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Chemical Composition and Phytotoxic Activity of *Rosmarinus officinalis* Essential Oil

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In this study we determined the chemical composition of *R. officinalis* essential oil and evaluated its possible phytotoxic activity. The chemical composition of the essential oil was studied by GC and GC-MS analyses. A total of 57 compounds were identified and the main components are α-pinene (24.9%), verbenol (8.5%), verbenone (8.5%), 1,8-cineol (8.2%) and isoborneol (8.1%). Moreover, the essential oil and its main constituents, α-pinene and 1,8-cineol,were evaluated for their possible *in vitro* phytotoxic activity against germination and initial radical growth of radish (*Raphanus sativus* L.), rue (*Ruta graveolens* L.), lettuce (*Lactuca sativa* L.) and tomato (*Solanum lycopersicum* L.). The results showed thatboth germination and radical elongation were sensitive to the oil but not in the same way to α-pinene and 1,8-cineol. The oil influences in different ways radical elongation of *R. sativus, R. graveolens*, and *L. sativa* and the germination of *S. lycopersicum.* Instead α-pinene influenced only radical elongation of lettuce.

Keywords: *Rosmarinus officinalis*, Essential oil, α-Pinene, 1,8-Cineol, Phytotoxic activity.

Rosmarinus officinalis L. (rosemary) is an aromatic plant belonging to Lamiaceae family used for culinary and medicinal proposes, due to its aromatic properties and health benefits [1]. Rosemary is claimed to possess many properties mainly related to the phenolic and the volatile constituents [2]. Different studies demonstrated the antibacterial activity of *R. officinalis* essential oil against both Gram-positive and Gram-negative bacteria [2,3]. Some of its components, such as 1,8-cineol, camphor, and α-pinene, showed different cytotoxic effects depending on the tumour cell type, oil concentration and time of exposition [2,4]. Moreover, rosemary extract presented better antioxidant activity than the ascorbic acid, and inhibits melanoma cell growth [4,5].

Few studies reported the phytotoxic activity of *R. officinalis* essential oil [6-9]. Today, the use of natural substances as biocontrol agents is receiving increased attention due to the potential human and environmental toxicity of synthetic pesticides and herbicides [10] and the available literature indicated the possible use of natural substances as safe phytotoxic agents [11]. In this study we analyzed the chemical composition of *R. officinalis* essential oil and evaluated its possible phytotoxic activity against seeds germination and seedling growth of *Raphanus sativus* L., *Ruta graveolens* L., *Lactuca sativa* L. and *Solanum lycopersicum* L. We evaluated also the possible phytotoxic activity of the main constituents of *R. officinalis* essential oil: α-pinene and 1,8-cineol.

The hydrodistillation of the leaves of *R. officinalis*, harvested in the Campus of the University of Salerno (Campania, Southern Italy) provided an essential oil characterized by a typical odour, in a yield of 0.4% calculated on the fresh weight.

Table 1 shows the chemical composition of the essential oil; compounds are listed according to their elution order on a HP-5MS column. Altogether, 57 compounds were identified and the main components are α-pinene (24.9%), verbenol (8.5%), verbenone (8.5%), 1,8-cineol (8.2%), isoborneol (8.1%), (*Z*)-caryophyllene (7.5%). Other compounds, in a lesser amount are limonene (4.0%), myrtanol (3.5%), *trans*-thujone (3.3%), mentha-2,8-diene (2.5%), dihydrocarveol (2.5%), octen-2-ol (2.0%), *trans*-pinocamphone (1.5%), terpinen-4-ol (1.3%), dihydrocarvone (1.3%), (*Z*)-β-ocimene (1.3%), *p*-cymene (1.1%), mentha-2,4(8)-diene (1.1%).

Rosmarinus officinalis essential oil was evaluated for its activity against germination and radical elongation of radish, rue, lettuce and of tomato, species frequently utilized in biological assays. The oil influences in different ways radical elongation of *R. sativus*, *R. graveolens,* and *L. sativa* but not their germination. In particular, the treatment of *R. graveolens* seeds with a concentration of 100 µg/mL of the essential oil increased radical elongation (Figure 1A). Instead, the treatment of *R. sativus* seeds with the same concentration significantly inhibited the radical elongation (Figure 1B). Moreover, the essential oil affected significantly, at all doses tested, the radical elongation in *L. sativa* (Figure 1C). *R. officinalis* essential oil is not active against radical elongation of *S. lycopersicum* seeds but inhibits their germination at concentrations of 0.1 and 1.0 µg/mL (Figure 3).

The main component of Italian rosemary essential oil is 1,8-cineol with over 40%, in this study we studied another of three chemotypes reported for rosemary (α-pinene, 1,8-cineol and camphor) [12]. In the composition of our *R. officinalis* essential oil, α-pinene (24.9%), verbenol (8.5%), verbenone (8.5%), and 1,8-cineol (8.2%) are the main components. Our results agree with many different studies where it has been reported that α-Pinene and 1,8-cineol dominated in *R. officinalis* essential oil [7, 13-17]. Farhat have found also camphor (14.5-20.4%) and myrtenal (4.3-7.4%) that in our essential oil are present in low percentage or are absent[13]. Odjeda-Sana have reported α-pinene (31.2%) as the main constituent of *R. officinalis* (wide phenotype) essential oil from Argentina; other compounds were 1,8-cineol (21.6%), borneol (4.2%) e camphor (7.0%) [14]. Alipour and Gachkar have reported in two different studies percentages of α-pinene and 1,8-cineol in Iranian *R. officinalis* essential oil similar to those in our essential oil, respectively 27.7% and 14.9% for α-pinene and 9.4% and 7.4% for 1,8-cineol [7,15]. Gachkar *et al.* found also a higher quantity of linalool (14.9%) respect to our essential oil (0.1%) [15]. Hussain have demonstrated that in rosemary essential oil native to Pakistan, there is a major content of 1,8-cineol (38.5%) than α-pinene (12.3%),

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

No.	Compound	%	Ki^a	Ki^b	Identification
1	3-Methyl-pentanol	0.4	759	841	1,2
2	4-Methyl-pentanol	0.1	771	838	1,2
3	Isopentyl formate	T	791	791	1,2
4	*n*-Octane	0.3	746	800	1,2
5	Santolina triene	0.3	897	908	1,2
6	α-Pinene	24.9	917	939	1,2,3
7	4(*Z*)-Hepten-1-ol	0.2	964	966	1,2
8	3(*Z*)-Octen-2-ol	2.0	970	978	1,2
9	*trans-m*-Mentha-2,8-diene	2.5	974	983	1,2
10	3-Octanol	0.1	981	991	1,2
11	δ-2-Carene	0.3	984	1002	1,2
12	α-Terpinene	0.6	990	1017	1,2,3
13	*p*-Cymene	1.1	997	1024	1,2,3
14	Limonene	4.0	1007	1029	1,2
15	1,8-Cineol	8.2	1009	1031	1,2,3
16	(*Z*)-β-Ocimene	1.3	1018	1037	1,2
17	*cis*-Sabinene hydrate	0.1	1048	1070	1,2
18	*p*-Mentha-2,4(8)-diene	1.1	1061	1088	1,2
19	*p*-Cymenene	0.1	1017	1091	1,2
20	Linalool	0.1	1074	1096	1,2,3
21	1,3,8-*p*-Menthatriene	0.5	1079	1110	1,2
22	*trans*-Thujone	3.3	1084	1114	1,2,3
23	dehydro-Sabina ketone	0.2	1091	1120	1,2
24	*exo*-Fenchol	0.1	1095	1121	1,2
25	1-Undecyne	T	1098	1125	1,2
26	*allo*-Ocimene	0.1	1097	1132	1,2
27	Eucarvone	0.6	1100	1150	1,2
28	*neo-allo*-Ocimene	0.5	1106	1144	1,2
29	Pentyl-benzene	T	1113	1156	1,2
30	2-(1Z)-propenyl-phenol	0.1	1115	1150	1,2
31	trans-verbenol	8.5	1120	1144	1,2
32	*p*-Mentha-1,5-diene-8-ol	T	1131	1170	1,2
33	*iso*-3-thujanol	T	1138	1138	1,2
34	Camphor	0.3	1141	1146	1,2,3
35	Isoborneol	8.1	1149	1160	1,2
36	*trans*-Pinocamphone	1.5	1155	1162	1,2
37	Terpinen-4-ol	1.3	1159	1177	1,2,3
38	*p*-Cymen-8-ol	0.1	1170	1182	1,2
39	Myrtenol	0.3	1180	1195	1,2
40	cis-Dihydro-carvone	1.3	1188	1192	1,2
41	Verbenone	8.5	1192	1205	1,2
42	iso-Dihydro-carveol	2.5	1217	1214	1,2
43	(*E*)-Ocimenone	0.1	1234	1238	1,2
44	Carvone	0.8	1253	1243	1,2,3
45	Car-3-en-2-one	0.2	1258	1248	1,2
46	*cis*-Myrtanol	3.5	1267	1253	1,2
47	Thymol	0.2	1278	1290	1,2
48	γ-Terpinen-7-al	T	1300	1291	1,2
49	*transP* acetate	0.1	1306	1298	1,2
50	Dihydro carveol acetate	0.1	1315	1307	1,2
51	Limonene aldehyde	0.4	1343	1328	1,2
52	Isoledene	T	1355	1376	1,2
53	Geranyl acetate	0.9	1365	1381	1,2,3
54	(*Z*)-Caryophyllene	7.5	1398	1408	1,2,3
55	Caryophyllene oxide	0.6	1560	1583	1,2,3
56	Humulene epoxide	0.1	1577	1608	1,2,3
57	Selina-3,11-dien-6-ol	0.2	1635	1644	1,2
	Total	**99.9**			
	Monoterpenes	**40.9**			
	Oxygenated monoterpenes	**46.6**			
	Sesquiterpenes	**7.6**			
	Oxygenated sesquiterpenes	**0.8**			
	Other compounds	**3.8**			

^{a} Linear retention index on a HP-5MS column; ^{b} Linear retention index on a HP Innowax column; ^{c} Identification method: 1 = linear retention index; 2 = identification based on the comparison of mass spectra; 3 = Co-injection with standard compounds.

and an higher percentage of camphor (17.1%) respect to our essential oil [2], instead, *R. officinalis* essential oil analyzed by Spagnoletti *et al*. was mainly characterized by γ-terpinene (35.1%), *p*-cymene (19.8%) and thymol (21.8%) [18], compounds that are present in our essential oil in very low percentage (*p*-cymene 1.1%; thymol 0.2%) or are totally absent such as γ-terpinene.

Also, the chemical composition of Moroccan *R. officinalis* essential oil, analyzed by Bouyahya highlights that the major chemical components are represented by 1,8-cineol (23.6%), camphor (18.7%), borneol (15.5%) and α-pinene (14.1%) [19].

Angelini *et al*. have studied the chemical composition of essential oils of two rosemary ecotypes collected in two divergent Italian hilly environments. The essential oil of rosemary type A contained mainly α-pinene (37.2%), 1,8-cineol (22.6%), camphor (7.1%), camphene (7.2%), and borneol (4.6%), whereas the oil obtained from the type B was characterized by a lesser amount of α-pinene (13.5%), a 2-fold concentration of 1,8-cineol (46.8%), and 3 times as much borneol (12.9%) [6]. The differences between the composition of our essential oil and previous studies could be determined by different genotype and the chemotype can be influenced by several factors such as geographical origin, environmental and agronomic conditions [20]. Moreover, the composition of essential oils from a particular species of plant can differ between harvesting seasons and between different extraction methods [21].

Our data showed that *R. officinalis* essential oil is active on radical elongation of *R. sativus*, *R. graveolens*, and *L. sativa* seeds and on germination of *S. lycopersicum* L. seeds. The concentration of 100 µg/mL increases elongation of rue radicals and inhibits that of radish. Instead, all concentrations inhibited the radical elongation of lettuce oil-concentration-dependently. Only concentrations of 10 and 1 µg/ml inhibited the germination of *S. lycopersicum* L. seeds.

In literature few studies evaluated the phytotoxic activity of *R. officinalis* essential oil [6-9]. Only two of these reports have considered some species that we studied [6,7]. Their results corroborate with ours, in fact *R. officinalis* essential oil reduces root growth in radish and lettuce, but they showed also a decrease of germination percentage [6,7]. Moreover, *R. officinalis* essential oil can reduce significantly germination rate and seedling length of bermudagrass (*Cynodon dactylon* L.), tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perenne* L.) [9].

Instead, as in our experiments, Azirak and Karaman showed that *Rosmarinus officinalis* essential oil was generally ineffective at low concentrations in inhibiting germination of some species (*Alcea pallid* Waldst. & Kit., *Amaranthus retroflexus* L., *Centaurea salsotitialis* L., *Raphanus raphanistrum* L., *Rumex nepalensis* Spreng., *Sinapis arvensis* L. and *Sonchus oleraceus* L.) that grow in field and horticultural crops [8].

In this study we have tested also phytotoxicity of the essential oil main components, α-pinene and 1,8-cineol. In fact, many monoterpenes are potent inhibitors of seed germination and growth of several plant species [22]. In our study α-pinene have reduced radical grow of *R. sativus* (Figure 3) at all concentration tested, but not influenced radical elongation of the other species and their seed germination (data not shown). Instead, other previous studies demonstrated that α-pinene inhibits in species- and concentration-dependent manner seed germination of *Cassia occidentalis*, *Amaranthus viridis*, *Triticum aestivum* [23], the electron transfer chain affecting in this way the energy metabolism of isolated mitochondria from maize coleoptiles and primary roots [24].

Another constituent of essential oils that can influence roots growth and germination rates is 1,8-cineol. This compound decreases germination, inhibits mitosis and may affect plant growth at different stages of early development. Moreover 1,8-cineol can increase stress on the surrounding plants, rendering them more susceptible to other biotic and abiotic stresses [25].

In our experiments 1,8-cineol have not influenced germination and radical elongation of *R. sativus* L., *R. graveolens* L., *L. sativa* L. and *S. lycopersicum* L. (data not shown). Probably the phytotoxic activity of *R. officinalis* essential oil on plant species studied is due to these or other compounds that act synergistically.

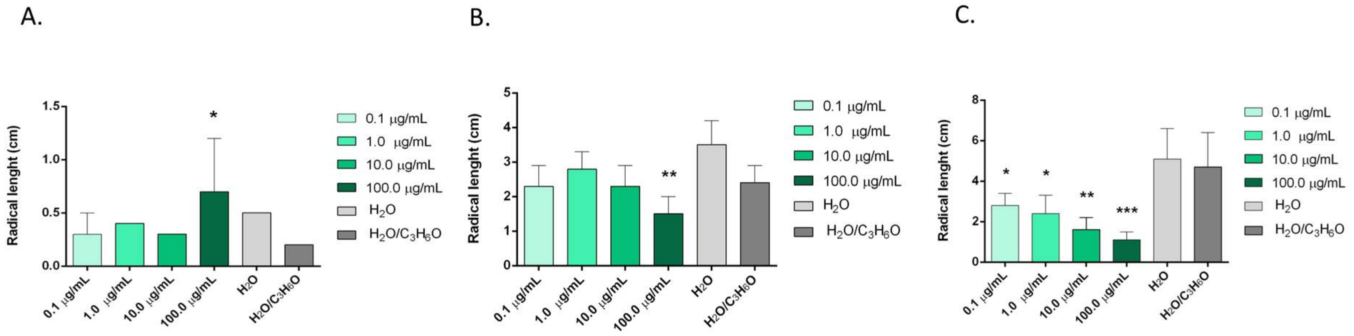

Figure 1: Phytotoxic activity of *R. officinalis* essential oil against radical elongation of *Ruta graveolens* L. (A) and *Raphanus sativus* L. (B), *Lactuca sativa* L. (C),120 h after sowing. Results are expressed as the mean of three experiments ± standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control (ANOVA followed by Dunnett's multiple comparison test).

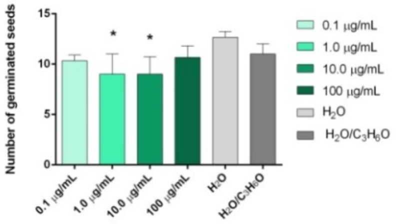

Figure 2: Phytotoxic activity of *R. officinalis* essential oil against seeds germination of *S. lycopersium* L., 120 h after sowing. Results are expressed as the mean of three experiments ± standard deviation. * $p < 0.05$, compared to control (ANOVA followed by Dunnett's multiple comparison test).

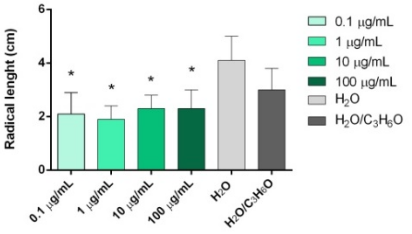

Figure 3: Phytotoxic activity of α-pinene against radical elongation of *Raphanus sativus* L. 120 h after sowing. Results are expressed as the mean of three experiments ± standard deviation. * $p < 0.05$, compared to control (ANOVA followed by Dunnett's multiple comparison test).

Experimental

Plant material: *R. officinalis* aerial parts were collected in September 2017, in the Campus of the University of Salerno. Representative homogeneous samples of this population werecollected during the balsamic time. The plant was identified by Prof. V. De Feo, on the basis of Flora d'Italia [26] and a voucher specimen has been deposited in the herbarium of the Medical Botany Chair of the University of Salerno.

Essential Oil Extraction: One kilogram of fresh aerial parts were ground in a Waring blender and then subjected to hydrodistillation for 3 h according to the standard procedure described in the European Pharmacopoeia [27]. The oil was solubilized in *n*-hexane, filtered over anhydrous sodium sulphate and stored under N_2 at +4°C in the dark, until analyzed and tested.

GC-FID Analysis: Analytical gas chromatography was carried out on a Perkin-Elmer Sigma-115 gas-chromatograph (Perkin-Elmer, Waltham, MA, USA) equipped with a FID and a data handling processor. The separation was achieved using a HP-5 MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Column temperature: 40°C, with 5 min initial hold, and then to 270°C at 2°C/min, 270°C (20 min); injection mode splitless (1 µL of a 1:1000 *n*-hexane solution). Injector and detector temperatures were 250°C and 290°C, respectively. Analysis was also run by using a fused silica HP Innowax polyethylene glycol capillary column (50 m × 0.20 mm i.d., 0.25 µm film thickness). In both cases, helium was used as carrier gas (1.0 mL/min).

GC/MS Analysis: Analysis was performed on an Agilent 6850 Ser. II apparatus (Agilent, Roma, Italy), fitted with a fused silica DB-5 capillary column (30 m × 0.25 mm i.d., 0.33 µm film thickness), coupled to an Agilent Mass Selective Detector MSD 5973 (Agilent); ionization energy voltage 70 eV; electron multiplier voltage energy 2000 V. Mass spectra were scanned in the range 40–500 amu, scan time 5 scans/s.Gas chromatographic conditions were as reported in the previous paragraph; transfer line temperature, 295°C.

Identification of the Essential Oil Components: Most constituents were identified using gas chromatography by comparison of their Kovats retention indices (RI) (determined relative to the tR of *n*-alkanes (C_{10}–C_{35})), with either those of the literature [28-31] and mass spectra on both columns or those of authentic compounds available in our laboratories by means of NIST 02 and Wiley 275 libraries [32]. The components' relative concentrations were obtained by peak area normalization. No response factors were calculated.

Phytotoxic Activity: The phytotoxic activity was evaluated on germination and root elongation of four plant species: radish (*Raphanussativus* L.), rue (*Rutagraveolens* L.), lettuce (*Lactuca sativa* L.) and tomato (*Solanum lycopersicum* L.).These seeds are usually used in assays of phytotoxicity because they easily germinate and are well known from the histological point of view. The seeds of radish and rue were purchased from Tesoro della terra srl (Andria, Bari), the seeds of lettuce from Pagano srl (Scafati, Salerno), the seeds of tomato from Euroselect (Bari). The seeds were surface sterilized in 95% ethanol for 15 s and sown in Petri dishes (Ø = 90 mm), containing three layers of Whatman filter paper, impregnated with distilled water (7 mL, control) or the tested solution of the essential oil (7 mL), at the different doses. The germination conditions were 20 ± 1°C, with natural photoperiod. The essential oils, in water–acetone mixture (99.5:0.5), were assayed at the doses of 100, 10.0, 1.0 and 0.1 µg/mL. Controls performed with the water-acetone mixture alone showed no differences in comparison to controls. Seed germination was observed directly in Petri dishes every 24 h. A seed was considered germinated when the protrusion of the root became evident [33]. After 120 h (on the fifth day), the effects on radical elongation were measured in cm. Each determination was repeated three times, using Petri dishes containing 10 seeds each. Data are expressed as the mean ± SD for both germination and radical elongation.

Statistical Analysis: All experiments were carried out in triplicate. Data of each experiment were statistically analysed using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA), followed by comparison of means (one-way ANOVA) using Dunnett's multiple comparisons test, at the significance level of $p < 0.05$.

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