Composition and Antifungal Activity of Two Essential Oils of Hyssop (*Hyssopus officinalis* L.)

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Abstract

The two essential oils isolated from plants of hyssop (*Hyssopus officinalis* L. ssp. *officinalis*) grown in two different localities near Urbino (Marche, Italy) were analyzed by GC and GC/MS. The major components were pinocamphone (34% and 18.5%), isopinocamphone (3.2% and 29%) and β-pinene (10.5% and 10.8%). The major differences in the composition of the oils were detectable in the ratio of pinocamphone/isopinocamphone, in the percentage of linalool (0.2% and 7.9%) and camphor (0.3% and 5.3%).The oils showed antifungal activity against 13 strains of phytopathogenic fungi; the activity of the oil extracted from plants grown at 1000 m above sea level showed higher activity.

Key Word Index

Hyssopus officinalis, Lamiaceae, hyssop, essential oil composition, β-pinene, pinocamphone, isopinocamphone, antifungal activity.

Introduction

Hyssop, *Hyssopus officinalis* L., is a perennial and polimorphous species belonging to the Lamiaceae family, native to the Mediterranean area, southern Europe and Asia Minor and cultivated in the United States and Russia (1).

The flowering tops produce a pleasant essential oil responsible for most of the biological activities of the plant. The aerial part of the plant is used for its aromatic scent and is generally employed in perfumery, in the food industry as flavoring agent, in liqueurs and cosmetic products, and in the pharmaceutical field in several antiseptic preparations (2).

A crude extract of dried leaves has been reported to have an anti-HIV activity (3), and the aerial parts are ingredients in blood-pressure-increasing preparations and tea blends for cough and asthma relief. The internal use of *H. officinalis* preparations is limited by their irritating action on mucous membranes; some caution is necessary in its use because of the presence of terpenic ketones such as pinocamphone and isopinocamphone, which have been linked to convulsive poisonings (4). These bicyclic monoterpene ketones are generally known as the main characteristic components of the oil of the *Hyssopus* genus (1).

Literature reports that the chemical composition of *H. officinalis* oil shows a great variability that can be related to many factors (5-11).

The in vitro antimicrobial and cytotoxic activity of the oil of *H. officinalis* was shown on *Staphylococcus aureus* and *Streptococcus sanguis* by Renzini et al. (12). In another study conducted by Mazzanti et al. (13), the effect of *H. officinalis* oil was tested on several Gram positive and Gram negative bacteria, and on strains of *Candida albicans*, *C. krusei*, and *C. tropicalis*: the antimicrobial activity of the oil was negligible, while all yeasts were strongly inhibited. The antifungal activity of *H. officinalis* oil was also tested on *Pyrenophora avenae* and *Pyricularia oryzae*, phytopatogenic fungi: the mycelial growth of the two fungi was completely inhibited by 0.4% hyssop oil (14). The same oil induced alterations in both growth and lipid composition in *Aspergillus fumigatus* (15).

Considering these results, in our study we assayed the composition of the oils of two specimens of *H. officinalis* grown in different conditions and tested the antifungal activity of these oils on several phytopatogenic fungi. The growth of some of these same fungi were found to be inhibited by the oils obtained from several aromatic plants of the Lamiaceae family in a previous study conducted in our laboratory (16).

Experimental

Plant material: Seeds of *H. officinalis* L. ssp. *officinalis*, kindly supplied by the "Giardino delle piante officinali di

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Casola Valsenio (Ravenna, Italy)," were allowed to germinate in the Botanical Garden of the University of Urbino. After two months, the plantlets were transferred to experimental fields located, respectively, 100 m and 1000 m above sea level (loc. Pesaro and Monte Nerone, Pesaro-Urbino). The flowering tops of the three-year-old plants were collected in the middle of September at full flowering stage. A specimen of each collection has been deposited in the Herbarium of the Botanical Garden of the University of Urbino.

Oil isolation: The fresh flowering tops were steam distilled for 3 h in a Clevenger-type apparatus, until the material was exhausted. The yields (v/w) were 2.30 and 3.1 mL/kg for plants grown at 100 m and 1000 m, respectively.

Oil analysis: Analyses of the oils were carried out by GC and GC/MS. GC analyses were performed on a Hewlett-Packard gas chromatograph, model 5890, equipped with a flame ionization detector (FID) and coupled to an electronic integrator. The chromatograph was fitted with a methyl silicone column (12.5 m x 0.25 mm, 0.25 μm film thickness). GC analytical conditions were: carrier gas, He; flow rate, 0.9 mL/min; and injector and detector temperatures, 280°C and 250°C, respectively. The oven temperature was programmed from 50°-270°C at a rate of 4°C/min. Quantitative data were obtained by electronic integration of FID area data without the use of response factor correction.

GC/MS analyses were performed using a Hewlett-Packard 6890 chromatograph combined with HP ChemStation Software, equipped with an HP 5973 mass selective detector. Operation conditions were: carrier gas, He; ionization voltage, 70 eV; scanning speed, 1 sec over 40-300 amu range; and an ion source temperature, 180°C. The column and conditions of use were the same as above.

Compounds were identified by comparison of their GC retention indices calculated by linear interpolation relative to retention times of a series of n-alkanes (C_7 - C_{25}) with those reported in the literature (16), and by comparison of mass spectra from the Nist98 Mass Spectral Database.

The analyses were repeated three times with the same quantity of material and the same analytical method and values reported represent the average of three experiments.

Test organisms: Fungal plant pathogens used in the tests were: *Fusarium culmorum* (Smith) Saccardo (ATCC 12656), *Fusarium graminearum* Schwabe (ATCC 15624), *Fusarium poae* (Peck) Wollenweber (ATCC 24383), *Fusarium avenaceum* (Corda: Fries) Saccardo (ATCC 24362), *Fusarium equiseti* (Corda) Saccardo (ATCC 11853), *Fusarium semitectum* Berkeley et Ravenel (ATCC 15659), *Fusarium sporotrichoides* Sherbakoff (ATCC 24630), *Fusarium oxysporum* Schl., *Phytophtora capsici* Leon., *Botrytis cinerea* Pers., *Rhyzoctonia solani* Ell. et Mart., *Phytophtora cryptogea* Pethyb. et Laff. and *Alternaria solani* Ell. et Mart.

The last six strains of fungi were kindly supplied by Davide Pancaldi, DI. PRO. VAL (Facoltà di Agraria), Università di Bologna.

Antimicrobial testing: The agar dilution method for determining inhibitory concentration (MIC) is as follows. The phytopathogenic fungi were tested by an agar dilution method in the appropriate culture media (PDA, Difco). The oils were dissolved in absolute ethyl alcohol and 5% Tween 20 (Fluka), and added to the culture medium at a temperature of 40°-45°C, then poured into Petri dishes (9 cm diameter) (18) — concentrations of 50 ppm, 100 ppm, 200 ppm, 400 ppm, 600 ppm, 800 ppm, 1600 ppm, 3200 ppm and 5000 ppm were tested.

The fungi were inoculated as soon as the medium had solidified. A disc (0.5 cm diameter) of mycelial material, taken from the edge of seven-day-old fungal cultures, was placed at the center of each Petri dish. Controls consisted of 50 ppm, 100 ppm, 200 ppm, 400 ppm, 600 ppm, 800 ppm, 1600 ppm, 3200 ppm and 5000 ppm of ethyl alcohol + 5% Tween 20 mixed with PDA. The Petri dishes with the inoculum were placed in the dark under controlled temperature conditions of 22 ± 1°C.

The efficacy of treatment was evaluated after seven days by measuring the diameter of the fungal colonies when all the free surface of the medium in the control Petri dishes had been covered. The values were expressed in terms of percent inhibition of growth compared to control = 100 (19).

The fungicidal activity of the oils was determined using the technique of Thompson (20) and Carta and Arras (21): the mycelial disks were transferred from Petri dishes in which no growth was observed (total inhibition = 100) onto fresh plates of PDA, in order to verify, after seven days, the fungistatic or fungicidal activity of such inhibition. All experiments were carried out in triplicate.

Determination of minimal inhibitory concentration: The MIC values (μg/mL) were determined by the dilution method in solid medium. For oil A (*H. officinalis* grown at 100 m), dilutions of the emulsions of oil were made in the culture medium over the concentration range of 200 ppm (200 μg/mL) and 400 ppm (400 μg/mL) for *Rhyzoctonia solani*, of 400 ppm (400 μg/mL) and 600 ppm (600 μg/mL) for *Phytophtora capsici*, and of 800 ppm (800 μg/mL) and 1600 ppm (1600 μg/mL) for all other fungal strains. For oil B (*Hyssopus officinalis* grown at 1000 m), the conditions were the same. MICs were determined as the concentration with no visible growth. All experiments were performed in triplicate. If the MICs results were different, only the higher value obtained was noted.

Determination of minimal fungicidal concentrations: MCFs were obtained using a membrane filtration method (22) and were calculated from 400 ppm to 5000 ppm with steps of 400 ppm using the following concentrations: 0.8 mg/mL, 1.2 mg/mL, 1.6 mg/mL, 2.0 mg/mL, 2.4 mg/mL, 2.8 mg/mL, 3.2 mg/mL, 3.6 mg/mL, 4.0 mg/mL, 4.4 mg/mL and 4.8 mg/mL. After homogenization, each fungal inoculum (1 mL) (10^8 spores/mL) was added to 10 mL of oil emulsion; the mixture was kept at room temperature for a contact time of 15 min. After the contact time, 1 mL of the mixture was transferred into a sterile filtration apparatus (MILLIPORE SWINNEX-25), washed twice with 100 mL of the neutralizing solution consisting of distilled water containing 10% Tween 20 sterilized at 121°C for 20 min, filtered, and rinsed with sterile distilled water. The membranes were separately laid out on the middle of the Petri dishes containing the corresponding agar medium (PDA). After incubation, the colonies were counted on the membranes. Previously, we

Table I. Percentage composition of two samples of hyssop oil

Compound	RI	Oil A	Oil B	Compound	RI	Oil A	Oil B
α-pinene	942	2.1	2.1	α-terpineol	1189	0.1	0.5
sabinene	973	0.4	0.4	linalyl acetate	1257	2.9	0.8
E-pinene	980	10.5	10.8	E-caryophyllene	1418	5.6	2.4
myrcene	986	3.5	1.6	α-humulene	1453	3.2	1.9
α-phellandrene	1003	7.4	9.6	germacrene D	1480	5.1	3.3
α-terpinene	1016	1.9	0.7	α-cadinene	1513	2.9	t
linalool	1097	0.2	7.9	*cis*-calamenene	1521	0.8	t
camphor	1136	0.3	5.3	E-cadinene	1537	3.8	2.4
pinocamphone	1152	34.0	18.5	bicyclogermacrene	1556	1.6	1.4
isopinocamphone	1160	3.2	29.0	spathulenol	1576	2.3	1.4

t = trace

checked that the mixture ethanol plus 5% Tween 20 did not inhibit the germination of spores. For fungi that did not produce spores in vitro (*Fusarium oxysporum* and *Phytophtora capsici*), MFCs were determined by putting them in contact with the oil emulsion discs of mycelial material (0.5 mm diameter) taken from the edge of seven-day-old fungal colonies. Each disc was kept for 15 min in 1 mL of oil emulsion at room temperature, washed with 2 mL of the neutralizing solution (see above), and rinsed with sterile distilled water in order to remove excess Tween 20. MFCs were calculated as the lowest concentrations of oil, which inhibited the recovery of fungus. Previously, we checked that the mixture of ethanol plus 5% Tween 20 did not inhibit the mycelial growth of the two fungi.

Linalool and camphor (used to determine MICs and MFCs of the pure major components of the oil) were purchased from Sigma. (±) Isopinocamphone was obtained by PDC (pyridinium dichromate) (23) mediated oxidation of commercially available (±)-isopinocampheol (Fluka). In order to obtain pure pinocamphone, isopinocamphone was treated at reflux with 5% methanolic KOH. GC/MS analysis of the reaction mixture revealed the presence of 75:25 mixture of pinocamphone/isopinocamphone.

Results and Discussion

Oil composition: The results of the oil analyses are given in Table I. The oils obtained from plants grown at 100 m and 1000 m were indicated as oil A and oil B, respectively. The major components of the oils were pinocamphone (34% and 18.5%, respectively in oil A and B), isopinocamphone (3.2% and 29%), β-pinene (10.5% and 10.8%), α-phellandrene and germacrene D. The major differences were detectable in the ratio of pinocamphone/isopinocamphone, and in the percentage of linalool (0.2% in A, 7.9% in B) and camphor (0.3% in A, 5.30% in B).

The composition of our oils was in agreement with data reported in literature. In fact, pinocamphone and isopinocamphone are generally the compounds characterizing the oils of *H. officinalis*: the ratio between these compounds is not constant (24).

Moreover, hyssop oils from different phenotypes or from different areas show a great variability in chemical composition (8,9,25). The chemical composition can be related to many factors, such as the geography, climate and technological influence, origin, stages of development, parts used, and harvesting time, as well as the presence of chemotypes (5-11). In some cases, compounds such as β-phellandrene (25), 1-8 cineole (9), limonene and methyl eugenol (8), rather than pinocamphone and isopinocamphone, were found to be the main constituents of the oils.

The differences detected in our oil compositions can be attributed in part to the differing climatic conditions of the localities where the plants were grown, as already observed by other authors (1).

The results obtained in assays of the antifungal activity of the oils are shown in Table II and III. At 400 ppm of oil A, we observed a total inhibition of *Rhyzoctonia solani*, while at the same concentration, oil B inhibits *Rhyzoctonia solani* and *Botrytis cinerea*. The inhibition of this last fungus is reached at 600 ppm for oil A. At 600 ppm, oils A and B showed a total inhibition of *Phytophtora capsici*. At 800 ppm, oil B inhibited *Fusarium oxysporum*, and at 1600 ppm both oils exerted a total inhibition of all the other fungi tested. The above-mentioned oil concentrations resulted in fungistatic.

A fungicidal activity was observed at 3200 ppm of both oils on *Phytophtora capsici*, *Botrytis cinerea* and *Rhyzoctonia solani*, while 5000 ppm exerted the same effect on all the other fungi.

Tables IV and V show the MIC and MFC values for the A and B oils and the major constituents (pinocamphone, isopinocamphone, camphor and linalool), respectively.

The MICs varied in a range from 400 ppm for *Rhyzoctonia solani* to 1600 ppm for *Alternaria solani* for A oil, while for B oil the range was from 300 ppm for *Rhyzoctonia solani* to 1400 ppm for *Alternaria solani*. The MFC values obtained for A oil varied in a range from 2.8 mg/mL (*Phytophtora capsici* and *Rhyzoctonia solani*) to 4.8 mg/mL (*Fusarium graminearum*). The values for B oil varied from 2.4 mg/mL (*Phytophtora capsici*, *Botrytis cinerea* and *Rhyzoctonia solani*) to 4.0 mg/mL (*Fusarium graminearum* and *F. poae*).

The MIC and MFC values obtained for A oil were higher than the values obtained for B: in media, considering all the tested fungi, the values of MFCs of the A oil were 600 ppm higher than values obtained for B.

Table V shows the results obtained with the pure main components: the strongest antifungal activity was shown by

Table II. Effect of hyssop oil A on in vitro growth of selected pathogens fungal (% inhibition)

Phytopathogenic fungus	50 ppm	100 ppm	200 ppm	400 ppm	600 ppm	800 ppm	1600 ppm	3200 ppm	5000 ppm
Fusarium culmorum	0	0	16.6	33.3	53.7	62.9	100*	100*	100°
Fusarium graminearum	0	6.3	14.8	40.4	53.2	68.0	100*	100*	100°
Fusarium poae	0	0	16.0	40.0	50.0	68.0	100*	100*	100°
Fusarium avenaceum	0	3.8	23.0	38.4	63.4	67.3	100*	100*	100°
Fusarium equiseti	0	3.8	21.1	34.6	69.2	73.0	100*	100*	100°
Fusarium semitectum	0	3.5	19.2	31.5	57.0	79.0	100*	100*	100°
Fusarium sporotrichoides	0	0	22.2	35.5	46.6	77.7	100*	100*	100°
Fusarium oxysporum	0	0	47.3	55.2	60.5	73.6	100*	100*	100°
Phytophtora capsici	0	0	17.7	40.0	100*	100*	100*	100°	100°
Botrytis cinerea	0	5.0	15.0	40.0	100*	100*	100*	100°	100°
Rhyzoctonia solani	16.2	31.2	66.2	100*	100*	100*	100*	100°	100°
Alternaria solani	0	9.0	18.1	18.2	22.7	50.0	100*	100*	100°
Phytophtora cryptogea	0	0	27.0	52.0	66.6	77.0	100*	100*	100°

Different symbols indicate the different activity exerted by the oil tested = * fungistatic, ° fungicidal

Table III. Effect of hyssop oil B on in vitro growth of selected pathogens fungal (% inhibition)

Phytopathogenic fungus	50 ppm	100 ppm	200 ppm	400 ppm	600 ppm	800 ppm	1600 ppm	3200 ppm	5000 ppm
Fusarium culmorum	10.0	22.2	44.4	62.9	76.0	93.3	100*	100*	100°
Fusarium graminearum	5.7	14.8	36.1	53.2	70.2	90.8	100*	100*	100°
Fusarium poae	9.4	22.0	38.0	60.0	74.0	96.0	100*	100*	100°
Fusarium avenaceum	11.2	23.0	46.1	61.5	71.1	98.8	100*	100*	100°
Fusarium equiseti	9.2	21.1	46.1	65.4	75.0	98.8	100*	100*	100°
Fusarium semitectum	11.3	29.8	49.1	65.0	77.2	96.0	100*	100*	100°
Fusarium sporotrichoides	5.7	13.3	40.1	64.4	75.5	98.0	100*	100*	100°
Fusarium oxysporum	6.2	15.7	51.6	73.5	91.0	100*	100*	100*	100°
Phytophtora capsici	10.1	15.5	40.0	60.0	100*	100*	100*	100°	100°
Botrytis cinerea	37.4	63.7	87.5	100*	100*	100*	100*	100°	100°
Rhyzoctonia solani	31.5	60.3	88.0	100*	100*	100*	100*	100°	100°
Alternaria solani	15.5	39.0	30.1	45.4	65.2	90.7	100*	100*	100°
Phytophtora cryptogea	10.7	23.0	49.6	64.5	80.0	100*	100*	100*	100°

Different symbols indicate the different activity exerted by the oil tested = * fungistatic, ° fungicidal

Table IV. Screening of minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of hyssop oils A and B

	Oil A		Oil B	
Phytopathogenic fungus	MIC μg/mL	MFC mg/mL	MIC μg/mL	MFC mg/mL
Fusarium culmorum	1500	4.4	1200	3.6
Fusarium graminearum	1500	4.8	1200	4.0
Fusarium poae	1500	4.4	1200	4.0
Fusarium avenaceum	1500	4.4	1200	3.6
Fusarium equiseti	1400	4.0	1200	3.6
Fusarium semitectum	1400	4.4	1200	3.6
Fusarium sporotrichoides	1400	4.4	1200	3.6
Fusarium oxysporum	1100	4.4	800	3.6
Phytophtora capsici	600	2.8	500	2.4
Botrytis cinerea	600	3.2	400	2.4
Rhyzoctonia solani	400	2.8	300	2.4
Alternaria solani	1600	4.0	1400	3.6
Phytophtora cryptogea	1000	4.0	800	3.6

Table V. Screening of minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of isopinocamphone, pinocamphone/isopinocamphone 75/25, linalool and camphor (major constituents) tested against fungal strains

	Isopinocamphone		Mix (75/25) pinocamphone/ isopinocamphone		Linalool		Camphor	
Phytopathogenic fungus	**MIC μg/mL**	**MFC mg/mL**	**MIC μg/mL**	**MFC mg/mL**	**MIC μg/mL**	**MFC mg/mL**	**MIC μg/mL**	**MFC mg/mL**
Fusarium culmorum	600	2.0	600	2.0	800	2.8	1000	3.6
Fusarium graminearum	600	2.0	600	2.0	800	2.8	1000	3.2
Fusarium poae	600	2.0	600	2.0	800	2.8	1100	3.2
Fusarium avenaceum	600	2.0	600	2.0	800	2.8	1000	3.6
Fusarium equiseti	600	2.0	600	2.0	1000	3.2	1200	4.0
Fusarium semitectum	600	2.0	600	2.0	1000	3.2	1200	4.0
Fusarium sporotrichoides	600	2.0	600	2.0	1000	3.2	1200	4.0
Fusarium oxysporum	600	2.0	600	2.0	800	2.8	1000	3.6
Phytophtora capsici	100	0.8	100	0.8	300	2.4	500	2.4
Botrytis cinerea	100	0.8	100	0.8	300	2.4	500	2.4
Rhyzoctonia solani	100	0.8	100	0.8	200	2.4	500	2.8
Alternaria solani	600	1.6	600	1.6	1000	3.2	1200	4.0
Phytophtora cryptogea	600	1.6	600	1.6	1000	3.2	1200	4.0

isopinocamphone and the mixture pinocamphone/ isopinocamphone (75/25). For both components, MIC values were found to be from 100 ppm, for *Phytophtora capsici*, *Botrytis cinerea* and *Rhyzoctonia solani*, to 600 ppm for the other tested fungi, with no differences between the two components.

Linalool showed antifungal activity against fungi of the genus *Fusarium*, as already verified in a previous work (26). Pure commercial camphor did not cause a strong inhibition of all fungi species.

The analysis of the oils showed that the amount of pinocamphone, isopinocamphone, linalool and camphor was higher in B oil than in A oil, and this difference was correlated to many factors, such as, in our plants, geographical and climatic influences (5-11).

The stronger antimicrobial activity of oil B was correlated to the higher percentages in this oil of compounds whose antimicrobial activity has been shown in our tests and reported in literature (14,27). Letessier found that pinocamphone and isopinocampheol, a precursor of isopinocamphone, alone or in combination, reduced growth of some phytopathogenic fungi (14). The antimicrobial activity of linalool has been reported in literature (27).

The activity of camphor as a pure component, verified by Carta et al. (28) on *Botrytis cinerea*, is moderate on our tested fungi, as showed also by Pitarokili et al. (29).

The highest effectiveness of oil B leads us to believe, in agreement with Pitarokili, that camphor can also exert, if not a direct activity, at least a synergistic effect with other components.

In conclusion, the oils of hyssop tested (A and B) showed significant antifungal activity. It would be useful to determine the mode of action of the oil, for the development of new antifungal agents.

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