata* (soursop), *A. squamosa* (sweetsop), and *A. x atemoya* are cultivated in more tropical regions and are very well accepted for consumption *in natura*, as juices or jams (Braga Sobrinho, 2014), the expansion of the Annonaceae market in Brazil depends either on the development of research to extend the shelf life of these fruits, allowing them to be processed and reach markets more distant from the productive areas (São José et al., 2014), or on the development of more marketable varieties, which could help to reduce the economic dependency on the monocultures. Fruits from Annonaceae species in the Brazilian Cerrado have been transformed into cereal bars and liquors (Oliveira, 2015, 2019), and several species were evaluated for propagation by cuttings (Scaloppi Junior and Martins, 2014). Specimens of *A. rugulosa* and *A. neosalicifolia* have a very similar habit, height (up to 20 m), and very similar leaf shape (Záchia and Irgang, 1996), differing by a characteristic glossiness in the abaxial surface of *A. rugulosa* and the shape of galls in the leaf, which are characteristic for each species. Globose (4 x 4 mm)

galls are usually found in the leaves of *A. rugulosa* while cylindric (1-2 x 6 mm) ones are found in the leaves of *A. neosalicifolia* (similar galls are sometimes found in *A. emarginata*). A possible link between these galls and the production of volatiles in *A. neosalicifolia* will be discussed elsewhere (de Souza, T.J.T., personal communication).

The volatile profile of a plant, besides acting in the defense strategy and attraction of pollinators for reproduction and survival, may also show pharmacological properties with economic importance. It was suggested that not only the parentals but also the rootstock used in propagation through cuttings could influence the volatile profile of a cultivated Annonaceae (Campos, Vieira, Baron, et al., 2019a,b). Also, despite the fruit volatiles being very different from the respective leaf volatiles (de Sousa Galvão et al., 2020; Neta et al., 2019; Pino, 2000; Santana et al., 2017; Xu et al., 2016), further analyses of the fruits from these two chemotypes of *A. neosalicifolia*, could reveal if the differences here reported are reflected in those edibles and if they somehow affect their sensorial properties. On the other hand, further study of the interaction of each chemotype with the species' characteristic galls and their gall-inducing insects could reveal important trophic or signaling systems possibly exploitable in the cultivation of Annonaceae through the aforementioned rootstock properties.

To the best of our knowledge, this is the first report on the chemical composition of the EO from leaves of two chemotypes of *A. neosalicifolia* presented with its seasonal variation.

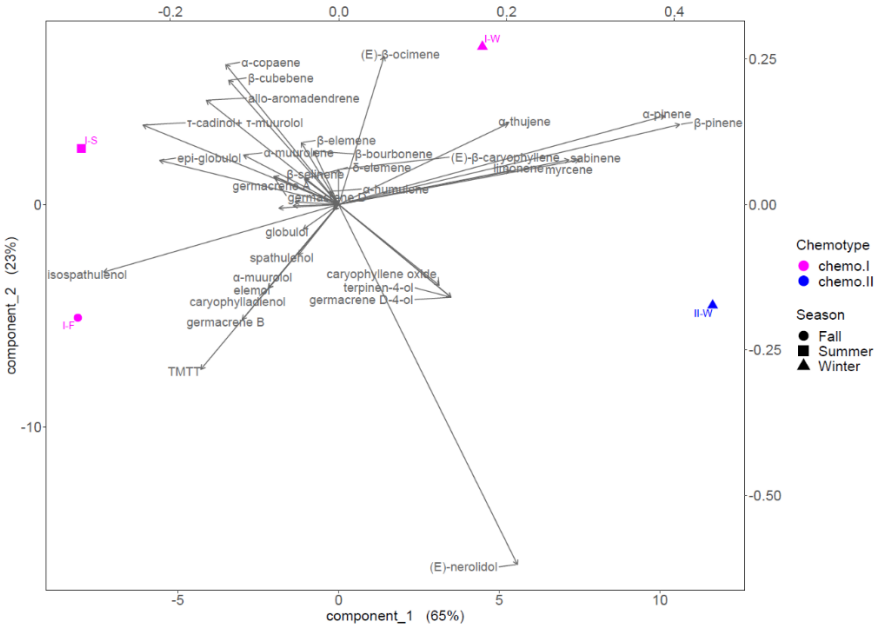
Table 1. Chemical composition of the essential oils from the leaves of *Annona neosalicifolia* H.Rainer.

				Chemotype I			**Chemotype II**
KI[a]	**RI[b]**	**IM [c]**	**Compound**	**Summer**	**Fall**	**Winter**	**Winter**
922	930	I,M	α-thujene	-	-	0.5	0.2
925	932	I,M	α-pinene	-	-	**6.1**	**7.4**
960	969	I,M	sabinene	-	-	0.7	1.4
963	974	I,M	β-pinene	-	-	**6.4**	**11.2**
979	988	I,M	myrcene	-	-	0.6	1.1
1017	1024	I,M	limonene	-	-	0.2	0.3
1037	1044	I,M	(*E*)-β-ocimene	-	-	0.3	-
1091	1095	I,M	linalool	-	tr [d]	-	tr
1107	1114	I,M	DMNT [e]	-	-	-	tr
1164	1174	I,M	terpinen-4-ol	-	-	-	0.2
1325	1335	I,M	δ-elemene	tr	0.1	0.1	tr
1358	1374	I,M	α-copaene	0.4	0.1	0.4	tr
1371	1387	I,M	β-bourbonene	tr	0.1	0.2	tr
1372	1387	I,M	β-cubebene	0.3	0.1	0.3	tr
1374	1398	I,M	β-elemene	2.9	1.2	3.0	0.6
1401	1417	I,M	(*E*)-β-caryophyllene	**26.3**	**9.3**	**23.9**	**15.1**
1433	1452	I,M	α-humulene	3.3	2.3	3.2	2.3
1439	1458	I,M	*allo*-aromadendrene	0.3	0.3	0.4	tr
1460	1484	I,M	germacrene D	**22.9**	**25.4**	**19.9**	**4.5**
1471	1489	I,M	β-selinene	-	0.1	0.1	-
1476	1500	I,M	bicyclogermacrene	**34.2**	**41.8**	**27.0**	**13.9**
1479	1500	I,M	α-muurolene	0.3	tr	tr	tr
1482	1508	I,M	germacrene A	2.2	3.6	2.8	0.5

1493	1513	I,M	γ-cadinene	tr	tr	tr	-
1598	1503	I,M	germacrene C [f]	-	tr	tr	-
1499	1522	I,M	δ-cadinene	0.9	1.6	0.7	0.3
1508	1533	I,M	(*E*)-cadina-1,4-diene	-	tr	tr	-
1514	1537	I,M	α-cadinene	-	tr	tr	-
1542	1559	I,M	germacrene B	-	0.3	tr	-
1543	1548	I,M	elemol	-	0.1	-	-
1549	1561	I,M	(*E*)-nerolidol	-	0.7	tr	**36.3**
1552	1574	I,M	germacrene D-4-ol	-	tr	tr	0.2
1561	1577	I,M	spathulenol	0.9	3.7	0.8	0.9
1563	1590	I,M	TMTT [g]	-	1.7	tr	-
1566	1582	I,M	caryophyllene oxide	tr	0.5	0.4	0.6
1573	1590	I,M	globulol	0.7	0.7	0.3	0.4
1582	1585	I,M	*epi*-globulol [h]	0.6	0.5	0.1	tr
1612	1639	I,M	caryophylladienol	-	0.1	-	-
1625	1637	I,M	Isospathulenol [h]	1.0	0.7	tr	tr
1629	1640	I,M	τ-cadinol+ τ-muurolol	1.0	1.3	0.4	tr
1634	1644	I,M	α-muurolol	tr	0.1	tr	tr
1642	1652	I,M	α-cadinol	1.0	1.2	0.7	0.4
		Hydrocarbon Monoterpenes		-	-	14.8	21.6
		Oxigenated Monoterpenes		-	-	-	0.2
		Hydrocarbon Sesquiterpenes		95.0	86.3	82.0	37.2
		Oxigenated Sesquiterpenes		5.2	11.3	2.7	38.8
		(%) Total identified		99.2	95.9	99.5	97.8
		(%) Yield [i]		0.12	0.10	0.16	0.14

[a] Retention index relative to C9–C22 n-alkanes in a DB5 column; [b] Literature Retention Index ([e,g] Snoeren, 2009; Vredenbregt, 2007; [h] Babushok et al, 2011; [f] de Souza et al, 2021; Adams, 2007 otherwise); [c] Identification method (I: Retention Index; M: Mass spectrum); [d] Traces; [i] Yield as a function of the volume of oil obtained and mass of fresh leaves (v/w). DMNT (4,8-dimethylnona-1,3,7-triene), TMTT (4,8,12-trimethyltrideca-1,3,7,11-tetraene).

Figure 1. Compositional PCA of Essential Oils from the Leaves of *Annona neosalicifolia* H.Rainer



Conclusion

This species produces EO with compounds that are common in the Annonaceae family, where hydrocarbon sesquiterpenes predominated, especially β-caryophyllene, germacrene D, and bicyclogermacrene. In the winter, changes occurred in the chemical composition, mainly with the rise in the percentage of the monoterpenes α-pinene and β-pinene. On the other hand, variations were observed on the percentages of the oxygenated sesquiterpene *(E)*-nerolidol which was very different between chemotypes.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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