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Article

Chemical Composition and Antifungal Activity of the Essential Oil of *Hyptis ovalifolia* Benth. (Lamiaceae)

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As folhas de *Hyptis ovalifolia* Benth. (Lamiaceae) foram submetidas à hidrodestilação e a fração volátil foi investigada por CG/EM. O componente principal, (*R*)-6-[(*Z*)-1-heptenila]-5,6-diidro-2*H*-piranona (**1**), representando 60% do óleo essencial, foi isolado por cromatografia em coluna e identificado por métodos espectroscópicos. O composto mostrou atividade *in vitro* contra 4 espécies (60 isolados clínicos) dos dermatófitos: *Microsporum canis*, *Microsporum gypseum*, *Tricophyton mentagrophytes* e *Tricophyton rubrum* com concentração inibitória mínima variando de 125-7,8 μg mL^{-1}.

The leaves of *Hyptis ovalifolia* Benth. (Lamiaceae) were subjected to hydrodistillation and the resulting volatiles were investigated by GC/MS. The main constituent representing 60% of the essential oil was isolated by column chromatography and identified by spectroscopic methods as (*R*)-6-[(*Z*)-1-heptenyl]-5,6-dihydro-2*H*-pyran-2-one (**1**). This compound showed strong *in vitro* activity against four dermatophyte fungi *Microsporum canis*, *Microsporum gypseum*, *Tricophyton mentagrophytes*, and *Tricophyton rubrum* (a total of 60 strains) with a minimal inhibitory concentration observed in the range of 125-7.8 μg mL^{-1}.

Keywords: *Hyptis ovalifolia*, essential oil, antifungal activity, Lamiaceae, (*R*)-6-[(*Z*)-1-heptenyl]-5,6-dihydro-2*H*-pyran-2-one

Introduction

The genus *Hyptis* Jacq. (Lamiaceae) includes about 400 species that mainly occur in tropical America.[1] This genus represents an important source of bioactive constituents possessing interesting biological effects, such as antimicrobial, cytotoxic and insecticidal activities.[2-4] In the course of our program aimed to isolate new antifungal compounds from Brazilian Cerrado plants, the essential oil of *Hyptis ovalifolia* Benth., popularly known in Brazil as 'malva do cerrado', was found to show a strong activity against dermatophytes. In this paper we report the isolation, structural determination and antifungal activity of the major constituent of this essential oil against *Microsporum canis*, *Microsporum gypseum*, *Tricophyton mentagrophytes*, and *Tricophyton rubrum* using the agar dilution method[5] to determine the minimal inhibitory concentrations (MICs).

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Experimental

General experimental procedures

The GC/MS analyses were carried out using a QP5050 GC/MS (Shimadzu), equipped with J&W Scientific DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 μm). The oven temperature was programmed as follows: 60 °C for 2 min with an increase of 3 °C min^{-1} to 240 °C, followed by 10 °C min^{-1} to 280 °C for 2 min. Carrier gas: Helium (1 mL min^{-1}). The injector was operated in split mode with ratio 1:50 at 220 °C and interface temperature at 240 °C. The retention index were obtained by co-injecting the oil

sample with an *n*-alkanes series[6] and used to identify the compounds present, together with the comparison to the NIST (NIST 21 and NIST 107) mass spectral library. NMR spectra were recorded with a Varian Gemini 2000 spectrometer to operating at 300 MHz for ^{1}H and at 75.5 MHz for ^{13}C. $CDCl_3$ was used as the solvent, with Me_4Si (TMS) as internal standard for the ^{1}H NMR spectrum and $CDCl_3$ as internal standard for the ^{13}C NMR spectrum. Methyl, methylene, methyne, and carbon non-bonded to hydrogen were discriminated using DEPT-135° and DEPT 90° spectra (Distortionless Enhancement by Polarization Transfer). 2D NMR spectroscopy was performed with standard H, H correlation and H, C correlation pulse sequences available in the spectrometer.

Extraction and isolation of the essential oil compounds

Hyptis ovalifolia Benth. (Lamiaceae) specimens were colleted in Goiânia, state of Goiás, Brazil. Voucher specimens (20.283) were deposited in the Herbarium of the Universidade Federal de Goiás (UFG).

The essential oil of *Hyptis ovalifolia* was obtained from fresh leaves by hydrodistillation in a Clevenger-type apparatus for five hours. The aqueous phase was extracted with diethyl ether. The organic phase was dried over anhydrous sodium sulfate and concentrated under N_2 to yield 0.05 % of the essential oil based on the weight of the fresh leaves. The identification of the compounds was based on the comparison of their retention indices and on mass spectra comparison with data of the literature[7] as well as by computerized matching of the acquired mass spectra with of the GC/MS data system. Separation of compound **1** was performed by CC using silica gel (Merck) and hexane - ethyl ether (4:1). Compound (**1**): $[\alpha]_D$: -21° (*c* 2.0, $CHCl_3$) (literature value of -21° (EtOH)).[8] IR (KBr) ν_{max}/cm^{-1}: 2928, 1723, 1380. EIMS *m/z* (rel. int.): 194 $[M]^{+\cdot}$ (3), 97 (20), 68 (100); ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H 6.90 (ddd, *J* 9.8, 5.4 and 3.0 Hz, 1H, H-4), 6.05 (ddd, *J* 9.8, 2.4 and 1.2 Hz, 1H, H-5), 5.66 (dtd, *J* 11.1, 7.5 and 0.9 Hz, 1H, H-2'), 5.56 (ddt, *J* 11.1, 8.4 and 1.5 Hz, 1H, H-1'), 5.22 (ddd, *J* 10.2, 8.4 and 5.5 Hz, 1H, H-2), 2.40 (m, 2H, H-3), 2.10 (m, 2H, H-3'), 1.40-1.30 (m, 6H, H-4', H-5' and H-6'), 0.89 (t, *J* 6.6 Hz, 3H, H-7'). ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ_C 164.3 (C-6), 145.0 (C-4), 135.6 (C-2'), 126.3 (C-1'), 121.6 (C-5), 73.7 (C-2), 31.1 (C-5'), 29.6 (C-3), 28.8 (C-4'), 27.5 (C-3´), 22.2 (C-6'), 13.7 (C-7´). These data are consistent with the literature.[8,9]

Dermatophyte strains

Dermatophytes used: *Microsporum canis* (10 strains), *Microsporum gypseum* (10 strains), *Trichophyton rubrum* (20 strains) and *Trichophyton mentagrophytes* (20 strains). The microorganisms were clinically isolated from patients with dermatophytosis and identified in the Laboratório de Micologia, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Brazil.

Assay for antifungal testing

In vitro antifungal activity of the compound **1** was evaluated against dermatophytes using a serial dilution agar technique. The compound **1** was solubilized in 1mL of dimethyl sufoxide (DMSO) and serially two-fold diluted in Mycobiotic agar medium (Difco, Detroit, Mich.) to obtain a concentration range of 3.9-1000 μg mL^{-1}. Dermatophyte suspensions in a sterile 0.85% saline solution containing Tween 80 (0.05%) were adjusted to give a final concentration of 1x10^6 CFU mL^{-1}. The holes in the solid agar media were inoculated with 10 mL of the dermatophyte suspensions and plates were incubated at 25 °C for 5 days. Negative control plates containing only diluted DMSO, as well as positive control plates impregnated with Itraconazole (0.06-250 μg mL^{-1}) were included in each assay. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the compound **1** that inhibited any visible fungus growth.

Results and Discussion

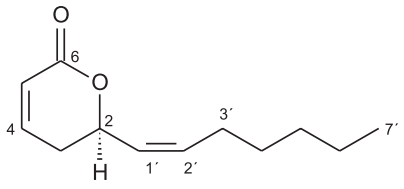
In a preliminary screening, the essential oil obtained from the leaves of *H. ovalifolia* was found to be an efficient antifungal agent against the dermatophytes *M. canis, M. gypseum, T. rubrum* and *T. mentagrophytes.* It inhibited these fungi at a concentration of 31.2 μg mL^{-1} and most often at even lower concentrations (Table 1). The analysis of the essential oil by GC/MS has enabled the identification of the following compounds (relative concentrations and retention index given in parentheses): α-copaene (0.84%; 1386), β-bourbonene (1.58%; 1405), (*Z*)-caryophyllene (0.74%; 1409), γ-elemene (4.38%; 1438), α-humulene (1.05%; 1474), γ-cadinene (6.60%; 1506), viridiflorol (6.08%; 1599), caryophyllene oxide (4.98%; 1606) and α-cadinol (0.74%; 1674), among other minor constituents. The identification of the compounds was based on the comparison of their mass spectra with those of the NIST and by comparison of their retention indices with those provided in the literature.[7] However, the main peak representing 60% of the total oil composition could not be identified by the mass spectra and retention index, thus it was isolated for further investigations. The unknown compound was obtained by the fractionation of the essential oil on a silica gel column. ^{13}C NMR and DEPT

Table 1. *In vitro* antifungal activity of compound **1** obtained from the essential oil of *H. ovalifolia* leaves

MIC	Dermathophytes														
	M. canis N (%)[b]			*M. gypseum* N (%)[b]			*T. rubrum* N (%)[b]			*T. mentagrophytes* N (%)[b]			Total N (%)[b]		
(μg mL^{-1})[a]	**1**	PC	EO	**1**	PC	EO	**1**	PC	EO	**1**	PC	EO	**1**	PC	EO
125	1 (10)	5 (100)	-	3 (30)	4 (80)	1 (20)	2 (10)	-	-	1 (5)	5 (50)	1 (10)	7 (11.7)	14 (46.7)	2 (6.7)
62.5	3 (30)	-	-	2 (20)	1 (20)	1 (20)	2 (10)	-	-	6 (30)	-	1 (10)	13 (21.7)	1(3.3)	2 (6.7)
31.2	0	-	-	3 (30)	-	2 (40)	4 (20)	-	1 (10)	7 (35)	-	5 (50)	14 (23.3)	-	8 (26.7)
15.6	5 (50)	-	5 (100)	1 (10)	-	1 (20)	10 (50)	-	7 (70)	5 (25)	-	3 (30)	21 (35)	-	16 (53.3)
7.8	1(10)	-	-	1 (10)	-	-	2 (10)	-	2 (20)	1(5)	1 (10)	-	5 (8.3)	1 (3.3)	2 (6.7)
3.9	-	-	-	-	-	-	-	2 (20)	-	-	1 (10)	-	-	3 (10)	
1.95	-	-	-	-	-	-	-	2 (20)	-	-	-	-	-	2 (6.7)	
0.97	-	-	-	-	-	-	-	1 (10)	-	-	2 (20)	-	-	3 (10)	
0.48	-	-	-	-	-	-	-	1 (10)	-	-	1 (10)	-	-	1 (3.3)	
0.12	-	-	-	-	-	-	-	4 (40)	-	-	-	-	-	5 (16.7)	
Total	10	5	5	10	5	5	20	10	10	20	10	10	60	30	30

[a] minimal inhibitory concentration; [b] N (number of strains); PC (positive control – Itraconazole); EO (Essential Oil); numbers into ( ) means percentage of strains.

spectra allowed the identification of 12 carbons: one methyl, five methylene, five methyne and one carbonyl group. The ^{1}H NMR spectrum indicated the presence of the primary methyl group (δ 0.89), four olefinic hydrogens (one double bond at δ 5.56 and 5.66 and a enone group at δ 6.05 and 6.90) and a sp^3 methyne group (δ 5.22). Two-dimensional NMR experiments (^{1}H x ^{1}H COSY and ^{1}H x ^{13}C HETCOR) allowed the assignment of all the ^{1}H NMR and ^{13}C NMR signals. The IR spectrum showed a band at 1723 cm^{-1} consistent with the presence of an α,β-unsaturated lactone moiety. These spectral features and the optical rotation $[\alpha]_D^{20}$ = -21° (*c* 2.0, $CHCl_3$) are in agreement with structure **1** [8,9] which was characterized as (*R*)-6-[(*Z*)-1-heptenyl]-5,6-dihydro-2*H*-pyran-2-one, previously isolated from *Chorisia crispiflora* (Bombacaceae),[8] *Aristolochia argentina* (Aristolochiaceae),[9] and *Annona haematantha* (Annonaceae)[10] and described here for the first time for the genus *Hyptis* (Lamiaceae). Other 6-substituted 5,6-dihydro-2*H*-pyran-2-ones containing acetoxy and hydroxy functions in the side-chain have been isolated from other species of *Hyptis*.[11] Natural products containing α,β-unsaturated lactone moiety are known to possess a wide range of biological activities including plant-growth inhibition,[12] insect antifeedant,[13] antifungal,[14] and antitumor activities.[11]



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The compound **1** was evaluated for its *in vitro* antifungal activity against 60 strains of four dermatophytes: *M. canis, M. gypseum*, *T. mentagrophytes*, and *T. rubrum* by the agar dilution method. The results of these assays are summarized in Table 1. According to the results obtained here, we can attribute the antifungal activity observed for the essential oil of *H. ovalifolia* to compound **1**. In addition, compound **1** showed antifungal activity similar to or better than that of itraconazole (positive control) against *M. canis* and *M. gypseum* with MICs ranging between 32.1-7.8 μg mL^{-1}, reinforcing the antifungal activity ascribed to pyrones.[14]

Therefore compound **1** could be an interesting compound for the development of new drugs against dermathophytes.

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