201500222

FULL PAPER

Bioactive Secondary Metabolites from *Schizogyne sericea* (Asteraceae) Endemic to Canary Islands

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Schizogyne sericea (Asteraceae) is a halophytic shrub endemic to the Canary Islands and traditionally employed as analgesic, astringent, anti-inflammatory, and vulnerary. A comprehensive phytochemical investigation was conducted on the flowering aerial parts by analyzing both essential oil constituents and polar compounds. The essential oil was dominated by *p*-cymene, with the noteworthy occurrence of *β*-pinene and thymol esters. From the EtOH extract, eight compounds were isolated and structurally elucidated. Essential oil, polar fractions, and isolates (**2**), (**4**), and (**5**) were separately *in vitro* assayed for antiproliferative activity on human tumor cell lines (A375, MDA-MB 231, and HCT116) by MTT assay, for antioxidant potential by DPPH, ABTS, and FRAP assays, and for antimicrobial activity by the agar disk diffusion method. Results revealed that essential oil and compounds **1** and **2** exert a strong inhibition on tumor cells, and in some cases, higher than that of cisplatin. Fractions containing thymol derivatives (**1** and **2**) and caffeoylquinic acid derivatives **4** and **5** displayed antioxidant activity comparable to that of Trolox, making *S. sericea* extract an interesting natural product with potential applications as preservative or in the treatment of diseases in which oxidative stress plays an important role.

Keywords: *Schizogyne sericea*, Asteraceae, Essential oil, Biological activities.

Introduction

The genus *Schizogyne* Cass., one of the three genera of the tribe Inulae of the family Asteraceae (subfamily Asteroideae), is endemic to the Selvagens and Canaries, which are Macaronesian Islands [1] and consists of two halophytic species: *S. glaberrima* DC. and *S. sericea* (L.f.) DC. These species differ in the hairiness and shape of leaves and also in their distribution, the former being endemic to Gran Canaria (Canary Islands) and the latter widespread in all the Canary and Selvagem Islands.

S. sericea (syn. *Chrysocoma sericea* L.f., *Inula schizogyne* Masf.), well-known as 'salado', is a shrub up to 1 m tall, with silky stems and leaves, the latter being linear, flat, 3 – 5 cm wide, obtuse, and white-grayish. Inflorescences are yellow capitula without ray florets. *S. sericea* is widespread in rocky coastal regions throughout all the Canary Islands, being more common in Tenerife and Gran Canaria [2].

In the folk medicine, the whole plant or the top flowering aerial parts were used in infusion as analgesic, astringent, anti-inflammatory, and vulnerary. Moreover, the plant is given to goats as forage [3].

From *Schizogyne* species a series of thymol derivatives was previously isolated and structurally elucidated, among which 10-acetoxy-8-hydroxy-9-isobutyryloxy-6-methoxythymol was shown to exert anti-inflammatory activity on carrageenan-induced edema in rats [4][5][6]. As far as the volatile fraction is concerned, only *S. glaberrima* was studied for the essential oil composition [7], whereas *S. sericea* has not been previously investigated.

In the present work, we carried out a comprehensive phytochemical analysis on both polar compounds and essential oil constituents obtained from the flowering aerial parts of *S. sericea* which were analyzed by

DOI: 10.1002/cbdv.201500222

NMR, and GC and GC/MS, respectively. The main compounds isolated from the EtOH extracts as well as the hydrodistilled essential oil were assayed for *in vitro* biological activity. In particular, the antioxidant activities were studied by DPPH (for free-radical scavenging activity), ABTS (for total radical scavenging capacity), and FRAP assay (for determination of antioxidant power); the antimicrobial activities were examined by agar disk diffusion; and the cytotoxicity on tumor cells was explored by MTT assay.

Results and Discussion

Essential Oil Analysis

A total of 62 volatile components, accounting for 89.6% of the total composition, were identified in the essential oil from *S. sericea* (*Table 1*). The majority of them belong to the group of monoterpenes (86.1%) and, to a minor extent, to sesquiterpenes (3.1%). Notably, in the monoterpene fraction the aromatic compounds represented the major group (54.8%), with the hydrocarbon *p*-cymene as the most representative (45%). Among them, noteworthy was the presence of thymol derivatives (9.8%) such as 8,9-dehydrothymol isobutyrate (2.8%) and thymol isobutyrate (3.2%). These compounds are of chemotaxonomic interest since they were found also in the essential oil of *S. glaberrima* from Gran Canaria [7] and in other Asteraceae such as *Arnica amplexicaulis* Nutt. (syn. of *A. lanceolata* subsp. *prima* (Maguire) Strother & S.J. Wolf) and *Carpesium divaricatum* Siebold & Zucc. [8][9]. In addition, we identified other thymol derivatives which were not found in the essential oil of *S. glaberrima* but in its EtOH extract such as 10-acetoxy-8,10-dehydro-9-isobutyryloxy-6-methoxythymol (0.8%) and 10-acetoxy-8,10-dehydro-9-isobutyryloxy-6-methoxythymol (1.4%) [4].

The second group of monoterpenes was made-up of hydrocarbons (22.1%), with β-pinene (6.0%), limonene (4.8%), camphene (4.8%), and α-phellandrene (4.4%) as the most abundant. Finally, the third group of monoterpenes consisted of oxygenated compounds (9.2%), which were represented mainly by isobornyl acetate (5.3%). Among sesquiterpenes, there were no components exceeding 1%.

Although several reports on the thymol derivatives have been published [5][6], the composition and biological activities of the essential oil of *S. sericea* are herein reported for the first time. Our results showed that its volatile chemical profile was qualitatively similar to that found for *S. glaberrima* from Gran Canaria, which contained *p*-cymene (64.9%), limonene (25.2%), and α-phellandrene (5.2%) as the major constituents [7]. Among other components, camphene (0.3%), β-pinene (0.3%), isobornyl acetate (0.1%), 8,9-dehydrothymol isobutyrate (1.0%), and thymol isobutyrate (0.9%) were found here at lower levels. Another difference between *S. sericea* and *S. glaberrima* was given by the number of essential oil components detected (62 *vs.* 29, respectively). We assume that the aforementioned differences can be attributed to genetic factors (*i.e.*, different species), geographic origin (Tenerife *vs.* Gran Canaria), microclimate, and harvesting period (September *vs.* April). Also, a different sensitivity in the GC method used may have influenced the results.

Polar Compounds

The analysis of the polar fraction led to the identification of several phenolic compounds (**1** – **8**, *Fig. 1*). In particular, the thymol derivatives, 2-{2-[(acetyloxy)methyl]oxiran-2-yl}-5-methylphenyl 2-methylpropanoate (**1**), 2-{2-[(acetyloxy)methyl]oxiran-2-yl}-4-methoxy-5-methylphenyl 2-methylpropanoate (**2**), and 3-(acetyloxy)-2-hydroxy-2-(2-hydroxy-5-methoxy-4-methylphenyl)propyl 2-methylpropanoate (**3**), were identified. Compound **2**, firstly isolated from *Ageratina glabrata* (Kunth) R.M.King & H.Rob. (Asteraceae) [8], and compound **3** were previously identified in this species as well as in *S. glaberrima* [4][5].

It is worth noting that compound 2-{2-[(acetyloxy)methyl]oxiran-2-yl}-5-methylphenyl 2-methylpropanoate (**1**) is reported for the first time in *Schizogyne*. This compound was first identified in *Wedelia forsteriana* Endl. [16]. Afterwards, it was found in the genera *Ageratina*, *Critonia*, *Fleischmannia* [17], and *Leontonyx* [18], all included in the family Asteraceae, thus demonstrating their taxonomical proximity. It should be mentioned that compound **1** is believed to be responsible for allergic phenomena of contact dermatitis caused by *Arnica sachalinensis* (Regel) A.Gray (Asteraceae) [19][20]. In addition, five phenolic acids, namely 3,5-dicaffeoylquinic acid (**4**), 1,3-dicaffeoylquinic acid (**5**), 3-caffeoylquinic acid (chlorogenic acid) (**6**), 4-caffeoylquinic acid (cryptochlorogenic acid) (**7**), and caffeic acid (**8**), were recognized for the first time in this species. These caffeoylquinic acid derivatives are endowed with interesting biological activities, *e.g.*, antioxidant [21][22] and hepatoprotective [23]. From the chemotaxonomical point of view, these phenolic compounds are highly distributed in the Asteraceae family. In particular, compounds **4** and **5** are constituents of *Achillea millefolium* L. [24] and together with caffeic (**8**) and caffeoylquinic acids (**6**, **7**) are present in the leaves of *Cynara syriaca* Boiss. [25]. Chlorogenic acid (**6**) was found to be a constituent of several species of the genus *Crepis* [26], *Baccharis retusa* DC. [27], and together with 3,5-dicaffeoylquinic acid (**4**) was found in other Asteraceae such as *Arnica montana* L., *Artemisia vulgaris* L., *Calendula officinalis* L., and *Chamomilla recutita* (L.) Rauschert [22].

Antioxidant Activity

The free-radical scavenging activity of essential oil and polar compounds from *S. sericea* was analyzed by three different *in vitro* assays, namely DPPH, ABTS, and FRAP, and results are presented in *Table 2*. The essential

Table 1. Chemical composition of the essential oil from the aerial parts of *Schizogyne sericea*

No.	Component[a])	*LRI*[b])	*LRI* lit[c])		%[d])	ID[e])
			ADAMS	NIST 08		
1	α-Thujene	917	924	917	tr[f])	*RI*, MS
2	α-Pinene	921	932	922	0.9	Std
3	Camphene	934	946	935	4.8	Std
4	Sabinene	961	969	961	0.1	*RI*, MS
5	β-Pinene	962	974	962	6.0	Std
6	Myrcene	983	988	983	0.2	Std
7	δ-2-Carene	993	1001	993	tr	*RI*, MS
8	α-Phellandrene	997	1002	998	4.4	Std
9	δ-3-Carene	1001	1008	1001	tr	Std
10	α-Terpinene	1010	1014	1010	0.1	*RI*, MS
11	*p*-Cymene	1019	1020	1018	45.0	Std
12	Limonene	1021	1024	1021	4.8	Std
13	(*E*)-β-Ocimene	1043	1044	1043	0.1	*RI*, MS
14	γ-Terpinene	1051	1054	1051	tr	Std
15	Terpinolene	1080	1086	1080	0.4	Std
16	*p*-Cymene	1088	1089		tr	*RI*, MS
17	Linalool	1097	1095	1097	tr	Std
18	(*E*)-4,8-Dimethyl-1,3,7-nonatriene	1112	1106[g])		0.2	*RI*, MS
19	*cis*-*p*-Menth-2-en-1-ol	1113	1118		0.2	*RI*, MS
20	*trans*-*p*-Menth-2-en-1-ol	1132	1136		0.1	*RI*, MS
21	Camphor	1134	1141	1136	tr	Std
22	Camphene hydrate	1136	1145	1136	0.1	*RI*, MS
23	Isoborneol	1155	1155		0.1	Std
24	Terpinen-4-ol	1168	1174	1169	0.1	Std
25	Dill ether	1181	1184		0.1	*RI*, MS
26	α-Terpineol	1182	1186	1182	0.1	Std
27	*cis*-Piperitol	1187	1195	1187	0.1	*RI*, MS
28	*trans*-Piperitol	1200	1207	1201	0.1	*RI*, MS
29	Coahuilensol, methyl ether	1220	1221[h])		1.2	*RI*, MS
30	Piperitone	1246	1249	1247	0.1	*RI*, MS
31	Isobornyl acetate	1276	1283	1277	5.3	Std
32	Lavandulyl acetate	1288	1288		0.5	*RI*, MS
33	Thymol	1291	1289	1291	0.1	Std
34	Myrtenyl acetate	1317	1324	1316	tr	*RI*, MS
35	δ-Elemene	1325	1335	1327	tr	*RI*, MS
36	Neryl acetate	1360	1359	1360	1.1	Std
37	β-Elemene	1380	1389	1381	tr	*RI*, MS
38	Cyperene	1385	1398	1385	tr	*RI*, MS
39	Methyl eugenol	1400	1403	1400	0.3	*RI*, MS
40	Cuminyl acetate	1409		1409	0.2	*RI*, MS
41	Isobornyl isobutanoate	1431	1431		0.8	*RI*, MS
42	α-Humulene	1437	1452	1440	0.1	Std
43	8,9-Dehydrothymol isobutyrate	1456	1456		2.8	*RI*, MS[i])
44	Thymol isobutyrate	1460	1460		3.2	*RI*, MS[i])
45	Germacrene D	1465	1484	1466	0.1	*RI*, MS
46	α-Selinene	1481	1498	1481	tr	*RI*, MS
47	Bicyclogermacrene	1481	1500	1482	0.3	*RI*, MS
48	Neryl isobutyrate	1486	1490	1487	0.3	*RI*, MS
49	α-Muurolene	1487	1500	1488	0.1	*RI*, MS
50	δ-Cadinene	1510	1523	1511	0.3	*RI*, MS
51	(*E*)-Nerolidol	1556	1561	1556	0.3	Std
52	Spathulenol	1572	1577	1572	0.4	*RI*, MS
53	Neryl isovalerate	1580	1582		0.1	*RI*, MS
54	β-Oplopenone	1596	1607	1596	0.1	*RI*, MS
55	*epi*-α-Cadinol	1635	1638		0.6	*RI*, MS
56	*epi*-α-Muurolol	1639	1640		0.5	*RI*, MS
57	α-Cadinol	1640	1652	1640	0.6	*RI*, MS
58	6-Methoxythymol isobutyrate	1671	1671		0.1	*RI*, MS[i])
59	Unknown[j])	1790			2.9	
60	Unknown[j])	1903			1.3	

Table 1. (cont.)

No.	Component[a])	*LRI*[b])	*LRI* lit[c])		%[d])	ID[e])
			ADAMS	NIST 08		
61	10-Acetoxy-8,9-dehydro-6-methoxythymol isobutyrate	1984			0.8	MS[k])
62	10-Acetoxy-8,10-dehydro-9-isobutyryloxy-6-methoxythymol	2084			1.4	MS[k])
63	10-Acetoxy-8,9-epoxy-2-methoxythymol	2116			tr	MS[k])
64	Abienol	2148	2149		0.3	*RI*, MS
	Oil yield (%)				1.7	
	Total identified (%)				89.6	
	Grouped compounds					
	Aromatic monoterpenes				54.8	
	Monoterpene hydrocarbons				22.1	
	Oxygenated monoterpenes				9.2	
	Sesquiterpene hydrocarbons				0.8	
	Oxygenated sesquiterpenes				2.3	
	Others				0.4	

[a]) Compounds are listed in order of their elution from a *HP-5MS* column. [b]) Linear retention index on *HP-5MS* column, experimentally determined using homologous series of C_8 – C_{30} alkanes. [c]) Relative retention index taken from *Adams* [10] and *FFNSC2* [11] for apolar capillary column. [d]) Percentage values are means of three determinations, with a RSD% for the main components below 5% in all cases. [e]) Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries Wiley, Adams, FFNSC2, and NIST 08; *RI*, by comparison of *RI* with those reported in the literature [10 – 12]; Std, by comparison of the retention time and mass spectrum of available authentic standard or. [f]) Tr, traces (mean value below 0.1%). [g]) Retention index value taken from *Vichi et al.* [13]. [h]) Retention index value taken from *Venditti et al.* [14]. [i]) *RI*s and MS fragmentations according to *Weyerstahl et al.* [15]. [j]) MS data for unidentified components: *RI* 1790 *m/z* (%) = 276(4, *M*+), 148(100), 146(87), 145(84), 43(73), 135(46), 71(30), 133(30), 206(24), 147(17), 149(14); *RI* 1903 *m/z* (%) = 276(1, *M*+), 149 (100), 43(97), 162(77), 145(62), 71(46), 161(39), 133(31), 150(24), 135(21), 146(19). [k]) MS fragmentations according to *Gonzalez et al.* [4][5].

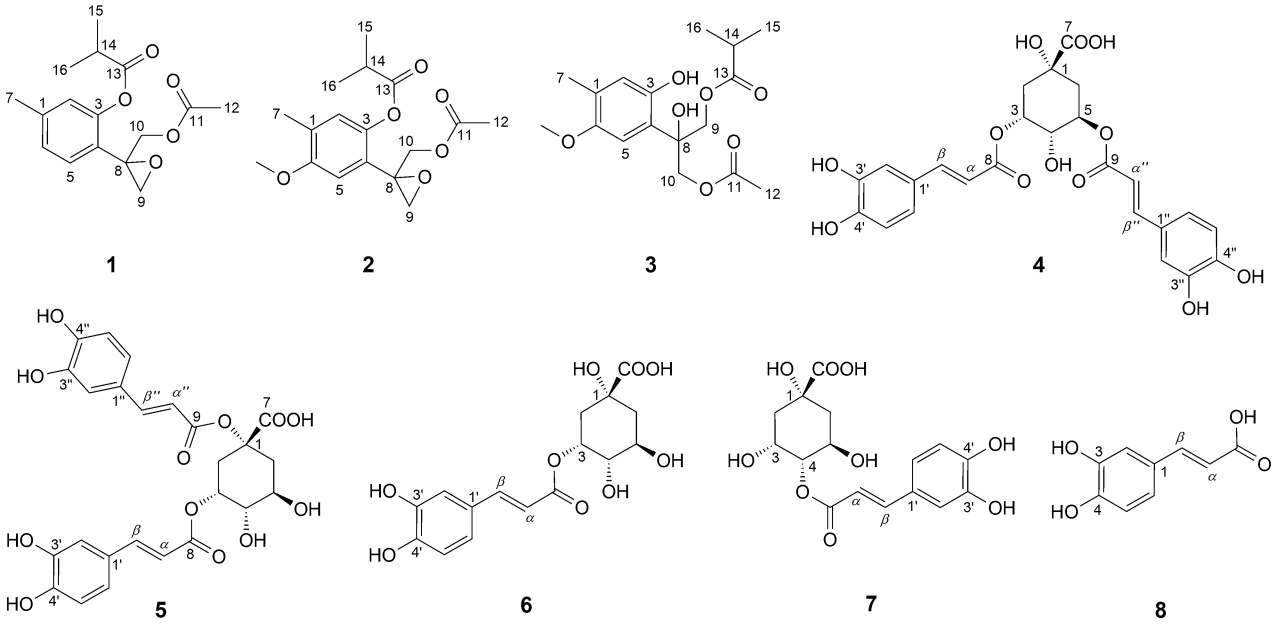

Fig. 1. Structures of compounds isolated from the polar fraction of *Schizogyne sericea*.

oil showed a weak antioxidant capacity, with TEAC values of 43.7 and 39.6 μmol TE/g toward DPPH and ABTS, respectively, and a TEAC value of 66.2 μmol TE/g in the FRAP assay. This weak free-radical scavenging capacity may be in part attributed to one of its main components, *p*-cymene. This aromatic monoterpene has been reported to exhibit low antioxidant activity *in vitro* [28], but high antioxidant potential *in vivo* and may act as a neuroprotective agent in the brain [29].

However, the caffeoylquinic acid derivatives compound **4** and **5**, showed significant antioxidant activity in the three different assays tested, which is in good agreement with the results obtained in previous work [30][31][32]. Although both compounds exhibited similar metal

Table 2. *In vitro* radical scavenging activities of essential oil, fractions and isolated compounds from *Schizogyne sericea*

Schizogyne sericea	DPPH		ABTS		FRAP
	TEAC[a]) [μmol TE/g]	IC_{50}[b]) [μg/ml]	TEAC[a]) [μmol TE/g]	IC_{50}[b]) [μg/ml]	TEAC[a]) [μmol TE/g]
Essential oil	43.7 ± 3.5	391 ± 31	39.6 ± 1.3	150 ± 4.9	66.2 ± 3.5
Fractions					
Compounds **1** + **2** (1:1)	196 ± 6.5	71.0 ± 2.4	363 ± 12.1	23.1 ± 0.8	568 ± 6.6
Compounds **1** + **2** (1:2)	2864 ± 97.0	4.9 ± 0.2	4564 ± 360.4	1.8 ± 0.1	850 ± 43.3
Isolated compounds					
Compound **2**	249 ± 19.7	56.1 ± 4.4	1471 ± 8.7	5.7 ± 0.0	775 ± 13.3
Compound **4**	1468 ± 45.5	9.5 ± 0.3	1720 ± 165.7	4.9 ± 0.5	803 ± 32.2
Compound **5**	728 ± 10.1	19.2 ± 0.3	942 ± 20.3	8.9 ± 0.2	803 ± 5.3
Positive control					
Trolox		3.5 ± 0.2		2.1 ± 0.0	

[a]) TEAC = Trolox equivalent (TE) antioxidant concentration. [b]) IC_{50} = The concentration of compound that affords a 50% reduction in the assay.

ion chelating activity in the FRAP assay (TEAC values in the range of 803.1 – 803.5 μmol TE/g), compound **4** showed higher radical scavenging activity toward DPPH and ABTS radicals with TEAC values of 1468 and 1720 μmol TE/g, respectively, when compared to compound **5** (728 and 942 μmol TE/g, respectively). High antioxidant activity was previously reported for the thymol derivatives compound **1** and **2** [33][34]. In our assays, these molecules were tested as such (**2**) or as a mixture containing them (**1** + **2**) in different ratios. The fraction containing compounds **1** and **2** (1:2) showed the highest radical scavenging activity, with TEAC values of 2864 μmol TE/g and 4564 μmol TE/g toward DPPH and ABTS radicals, respectively, and a TEAC value of 850 μmol TE/g in the FRAP assay (*Table 2*).

The good antioxidant activity displayed by *S. sericea* fractions and isolated compounds may support the traditional uses of the plant in the Canary Islands in the treatment of diseases, such as inflammations and wounds, in which oxidative stress plays an important role. In addition, these properties may be exploited also at industrial level in the manufacturing of food preservatives.

Cytotoxic Activity

The cytotoxic activity of *S. sericea* essential oil was evaluated on human tumor cell lines: A375 human malignant melanoma cell line, MDA-MB 231 human breast adenocarcinoma cell line, and HCT116 human colon carcinoma cell line by MTT assay. All cell lines were subjected to increasing concentrations of essential oil for 72 h. The results, collected in *Table 3*, show that essential oil exhibited a significant cytotoxic activity against the human cancer cells examined and induced a concentration-dependent inhibitory effect in the dilutions ranging from 0.8 to 200 μg/ml. The IC_{50} values of the essential oil were 3.5, 6.6, and 3.4 μg/ml for A375, MDA-MB 231, and HCT116 cell lines, respectively. In literature, the main compound of the essential oil, *p*-cymene (40.8%), is reported to have low cytotoxic activity on two tumor cell lines, A-549 lung carcinoma cells and DLD-1 colon adenocarcinoma cells [35]. This compound is highly hydrophobic and causes swelling of the cytoplasmic membrane [36]. Being easily incorporated in the lipid bilayer, it may facilitate transport of other effective active compounds. Among them, thymol derivatives might contribute to the cytotoxic activity or be responsible for synergism together with other compounds. Notably, 8,9-dehydrothymol isobutyrate is reported to be a component of a pharmaceutical formulation patented as anticancer agent [9].

Fractions and isolated compounds were investigated for their cytotoxic activity (*Table 3*) against the same human tumor cell lines. Data show highly significant antiproliferative activity for compound **2**, with IC_{50} values of 0.5 (1.4 μM), 0.5 (1.5 μM), and 1.2 (3.6 μM) μg/ml toward MDA-MB 231, A375, and HCT116 cell line, respectively. Compound **1** mixed with compound **2** showed a minor effect on tumor cells. These data agree with those reported in literature by *Aponte et al.* [37] where compound **2** and **3** were isolated from *Mikania decora* Poepp. and exhibited significant cytotoxic activity against a panel of human tumor cell lines. The authors supposed that the cytotoxic activity was due to the presence of the 8,9-epoxy-10-acetoxy groups. Analogous thymol derivatives showed similar cytotoxic activity [9]. As regards compounds **4** and **5**, they showed low cytotoxic effects against all tumor cell lines tested. *Puangpraphant et al.* [38] reported that dicaffeoylquinic acids isolated from *Ilex paraguariensis* induced apoptosis by increasing the activation of caspase-8 in both RKO and HT-29 colon cancer cells, but with IC_{50} values higher than those obtained in our experiments.

Antimicrobial Activity

Table 4 shows the results of the antimicrobial activity of *S. sericea* essential oil by the disk diffusion method. We found that the antimicrobial activity of the essential oil

Table 3. *In vitro* cytotoxic activity of essential oil, fractions and isolated compounds from *Schizogyne sericea*

Schizogyne sericea	Cell line [IC_{50} μg/ml][a])		
	A375[b])	MDA-MB 231[c])	HCT116[d])
Essential oil	3.5	6.6	3.4
95% C.I.[e])	3.1 – 4.0	6.1 – 7.2	3.0 – 3.8
Fractions			
Compounds **1** + **2** (1:1)	4.6	7.6	8.3
95% C.I.	3.8 – 5.5	6.7 – 8.7	7.0 – 9.9
Compounds **1** + **2** (1:2)	0.4	1.7	1.3
95% C.I.	0.4 – 0.5	1.6 – 1.8	1.2 – 1.4
Isolated compounds			
Compound **2**	0.5	0.5	1.2
95% C.I.	0.4 – 0.5	0.4 – 0.5	1.1 – 1.3
Compound **4**	> 100	> 100	> 100
Compound **5**	> 100	> 100	> 100
Positive control			
Cisplatin	0.4	2.9	2.4
95% C.I.	0.3 – 0.5	2.2 – 3.1	2.0 – 2.9

[a]) IC_{50} = The concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation). [b]) Human malignant melanoma cell line. [c]) Human breast adenocarcinoma cell line. [d]) Human colon carcinoma cell line. [e]) Confidence interval.

Table 4. Antimicrobial activity of essential oil, fractions and isolated secondary metabolites from *Schizogyne sericea* determined by the disk diffusion. Values indicate the diameter of the growth inhibition zone (mm) around the disk containing the indicated material and are averages of at least two determinations

Schizogyne sericea	*S. aureus*	*E. faecalis*	*E. coli*	*P. aeruginosa*	*C. albicans*
Essentialoil	11.0 ± 1.0	6.5 ± 0.5	8.0 ± 0.0	6.0 ± 0.0	8.5 ± 0.0
Fractions					
Compounds **1** + **2** (1:1)	9.3 ± 1.2	8.2 ± 1.0	8.3 ± 6.6	7.2 ± 1.6	8.8 ± 0.3
Compounds **1** + **2** (1:2)	9.3 ± 0.6	9.2 ± 1.3	8.3 ± 0.6	8.2 ± 0.8	9.0 ± 0.0
Isolated compounds					
Compound **2**	9.3 ± 1.2	9.0 ± 1.0	9.2 ± 1.0	8.0 ± 1.0	10.0 ± 0.0
Compound **4**	8.7 ± 1.5	6.7 ± 1.2	8.0 ± 1.0	6.0 ± 0.0	9.7 ± 0.6
Compound **5**	8.8 ± 0.8	7.7 ± 0.6	8.7 ± 0.6	7.5 ± 0.9	8.3 ± 0.6
Reference antibiotics					
Ciprofloxacin	22 – 24	n.r.[a])	28 – 30	28 – 32	n.r.
Nystatin	n.r.	n.r.	n.r.	n.r.	15 – 16

[a]) n.r.: not recommended for this species.

on the growth of bacterial pathogens was positive, albeit low, toward *Staphylococcus aureus* and *Escherichia coli* strains, but was ineffective in the case of *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Hence, it could be observed that this activity did not correlate with the subdivision of bacteria into Gram-positives and Gram-negatives. Nevertheless, *E. faecalis* and *P. aeruginosa* are well-known for their natural resistance to many antibiotics. However, antifungal activity against *Candida albicans* was also found (8.5 mm inhibition zone diameter).

The low or no antimicrobial activity showed by the essential oil against the test organisms considered may be explained by the presence of *p*-cymene, one of the main constituents, that has been described as nonactive [39]. Nevertheless, *p*-cymene is able to increase cell membrane permeability toward other compounds (*e.g.*, carvacrol), which are then favored in exerting their intrinsic antimicrobial activity [40]. In this respect, we have to mention that carvacrol or similar compounds were not present in the essential oil investigated. Other representative components, such as limonene, camphene, β-pinene, and isobornyl acetate, have been reported to have low or no activity in antimicrobial susceptibility testing [41]. All these data together may well explain the overall activity measured for *S. sericea* essential oil in the present work.

Activity of fractions and isolated compounds were in the range of 6.0 (no activity) to 10.0 mm (low activity) inhibition zone diameter. 2-{2-[(acetyloxy)methyl]oxiran-2-yl}-4-methoxy-5-methylphenyl 2-methylpropanoate (**2**) was undoubtedly the most active compound. All microbial species were affected by compound **2** with inhibition zone

diameters above 8 mm. Also *E. faecalis* and *P. aeruginosa* were inhibited to some extent. Compound **2** is also the main contributor to inhibition of microbial growth observed for the fractions. Inhibition zone diameters produced by compound **2**, when tested alone, were comparable to those obtained in combination with compound **1** (*Table 4*, Fractions). Actually, compound **1** seemed to be inactive. Reduced diameters were obtained by the fraction combining compounds **1** and **2** at 1:1 molar ratio against all microbes, except for *S. aureus*, probably due to a dilution effect on compound **2**. Accordingly, once the relative amount of compound **2** was doubled (fractions with compounds **1** + **2** at 1:2 molar ratio) the activity was almost completely restored reaching that of compound **2** alone. Compounds **4** and **5** showed a measurable activity (inhibition zone diameters 6.0 – 9.7 mm). 3,5-dicaffeoylquinic acid (**4**) was inhibiting all microorganisms tested, while 1,3-dicaffeoylquinic acid (**5**) produced a wider inhibition zone in the yeast than against bacteria, especially *E. faecalis* and *P. aeruginosa*. The results obtained with phenolic acids agree with previous reports on the antimicrobial activity by these compounds [42][43].

In conclusion, even if results of tested isolated compounds from polar fractions of *S. sericea* did not show potent antimicrobial inhibition, this work provides the first report on the antibacterial and antifungal activity of isolated compounds 2-{2-[(acetyloxy)methyl]oxiran-2-yl}-5-methylphenyl 2-methylpropanoate and 2-{2-[(acetyloxy)methyl]oxiran-2-yl}-4-methoxy-5-methylphenyl 2-methylpropanoate, in addition to that of the essential oil. Obtained data were not considered sufficiently promising to deserve further investigation by other susceptibility testing methods, such as the microdilution method.

Conclusions

This work represented the first phytochemical and biological investigation of the essential oil from *S. sericea.* The chemical profile evidenced similarities with that already reported for *S. glaberrima*. In this regard, the occurrence of thymol derivatives may be considered of chemotaxonomic interest. These molecules were also found in the polar fraction and they exhibited a strong cytotoxicity on tumor cells, thus having promising applications as natural anticancer agents. In this respect, future studies will be performed in our group to investigate the cytotoxic mechanisms and *in vivo* antitumor properties of these constituents. Moreover, polar fractions and isolated constituents obtained from the EtOH extract of *S. sericea* displayed a strong antioxidant activity making this plant an interesting source of natural products with potential applications as food preservative or in the treatment of diseases in which oxidative stress plays an important role.

This work was supported by the *FAR* 2014-2015 (*Fondo di Ateneo per la Ricerca*, FPI000044) of the University of Camerino.

Experimental Part

General

Solvents of RPE grade were purchased from *Sigma–Aldrich* (Saint Louis, MO, USA) or *Carlo Erba Reagenti* (Milan, Italy), silica gel *60* (70 – 230 mesh ASTM) from *Fluka* (Buchs, Switzerland). NMR Spectra: *Varian Mercury 300* MHz (now *Agilent Technologies*, Santa Clara, CA, USA) and/or on *Bruker Avance III 400* MHz instrument (*Bruker*, Billerica, MA, USA) using $CDCl_3$, CD_3OD, or D_2O (*Sigma–Aldrich*) as deuterated solvents; δ in ppm rel. to Me_4Si as internal standard, J in Hz. Internal solvent signal was used to set spectra in CD_3OD (*q*, 3.31 ppm) and D_2O (*s*, 4.79 ppm). MS: *Q-TOF MICRO* spectrometer (*Micromass*; now *Waters*, Manchester, UK) equipped with an ESI source that operated in the negative and/or positive ion mode; in *m*/*z*. The flow rate of sample infusion was 10 μl/min with 100 acquisitions per spectrum. Data were analyzed using the MassLynx software (*Waters*).

Plant Material

Aerial parts of *S. sericea* were collected in Montaña de Guaza (Tenerife, 428 m, Canary Islands, Spain) during blooming in September 2013. A voucher specimen of the plant was deposited with the *Herbarium* of the University of La Laguna (identified by Dr. *Consuelo Hernández Padrón*, Department of Plant Biology, Tenerife, Spain) and in the *Herbarium Universitatis Camerinensis* (included in the online edition of *Index Herbariorum* c/o School of Biosciences and Veterinary Medicine, University of Camerino, Italy) under the code TFC no. 50941 and CAME 26323, respectively, and archived and published in the anArchive system for botanical data (anArchive system, http://www.anarchive.it). Just after its collection, plant material was dried in an airy oven at 36 °C; its storage was at r.t. in darkness.

Essential Oil Isolation

Plant material (180 g) was reduced in small pieces and then hydrodistilled in a *Clevenger*-type apparatus using 5 l of distilled water for 3 h until no more volatile oil was obtained. The essential oil was stored in a sealed vial protected from light at −20 °C before chemical analysis and biological assays. The oil yield (1.7%, $n = 3$) was estimated on a dry weight basis.

GC/FID and GC/MS Analyses

For GC separations, an *Agilent 4890D* gas chromatograph coupled to a flame-ionization detector (FID) was used. Volatile components were separated on a *HP-5* capillary column (5% phenylmethylpolysiloxane, 30 m, 0.32 mm i.d.; 0.25 mm film thickness; *J and W Scientific*, Folsom,

CA, USA), with the following temp. program: 5 min at 60 °C, subsequently 4 °C/min up to 220 °C, then 11 °C/min up to 280 °C, held for 15 min, for a total run of 65 min. Injector and transfer line temp. were 280 °C; He was used as the carrier gas, at a flow rate of 1.8 ml/min; split ratio, 1:34. A mixture of aliphatic hydrocarbons (C_8 – C_{30}; *Sigma*, Milan, Italy) in hexane was directly injected into the GC under the above temp. program, in order to calculate the temp.-programmed *RI*s of peaks in the chromatograms. Oil samples were diluted to 1:100 in *n*-hexane and injected at a volume of 1 µl. Analysis was repeated three times for each sample and the mean value was reported. Data were collected using HP3398A GC Chemstation software (Hewlett Packard, Rev. A.01.01). Quantification of essential oil components was achieved by FID peak-area internal normalization without using correction factors. GC/MS analysis was performed on an *Agilent 6890N* gas chromatograph coupled to a *5973N* mass spectrometer using a *HP-5* MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 mm film thickness; *J & W Scientific*) capillary column. The temp. program was the same as described above. Injector and transfer line temp. were 280 °C; He was used as the carrier gas, at a flow rate of 1 ml/min; split ratio, 1:50; acquisition mass range, 29 – 400 m/z. Mass spectra were acquired in electron-impact (EI) mode with an ionization voltage of 70 eV. Oil sample was diluted to 1:100 in *n*-hexane, and the volume injected was 2 µl. Data were analyzed using MSD ChemStation software (*Agilent*, Version G1701DA D.01.00). Major oil constituents were identified by coinjection with authentic standards purchased from *Sigma–Aldrich* (Milan, Italy). Otherwise, the peak assignment was carried out according to the recommendations of the International Organization of the Flavor Industry (IOFI, http://www.iofi.org/), *i.e.*, by the interactive combination of chromatographic linear retention indices that were consistent with those reported in literature for apolar stationary phases [10][11][12], and MS data consisting in the computer matching with the WILEY275, NIST 08 ADAMS, FFNSC 2, and a homemade (based on the analyses of reference oils and commercially available standards) libraries.

Isolation of Compounds

A portion of 122.5 g of dried plant materials (aerial parts) was extracted three times by maceration for 24 – 48 h, with an 80% EtOH/H_2O solution (800 ml). The three extracts were gathered, the EtOH was distilled off at reduced pressure on a rotatory evaporator, and the resulting water suspension was lyophilized obtaining 29.0 g of crude extract. The chromatographic purification, on silica gel (SiO_2) column chromatography (CC), was conducted on 3.0 g of the crude extract, using BuOH saturated with H_2O as eluent to provide two low polarity fractions; *Fr-2 – Fr-5* and a mixture of waxes, chlorophylls, and other low polarity components (700.2 mg), (*Fr-6*) (86.4 mg) containing compounds **1** and **2** in mixture (1:1), 3,5-dicaffeoylquinic acid **4** (*Fr-17*) (23.3 mg), and several polar fractions containing mixtures of caffeoyl derivatives (**5**, **6**, **7**, and **8**) which were gathered by similarity in *Fr-13 – Fr-23*, excluding *Fr-17* (177.6 mg), and *Fr-24 – Fr-70* (393.3 mg). *Fr-13 – Fr-23* were further purified on a SiO_2 CC (4.5 g) eluted using a mixture of $CHCl_3$/MeOH, previously saturated with CO_2, (9:1 – 7:3) to give 1,3-dicaffeoylquinic acid **5** (*Fr-32*) (19.6 mg) and again 3,5-dicaffeoylquinic acid **4** in mixture 1:2 with cryptochlorogenic acid **7** (*Fr-44 – Fr-50*) (6.5 mg). Fraction *Fr-24 – Fr-70* was purified on acidic SiO_2 CC (7.0 g) with $CHCl_3$/MeOH (8:2 – 6:4) to afford caffeic acid **8** (*Fr-22*) (8.9 mg), chlorogenic acid (**6**), and cryptochlorogenic acid **7** (2:1) (*Fr-25 – Fr-33*) (18.3 mg). The low polarity fraction (*Fr-2 – Fr-5*) was rechromatographed on SiO_2 (35.0 g) with $CHCl_3$/AcOEt (99:1 – 9:1) to give 2-{2-[(acetyloxy)methyl]oxiran-2-yl}-4-methoxy-5-methylphenyl 2-methylpropanoate **2** (35.5 mg) (*Fr-30 – Fr-31*) and 3-(acetyloxy)-2-hydroxy-2-(2-hydroxy-5-methoxy-4-methylphenyl)propyl 2-methylpropanoate **3** (3.5 mg) (*Fr-40 – Fr-41*).

All the isolated compounds were identified by comparison with literature data and/or direct comparison with standard compounds available in our laboratory.

2-{2-[(Acetyloxy)methyl]oxiran-2-yl}-5-methylphenyl 2-methylpropanoate (**1**). ^{1}H-NMR (400 MHz, $CDCl_3$): 7.35 (*d*, *J* = 8.0, H–C(5)); 7.04 (*dd*, *J* = 8.5, 1.6, H–C(6)); 6.86 (br. *s*, H–C(2)); 4.52 (*d*, *J* = 12.3, H_a–C(10)); 4.21 (*d*, *J* = 12.2, H_b–C(10)); 3.02 (*d*, *J* = 4.3, H_a–C(9)); 2.81 (*d*, *J* = 4.2, H_b–C(9)); 2.34 (*s*, H–C(7)); 2.01 (*s*, H–C(12)); 1.30 (*d*, *J* = 7.0, Me(15,16)). ^{13}C-NMR (75 MHz, $CDCl_3$): 175.8 (C(13)); 170.7 (C(11)); 140.2 (C(3)); 130.4 (C(1)); 128.5(C(6)); 126.9 (C(2)); 123.1 (C(5)); 65.6 (C(10)); 57.0 (C(8)); 51.1 (C(9)); 34.4 (C(14)); 21.3 (C(12)); 19.2 (Me(15)); 19.1 (Me(16)); 14.3 (C(7)). ESI-MS: 315.10 ([*M*+Na]$^+$).

2-{2-[(Acetyloxy)methyl]oxiran-2-yl}-4-methoxy-5-methylphenyl 2-methylpropanoate (**2**). ^{1}H-NMR (300 MHz, $CDCl_3$): 6.89 (*s*, H–C(5)); 6.80 (*s*, H–C(2)); 4.55 (*d*, *J* = 12.2, H_a–C(10)); 4.20 (*d*, *J* = 12.2, H_b–C(10)); 3.82 (s, MeO); 3.04 (*d*, *J* = 5.3, H_a–C(9)); 2.81 (*d*, *J* = 5.3, H_b–C(9)); 2.80 (*q*, *J* = 7.0, H–C(14)); 2.18 (*s*, H–C(7)); 2.03 (*s*, H–C(12)); 1.30 (*d*, *J* = 7.0, Me(15,16)). ^{13}C-NMR (75 MHz, $CDCl_3$): 175.8 (C(13)); 170.5 (C(11)); 155.6 (C(6)); 141.5 (C(3)); 128.4 (C(4)); 126.8 (C(1)); 124.5 (C(2)); 109.7 (C(5)); 65.3 (C(10)); 56.9 (C(8)); 55.8 (MeO); 51.2 (C(9)); 34.2 (C(14)); 20.8 (C(12)); 19.1 (Me(15)); 19.0 (Me(16)); 16.2 (C(7)). ESI-MS: 345.17 ([*M*+Na]$^+$).

3-(Acetyloxy)-2-hydroxy-2-(2-hydroxy-5-methoxy-4-methylphenyl)propyl 2-methylpropanoate (**3**). ^{1}H-NMR (300 MHz, $CDCl_3$): 6.68 (*s*, H–C(2)); 6.48 (*s*, H–C(5)); 4.46 (*dd*, *J* = 22.4, 10.8, H–C(10), H–C(9)); 3.73 (*s*, *Me*O); 2.58 (*m*, H–C(14)); 2.15 (*s*, H–C(7)); 2.08 (*s*, H–C(12)); 1.12 (*d*, *J* = 7.0, Me(15,16)). ^{13}C-NMR (75 MHz, $CDCl_3$): 177.5 (C(13)), 171.3 (C(11)), 151.2 (C(6)), 150.0 (C(3)), 126.5 (C(1)), 120.7 (C(4)), 118.8 (C(2)), 108.8 (C(5)), 78.5

(C(8)), 67.4 (C(9)), 67.3 (C(10)), 56.3 (MeO), 34.1 (C(14)), 20.9 (C(12)), 19.0 (Me(15,16)), 15.9 (C(7)). ESI-MS: 363.19 ($[M+Na]^+$).

3,5-Dicaffeoylquinic Acid (= **(3*R*,5*R*)-3,5-Bis{[(2*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,4-dihydroxycyclohexanecarboxylic Acid**; **4**). ^{1}H-NMR, ^{13}C-NMR [44]. ESI-MS: 539.37 ($[M+Na]^+$), 515.32 ($[M-H]^-$).

1,3-Dicaffeoylquinic Acid (= **(1*R*,3*R*,4*S*,5*R*)-1,3-Bis{[(2*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-4,5-dihydroxycyclohexanecarboxylic Acid**; **5**). ^{1}H-NMR, ^{13}C-NMR [45]. ESI-MS: 539.33 ($[M+Na]^+$), 515.25 ($[M-H]^-$).

3-Caffeoylquinic Acid (= **Chlorogenic Acid**; = **(1*R*,3*R*,4*S*,5*R*)-3-{[(2*E*)-3-(3,4-Dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic Acid**; **6**). ^{1}H-NMR, ^{13}C-NMR [46]. ESI-MS: 353.08 ($[M-H]^-$), 377.17 ($[M+Na]^+$).

4-Caffeoylquinic Acid (= **Criptochlorogenic Acid**; = **(1*S*,3*R*,4*S*,5*R*)-4-{[(2*E*)-3-(3,4-Dihydroxyphenyl)prop-2-enoyl]oxy}-1,3,5-trihydroxycyclohexanecarboxylic Acid**; **7**). ^{1}H-NMR, ^{13}C-NMR [46]. ESI-MS: 353.19 ($[M-H]^-$).

Caffeic Acid (= **(2*E*)-3-(3,4-Dihydroxyphenyl)prop-2-enoic Acid**; **8**). ^{1}H-NMR, ^{13}C-NMR [47]. ESI-MS: 178.98 ($[M-H]^-$), 203.02 ($[M+Na]^+$).

Antioxidant Activity

DPPH free-radical scavenging activity was evaluated on a microplate analytical assay according to the previously described procedure by *Srinivasan et al.* [48]. A fresh working solution of DPPH was prepared by dissolving DPPH in EtOH to a final concentration of 100 μM. Incubation with serial dilutions of essential oil, fractions and isolated compounds was performed for 20 min at 37 °C in the dark. Discoloration was measured at 517 nm using the microplate reader FLUOstar Omega (*BMG Labtech GmbH*, Ortenberg, Germany). The free-radical scavenging activity of each solution was then calculated as percent inhibition according to the following equation:

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

Antioxidant activity of the essential oil, fractions, and isolated compounds from *S. sericea* was expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH concentration. Trolox was used as reference. Results are expressed in μM Trolox equivalents (TE)/g of product.

The ABTS stock solution was prepared according to the previously described procedure by *Iqbal et al.* [49]. ABTS (9.8 mg) was dissolved in 3.6 ml of distilled water and manganese oxide (0.6 g) was added to the ABTS solution and allowed to react for 20 min in the dark to form the radical $ABTS^+$. The stock solution was filtered and the working solution was then obtained by diluting $ABTS^+$ stock solution in EtOH to reach a final solution with absorbance of 0.700 OD at 734 nm. Trolox was used as reference. Results are expressed in μM Trolox equivalents (TE)/g of product. The capacity of free-radical scavenging (IC_{50}) was determined using the same previously used equation for the DPPH method. All data of antioxidant activity were expressed as means ± standard deviations (SD) of triplicate measurements.

The FRAP assay was performed on microplate according to *Firuzi et al.* [50], with some modifications. The stock solutions included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6\,H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3 \cdot 6\,H_2O$ solution and then warmed at 37 °C before using. Essential oil, fractions, and isolated compounds from *S. sericea* were allowed to react with FRAP solution for 30 min in the dark. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. Trolox was used as reference. Results are expressed in μM Trolox equivalents (TE)/g of product.

Cytotoxicity Assay

A375 (human malignant melanoma cells) and MDA-MB 231 cells (human breast adenocarcinoma cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS). HCT116 cells (human colon carcinoma cells), were cultured in RPMI 1640 medium with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and supplemented with 10% HI-FBS. Cells were cultured in a humidified atmosphere at 37 °C in the presence of 5% CO_2. The MTT assay was used as a relative measure of cell viability. Cell viability assays were carried out as described [51]. Briefly, cells were seeded at the density of 2×10^4 cells/ml. After 24 h, samples were exposed to different concentrations of essential oil (0.78 – 200 μg/ml) and isolated compounds from polar fraction (0.19 – 25 μg/ml). Cells were incubated for 72 h in a humidified atmosphere of 5% CO_2 at 37 °C. At the end of incubation, each well received 10 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (5 mg/ml in phosphate-buffered saline, PBS) and the plates were incubated for 4 h at 37 °C. The extent of MTT reduction was measured spectrophotometrically at 540 nm using a *Titertek Multiscan* microElisa (*Labsystems*, Helsinki, Finland). Experiments were conducted in triplicate. Cytotoxicity is expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}). The IC_{50} values were determined with GraphPad Prism 4 computer program (*GraphPad Software*, San Diego, CA, USA).

Antimicrobial Activity

Fraction, isolated compounds and essential oil were tested against a panel of microorganisms including

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Candida albicans* ATCC 24433. Bacterial strains were cultured overnight at 37 °C in blood agar plates. *C. albicans* was grown in *Sabouraud* dextrose agar. Tests by the paper disk diffusion method were performed following the *Clinical and Laboratory Standards Institute (CLSI)* guidelines [52]. Briefly, a suspension of the tested microorganism ($1 - 2 \times 10^8$ cells per ml in saline-10^6 per ml for *Candida*) was spread on the solid media plates using a sterile cotton swab. Sterile paper discs (6 mm in diameter) were placed on the surface of inoculated plates and spotted with 10 µl of the essential oil and 1 mg of fractions and isolated compounds dissolved in EtOH (1:1). The plates were incubated 24 h at 35 ± 1 °C (48 h for *C. albicans*). The diameters of zone inhibition (including the 6 mm disc) were measured with a caliper. A reading of more than 6 mm indicated growth inhibition. No zone inhibition was observed using EtOH alone. Ciprofloxacin (5 µg disc) and nystatin (100 Units disc) were used as reference antimicrobials against bacteria and fungi, respectively. Each test was repeated at least twice.

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Received July 13, 2015
Accepted January 25, 2016

