Essential Oil Composition and Allelopathic Effect of the Brazilian Lamiaceae *Hesperozygis ringens* (Benth.) Epling and *Hesperozygis rhododon* Epling†

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Samples of *Hesperozygis ringens* (Benth.) Epling and *Hesperozygis rhododon* Epling essential oils were analyzed by a combination of analytical techniques: capillary gas chromatography, liquid/solid chromatography, GC/MS coupling, and NMR spectroscopy. Twenty-four components have been identified representing altogether more than 95% of the oil content. The oil of *H. ringens* is constituted mainly by pulegone (79.2%) accompanied by several oxygenated derivatives (pulegone oxides, 1.2%; 8-hydroxy-*p*-menth-3-one, 1.3%; and 8-hydroxy-*p*-menth-4-en-3-one, 3.7%); that of *H. rhododon* contains menthone and pulegone as main compounds in almost comparable amounts (43.4% and 29.6%, respectively). Tests carried out on lettuce seeds using alcoholic extracts of the two species showed significant antigerminating properties mainly for *H. ringens*. The same activity was observed with its essential oil.

Keywords: *Hesperozygis ringens (Benth.) Epling; Hesperozygis rhododon Epling; Lamiaceae; essential oil composition; pulegone; menthone*

The subfamily Nepetoideae of the Lamiaceae is characterized by the presence of a high content of essential oils (Cantino and Sanders, 1986), several of them being commercially important sources of raw material for personal care, food, and cosmetic industries; the most common Lamiaceae oils are those obtained from the genera *Mentha*, *Rosmarinus*, *Thymus*, and *Lavandula* (Heinrich, 1992; Lawrence, 1992).

Hesperozygis Epling is a genus with about six species, five of them restricted to southeastern Brazil (Pereira and Pereira, 1973) and one cited for Mexico (Cantino and Sanders, 1986). *Hesperozygis ringens* is a woody herb native in rocky fields of southeastern Rio Grande do Sul; although it occurs sparsely in the region, it is the dominant species in the sites where it exists, a feature that may indicate some allelopathic action. The plant is employed for its antiparasitic properties and is known by the vernacular name "espantapulga" (a name that literally means "to keep fleas away"). *Hesperozygis rhododon* is a shrub that grows in rocky fields at the top of mountains along the Serra do Mar formation in the states of Paraná and São Paulo; no dominance was observed in the site of collection, although the species was very frequent. Neither vernacular name nor popular use are known.

The aim of this work is to compare the essential oil chemical composition of the two species and verify the allelopathic potential of them.

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EXPERIMENTAL PROCEDURES

Plant Material. Samples of *Hesperozygis* species were collected in 1993 in Caçapava do Sul, Rio Grande do Sul, and Quatro Barras, Paraná, Brazil. The plants were identified by M. Sobral, and voucher specimens were deposited at the herbarium of the Universidade Federal do Rio Grande do Sul (ICN).

Preparation of Essential Oils. Fresh leaves were subjected to hydrodistillation for 5 h using a Clevenger-type apparatus.

Gas−Liquid Chromatography. The compounds were first tentatively identified by peak enrichment and their GC retention indices on two fused silica gel capillary columns (25 m × 0.25 mm i.d. coated with OV-101, film thickness 0.25 μm; 25 m × 0.22 mm i.d. coated with Carbowax 20 M, film thickness 0.25 μm), using a chromatograph (Shimadzu GC-14A) equipped with a Shimadzu C-R4A Chromatopac integrator. Detector and injector temperatures were set at 250 and 210 °C, respectively; the oven temperature was programmed from 50 to 200 °C at 5 °C min^{-1}, with nitrogen as carrier gas. The percentage compositions were obtained from electronic integration measurements using flame ionization detection without taking into account relative response factors.

Gas−Liquid Chromatography/Mass Spectrometry. All the samples were then analyzed by GC/MS, using a Hewlett-Packard capillary GC quadrupole MS system (Model 5970) fitted with a 25 m × 0.23 mm i.d. fused silica gel column coated with DB-1, film thickness 0.25 μm; temperature was programmed as follows: 60 °C (1 min), 60−250 °C (5 °C min^{-1}). Helium was used as carrier gas at a flow rate of 0.9 mL min^{-1}; the mass spectrometer was operated at 70 eV.

The identification of nearly all compounds was based on a comparison of retention indices and mass spectra with those of commercial samples and with literature data (Stenhagen *et al.*, 1974; Adams, 1989; Jennings and Shibamoto, 1980); four of them were compared to authentic samples obtained by isolation or hemisynthesis, and their identification was confirmed on the basis of their NMR data.

Isolation. *8-Hydroxy-p-menth-4-en-3-one (**26**).* **26** was isolated by liquid/solid chromatography of *H. ringens* essential

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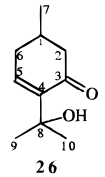
Table 1. Percentage Composition of *H. ringens* and *H. rhododon* Essential Oils from Brazil

peak	component	IK_{OV-101}	identification	relative abundance	
				H. ringens	*H. rhododon*
1	α-pinene	935	IK, MS	0.5	0.6
2	6-methylhept-5-en-2-one	968	IK, MS		0.2
3	sabinene	970	IK, MS	0.2	0.1
4	*β*-pinene	974	IK, MS	0.4	0.6
5	myrcene	985	IK, MS		0.9
6	*p*-cymene	1016	IK, MS		0.1
7	limonene	1025	IK, MS	0.7	4.2
8	1,8-cineole	1025	IK, MS	0.2	0.2
9	*cis*-linalool oxide	1063	IK, MS	0.1	
10	*trans*-linalool oxide	1078	IK, MS	0.1	
11	linalool	1088	IK, MS	1.4	0.3
12	unknown[a]	1096	IK, MS		5.7
13	unknown[a]	1100	IK, MS		0.3
14	unknown[a]	1138	IK, MS	0.5	
15	menthone	1148	IK, MS	0.3	43.4
16	isomenthone	1151	IK, MS		2.2
17	isopulegone	1157	IK, MS	1.2	2.4
18	terpinen-4-ol	1169	IK, MS		0.2
19	α-terpineol	1178	IK, MS	0.1	0.1
20	unknown[a]	1186	IK, MS	0.5	
21	unknown[a]	1200	IK, MS	0.5	
22	(+)-pulegone	1226	IK, MS, NMR	79.2	29.6
23	8-hydroxy-*p*-menthan-3-one	1231	IK, MS	1.3	
24	*trans*-pulegone oxide	1237	IK, MS, NMR	0.6	
25	unknown[a]	1247	IK, MS	0.4	
26	8-hydroxy-*p*-menth-4-en-3-one	1264	IK, MS, NMR	3.7	
27	menthyl acetate	1267	IK, MS		2.2
28	bornyl acetate	1267	IK, MS		0.7
29	*cis*-pulegone oxide	1275	IK, MS, NMR	0.6	
30	isomenthyl acetate	1282	IK, MS		0.1
31	unknown[a]	1334	IK, MS	1.1	
32	terpenyl acetate	1338	IK, MS		0.1
33	unknown[a]	1382	IK, MS	1.7	1.6
34	unknown[a]	1395	IK, MS	0.3	
35	caryophyllene oxide	1575	IK, MS	0.9	0.2

[a] Mass spectra data, Table 2.

oil on silica gel 60 (Merck; 70−230 mesh ASTM) eluted with a hexane−Et_2O gradient. MS *m/z* (rel intensity): 153 (100), 97 (30), 43 (28), 135 (15), 168 (1) $[M]^+$. ^1H NMR (200 MHz, $CDCl_3$): *δ* 6.9 (1 H, *m*, H_5), 4.4 (1 H, *s*, -O-H), 2.5 (2 H, *d*, H_2), 2.15 (2 H, *m*, H_6), 2.05 (1 H, *m*, H_1), 1.4 (6 H, *s*, H_{9-10}), 1.05 (3 H, *d*, H_7, $J = 5$ Hz). ^{13}C NMR ($CDCl_3$): *δ* 202.2 (C_3), 143.6 (C_4, C_5), 65.8 (C_8), 47.5 (C_2), 34.2 (C_6), 30.1 (C_1), 29.0 (C_{10}, C_9), 21.0 (C_7). MS spectral and ^1H NMR data were comparable with those obtained by Nagasawa *et al*. (1975) for the same compound isolated from essential oil of *Mentha gentilis* L.



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*Pulegone (**22**).* **22** was isolated by liquid/solid chromatography of *H. ringens* essential oil on silica gel 60 (Merck; 70−230 mesh ASTM) eluted with a hexane−Et_2O gradient. $[\alpha]_D$ (CH_2Cl_2) = 14.8° (lit. $[\alpha]_D$ (CH_2Cl_2) = 23.4°). The difference observed between the two values seems to indicate that the pulegone present in the essential oil is not enantiomerically pure.

Hemisynthesis. *trans-Pulegone Oxide (**24**) and cis-Pulegone Oxide (**29**).* These were prepared by epoxidation of pulegone; this compound (200 mg) was added to a chloroform solution of *m*-chloroperbenzoic acid (800 mg in 10 mL), and the reaction mixture was left for 24 h at room temperature with occasional stirring. This reaction mixture was washed with 10% sodium bicarbonate followed by water and then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure, and the residue was analyzed by gas chromatography and GC/MS. The crude product consisted of a mixture of the diastereoisomeric pulegone oxides (59% of *trans*-pulegone oxide, **24**, and 41% of *cis*-pulegone oxide, **29**). We assume, in agreement with the previous findings of Reusch and Johnson (1963) and Katsuhara (1967), that the most abundant diastereoisomer has the *trans* configuration.

24: MS *m/z* (rel intensity) 153 (100), 43 (40), 70 (35), 86 (34), 111 (32), 168 (22) $[M]^+$, 125 (20). **29**: MS *m/z* (rel. intensity) 153 (100), 86 (44), 111 (33), 43 (32), 70 (31), 168 (22) $[M]^+$; ^{13}C NMR (**24**−**29** mixture) *δ* 207.5, 206.5 (C_3), 70.2, 70.1 (C_4), 63.4, 63.2 (C_8), 51.3, 49.5 (C_2), 33.9, 30.6 (C_1), 32.9, 30.1 (C_5), 29.9, 26.2 (C_6), 22.0, 18.9 (C_7, C_9, C_{10}). The nuclear magnetic resonance spectrum of the mixture **24**−**29** may be correlated to those obtained by Reusch and Johnson (1963) for the two stereoisomers.

Isopulegol Oxides. These were obtained from commercial isopulegol (400 mg; Fluka) by epoxidation with *m*-chloroperbenzoic acid (see above). The crude product analyzed contained the four stereoisomers expected with two major components in almost equal proportion (∼90% of the mixture) characterized by identical mass spectra. MS *m/z* (rel intensity): 43 (100), 93 (98), 81 (80), 108 (72), 55 (70), 123 (60), 155 (25), 170 (3) $[M]^+$.

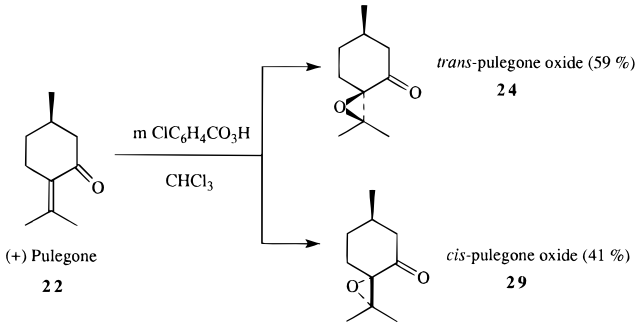
8-Hydroxymenthol. This was obtained by reduction of isopulegol oxides (300 mg) by a suspension of $AlLiH_4$ (30 mg) in 30 mL of anhydrous Et_2O. After heating at 40 °C for 1 h, the mixture was hydrolyzed, and the analysis was performed by GC/MS. 8-Hydroxymenthol (IK_{OV-101} = 1339) was the major component of the obtained mixture (70%). MS *m/z* (rel intensity): 81 (100), 59 (90), 43 (70), 96 (40), 41 (35), 54 (30).

*8-Hydroxy-p-menthan-3-one (**23**).* **23** was obtained by oxidation of 8-hydroxymenthol (200 mg) by 0.3 mL of a solution of chromium trioxide (10.3 g) in water (30 mL) and concentrated sulfuric acid (8.7 mL) (Bowden *et al*., 1946). MS *m/z* (rel intensity): 43 (100), 112 (80), 59 (70), 70 (65), 97 (40), 55 (35), 155 (15).

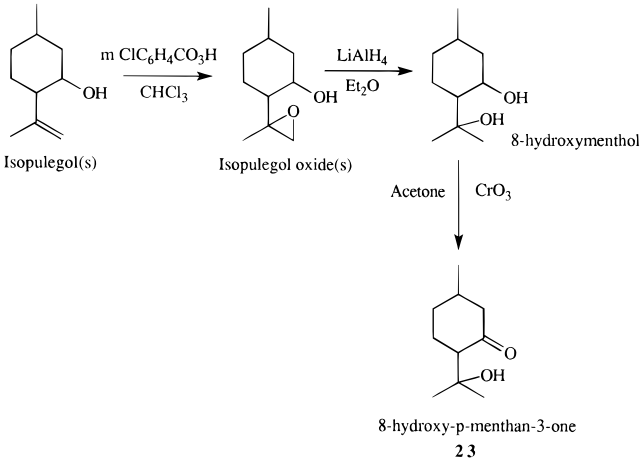
Table 2. Mass Spectral Data of Unidentified Compounds of *H. ringens* and *H. rhododon* Essential Oils

compd	IK_{OV-101}	MW	*m/z* (rel intensity, 70 eV)
12	1096	?	43 (100), 99 (58), 54 (20), 128 (17), 72 (14), 81 (14), 67 (13), 55 (12)
13	1100	?	43 (100), 101 (32), 83 (19), 112 (16), 41 (13), 55 (12)
14	1138	154	139 (100), 43 (89), 59 (31), 81 (30), 121 (27), 95 (19), 41 (15), 55 (14)
20	1186	150	150 (100), 79 (74), 35 (56), 80 (55), 91 (26), 107 (24), 108 (22), 77 (22)
21	1200	168	125 (100), 70 (69), 69 (61), 55 (55), 41 (55), 42 (46), 83 (37), 43 (35)
25	1247	168	153 (100), 43 (36), 86 (34), 41 (31), 70 (29), 101 (29), 83 (27), 168 (25)
31	1334	184	70 (100), 43 (56), 41 (42), 69 (40), 59 (38), 98 (34), 71 (32), 42 (31)
33	1382	?	43 (100), 152 (74), 109 (57), 81 (44), 69 (40), 112 (28), 137 (26), 41 (25)
34	1395	184	70 (100), 169 (98), 43 (85), 84 (82), 127 (78), 59 (68), 41 (63), 69 (54)

Scheme 1



Scheme 2



Germination and Growth Inhibitory Activity. Extracts of *H. ringens* and *H. rhododon* were obtained by maceration of dried and powdered leaves (20 g) in 200 mL of ethanol (95%) for 5 days. The solution was concentrated to 20 mL in vacuo. Test solutions for bioassays were prepared by dilutions of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 (v/v) with ethanol and distributed by lots of 2 mL in Petri dishes (diameter 6 cm) lined with filter paper. After solvent evaporation, 2 mL of water was added. Each dish was sown with 50 seeds of lettuce and incubated at 22 °C for 5 days. For each dilution tested, at least five Petri dishes were used. Germinated seeds were counted and compared with the reference. The hypocotyl growth and radicle growth were also observed. Distilled water was used instead of the test solution for the reference.

The same experiment was carried out with the essential oil of *H. ringens*. A volume of 1.4 mL of the essential oil was dissolved in 200 mL of *n*-hexane, and test solutions at final concentrations of 17, 14, 10, 7, 5, 3, and 2 μL in oil were distributed over paper filters in Petri dishes.

RESULTS AND DISCUSSION

Hydrodistillation of fresh leaves of *H. ringens* and *H. rhododon* gave essential oils in 4% and 1% yields, respectively. Altogether 26 components were identified in the samples analyzed, as can be observed in Table 1. The mass spectral data of nine unidentified constituents are given in Table 2.

The volatile oil of *H. ringens* is constituted mainly of pulegone (79.2%) accompanied by several oxygenated derivatives, the structures of which have been established by hemisynthesis and instrumental analysis; *cis*- and *trans*-pulegone oxides (**24** and **29**) and 8-hydroxy-*p*-menthan-3-one (**23**) were prepared as shown in Schemes 1 and 2, and their identification was confirmed on the basis of their NMR data. 8-Hydroxy-*p*-menth-4-en-3-one (**26**) was isolated by liquid/solid chromatography and characterized by MS as well as ^{13}C and ^{1}H NMR spectroscopy. The prevalence of pulegone, which shows potent insecticide activity against insects (Simmonds and Blaney, 1992), can explain the popular utilization of this plant for eliminating parasites.

The volatile oil of *H. rhododon* contains menthone (43.4%) as the major constituent, while pulegone represents only 29.6% of the chemical composition. Limonene is also present in 4.2%.

Table 3. Mean Germination of Lettuce Seeds ($n = 50$) Exposed to Different Dilutions of Alcoholic Extracts of *H. ringens* and *H. rhododon*[a]

	germination	
dilutions	*H. ringens*	*H. rhododon*
control	44.086 a	42.960 a
1:64	12.714 b	22.200 b
1:32	5.486 c	13.080 c
1:16	0.629 d	3.160 d
1:8	0.000 d	0.320 d
1:4	0.000 d	0.000 d
1:2	0.000 d	0.000 d

[a] Means within a column sharing the same letter are not significantly different at the 0.05 probability level according to the Tukey test.

After establishing the types of terpenes and their relative quantities in the essential oils of these two species, we investigated the potential of their alcoholic extracts in inhibiting the germination of lettuce seeds. The radicle protrusion was taken as parameter for the experiments.

In all concentrations tested, the extracts affected the lettuce radicles; even in the highest dilution (1:64) the tip of the radicles showed some necrosis. In lower dilutions (1:32 and 1:16) the radicles became shorter and died; at dilutions lower than 1:16, the radicles died immediately after emergence or do not emerge at all. Dilutions as low as 1:32 were inhibitory to hypocotyl growth.

The alcoholic extracts of the plants exhibited statistically significant inhibitory effect ($p > 0.05$) on seed germination in all concentrations assayed. The dilutions of 1:64, 1:32, and 1:16 give results significantly different from each other; dilutions 1:16 and smaller could not be statistically distinguished (Table 3). On the other hand, the two experiments displayed different inhibitory action; germination inhibition induced by *H. ringens* was significantly higher than that of *H. rhodo-*

Table 4. Comparison between the Inhibitory Effects of Alcoholic Extracts of *H. ringens* and *H. rhododon*[a]

species	mean germination of lettuce seeds
H. ringens	11.674 a
H. rhododon	7.037 b

[a] Means within a column sharing the same letter are not significantly different at the 0.05 probability level according to the Tukey test.

Table 5. Mean Germination of Lettuce Seeds ($n = 50$) Exposed to Different Concentrations of Essential Oil of *H. ringens*[a]

concn (μL)	germination	concn (μL)	germination
control	33.280 a	10	0.360 c
2	20.000 b	14	0.000 c
3	20.520 b	17	0.000 c
5	14.360 b		
7	6.360 c		

[a] Means within a column sharing the same letter are not significantly different at the 0.05 probability level according to the Tukey test.

don (Table 4). In the experiment with the essential oil of *H. ringens*, all the concentrations tested inhibited significantly lettuce seeds germination, as well as induced damages similar to those of the extracts in the seedlings that emerged (Table 5).

The above experiments suggest that the strong allelopathic effects in the germination and radicle growth in the species tested are probably due to the presence of high amounts of pulegone, a recognizedly allelopathic substance (Fischer, 1991). Moreover, the more pronounced effect of *H. ringens* extracts may be due not only to the high amount of pulegone but also to the higher concentrations of essential oils than *H. rhododon*. It is well known that monoterpene vapors may cause anatomical and physiological changes in plant seedlings and exposure to volatile terpenes can lead to accumulation of lipid globules in the cytoplasm, reduction in organelles including mitochondria, and disruption of membranes surrounding mitochondria and nuclei (Lorber and Muller, 1976). Comparative phytotoxic effects of aqueous solutions of a series of open-chain, monocyclic, and bicyclic monoterpenes upon lettuce seed germination showed that unsaturated hydrocarbons required higher concentration to reduce germination of lettuce. Open-chain alcohols and monocyclic and bicyclic alcohols were significantly more active, and the highest phytotoxic effects were observed for cyclic α,β-unsaturated ketones (Reynolds, 1987).

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