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Antifungal Activity of Essential Oil Isolated from *Ocimum gratissimum* L. (eugenol chemotype) against Phytopathogenic Fungi

Terezinha de Jesus Faria[1*], Rafael Sottero Ferreira[1], Lidiane Yassumoto[2], José Roberto Pinto de Souza[3], Noemia Kazue Ishikawa[4] and Aneli de Melo Barbosa[2]

[1]Departamento de Química; CCE; [2]Departamento de Bioquímica; CCE; [3]Departamento de Agronomia; CCA; [4]Departamento de Biologia Geral; CCB; Universidade Estadual de Londrina C. P. 6001; 85051-970; Londrina - PR - Brasil

ABSTRACT

An investigation of antifungal activity of the essential oil obtained by steam-distillation (1.1% w/w) of the aerial parts of *Ocimum gratissimum* and of an ethanolic extract from the steam-distillation residue was carried out using the agar diffusion method. The results revealed that the essential oil inhibited the growth of all fungi tested, including the phytopathogens, *Botryosphaeria rhodina*, *Rhizoctonia* sp. and two strains of *Alternaria* sp., while the extract from the residue was inactive. The essential oil was subjected to TLC bioautography used to detect fungitoxic constituents. The compound that showed antifungal activity was isolated and identified as eugenol. GC/MS analysis showed that eugenol was the main constituent of the essential oil studied. The antifungal activity of eugenol was evaluated against a species of *Alternaria* isolated from tomato (A_1) and *Penicillium chrysogenum*. The minimal inhibitory concentrations of eugenol were 0.16 and 0.31 mg/disc for *Alternaria* sp. (A_1) and *P. chrysogenum*, respectively.

Key words: *Ocimum gratissimum*, essential oil, antifungal activity, eugenol, phytopathogenic fungi

INTRODUCTION

Ocimum gratissimum, originating in the Orient, is widespread throughout tropical countries including Brazil, where it is popularly known as "alfavacão, alfavaca and alfavaca-cravo" (Lorenzi and Matos, 2002). This species is used in traditional medicines in South America and Africa for a variety of therapeutic purposes which includes its use in treating bacterial infections, diarrhoea, diabetes (Aguiyi et al. 2000), respiratory-tract infections, pneumonia, fever and coughs (Corrêa, 1926). Evaluation of the biological activities revealed that extracts of *O. gratissimum* exerted anti-diarrheal effects in experimental animals (Onajobi, 1986; Offiah and Chikwendu, 1999), showed high antiviral indices against HIV-1 and HIV-2 (Ayisi and Nyadedzor, 2003) and hypoglycemic activity in rats (Aguiyi, 2000). Atal et al. (1986) investigated the immunobiological activity of certain medicinal plants and observed that extracts of *O. gratissimum* appeared to improve the phagocytic function without affecting the humoral or cell-mediated immune system.

The essential oil of this species also presented interesting activities such as insecticidal (Kéita et al., 2001), antibacterial (Janssen et al., 1989;

* Author for correspondence

Nakamura et al., 1999; Cimanga et al., 2002; Ngassoum et al., 2003), antifungal (Dubey et al., 2000), and as a relaxant on isolated ileum from guinea pig (Madeira et al., 2002). The essential oil of *O. gratissimum* and its main component eugenol were efficient in inhibiting eclosion of *Haemonchus contortus*, a gastrointestinal parasite of small ruminants (Pessoa et al., 2002).

This paper describes the results of the evaluation of the antifungal activity of the essential oil obtained by steam-distillation of aerial parts of *Ocimum gratissimum*, the ethanolic extract from the steam-distillation residue, and the purified active compound eugenol, using the agar diffusion method and TLC bioautographic technique.

MATERIALS AND METHODS

General

^{1}H-NMR (200MHz) and ^{13}C-NMR (50.3MHz) spectra were recorded on a Bruker Spectrometer. Chemical shits were δ in (ppm) using tetramethylsilane as st,andard. IR spectra were obtained with a Shimadzu FTIR Model 8300 6AD, using KBr pellets. GC/MS-capillary gas chromatography was performed in a Shimadzu GC-17A chromatography coupled to a Shimadzu GC/MS-QP5000 using a DB-1 capillary column (cc, 30 m x 0.25 mm), temperature programmed at 60 ^{o}C per 4min over the range 60-250 ^{o}C at 10 ^{o}C/min, and 250-330 ^{o}C at 20 ^{o}C/min. Mass spectra were compared with spectra from the literature (Saito et al., 2004).

Plant material

Aerial parts of *O. gratissimum* were collected at the Horto de Plantas Medicinais, Department of Agronomy of the State, University of Londrina (Paraná). A voucher specimen (36554) has been deposited at the Herbarium of this University.

Steam distillation

The volatile oil was extracted from dried aerial parts of *O. gratissimum* by steam-distillation (100 g of plant material) for 5 h using a Clevenger apparatus. The distillate was extracted with dichloromethane, and the resulting extract dried over anhydrous sodium sulfate and concentrated under reduced pressure yielding 1.1 g of essential oil.

Extraction of the residue following steam-distillation

The plant material of, steam distillation was dried for three days and extracted with ethanol at room temperature. After filtration, the solvent was removed by rotary evaporation under reduced pressure yielding 7 g of extract.

Microbial cultures and growth conditions

The fungi used in the agar diffusion method were: *Aspergillus niger*, *Penicillium chrysogenum*, and the phytopathogens: two strains of *Alternaria* sp. (isolated from tomato and carrot and denoted as A_1 and A_2, respectively), *Botryosphaeria rhodina* and *Rhizoctonia* sp. Cultures of these fungi were grown on agar plate at 28 ^{o}C and maintained with periodic sub-culturing at 4 ^{o}C. All microorganisms were derived from stock cultures from the Department of Biochemistry of the State University of Londrina. The fungi used in the TLC bioautography technique was *Cladosporium herbarum* (NRRL 2175), maintained on agar slopes of PDA at 4 ^{o}C. The microorganism was derived from the Department of General Biology of the State University of Londrina.

Assay of antifungal activity

Antifungal activity of the essential oil and the extract of the steam-distillation residue was evaluated by the agar diffusion method. The test was performed in sterile Petri dishes (90 mm diameter) containing potato-dextrose-agar (PDA) medium (20 mL). The samples for evaluation were dissolved in ethyl acetate and aliquots at different concentrations applied to sterile paper discs (8 μL of solution per Whatmann No. 1 disc of 5 mm diameter, resulting in final concentrations of 7.50, 4.12, 0.08, 0.04, 0.02 and 0.01 mg/disc of the essential oil; 8μL of solution/disc of extract of steam-distillation residue and 4.40, 0.62, 0.31, 0.16 and 0.08 mg/disc of eugenol). The discs were placed on the surface at the center of agar plates inoculated with a fresh fungal suspension (one microorganism per Petri dish). The Petri dishes were then incubated at 28 ^{o}C for 48 h, followed by the measurement of the diameters of zones of inhibition. Inhibition of fungal growth was measured as zone diameters (mm) at 4-equidistant points taken from the centre of the inhibition zone, and the average value taken. All experiments were carried out in triplicate and the reported data

represents average values ±SD. Ethyl acetate was used as a control.

TLC bioautography

Essential oil was applied to 2 TLC plates and developed in dichloromethane as solvent. One plate was used as the reference chromatogram, and the other for bioautography. A spore suspension of *C. herbarum* was sprayed over the developed TLC plate, which was, in turn, incubated at 28 ^{o}C under humid conditions for 3 days (Homans and Fuchs, 1970). The inhibitory zones observed were then correlated with the spots seen on the T,LC plate used as the reference chromatogram, and visualized under UV light at 254 nm.

Isolation and identification of the major constituent

The essential oil obtained as described above (0.8 g) was chromatographed on a column of silica gel and eluted with a gradient of hexane-dichloromethane (95:5, 85:15 and 60:40) to 100% dichloromethane. One hundred and sixty five fractions each of 20 mL were collected and analysed by TLC to yield seven groups (G:1-7). Group G-2 (fractions 18-78) eluting with hexane was subjected to preparative TLC and eluted with dichloromethane. The material obtained was purified by flash chromatography as described by Still et al., (1978) using a mixture of petroleum ether/ethyl acetate (95:5) as eluent. Thirty fractions (5 mL) were collected. Fractions 13-25 contained the active compound (0.1 g), identified as eugenol.

RESULTS AND DISCUSSION

The present study showed that the essential oil was obtained in a yield of 1.12 % (w/w) from the dried aerial parts of *O. gratissimum*. Vieira et al. (2001) reported significant variations in the range and content of twelve accessions of *O. gratissimum* (0.29-1.66 %). Studies focused on characterizing the volatile oil from this species revealed variations in its chemical composition. Based upon the dominant constituents, volatile oils could be divided into various chemotypes: eugenol, thymol, citral, ethyl cinnamate, linalol or geraniol (Dubey et al., 2000; Vieira et al., 2001). The chemical composition also showed a great variation during the daytime (Silva et al., 1999). In the present work, the analysis by GC-MS showed eugenol to be the major constituent of the essential oil of *O. gratissimum* obtained from the Londrina region (Fig. 1). The essential oil and residue of steam-distillation were tested for antifungal activity. The steam-distillation residue was found to be inactive against all of the fungi tested, while the essential oil inhibited the growth of the six fungi screened. The results summarized in Table 1 showed that the essential oil produced a zone diameter of inhibition of 9-to-55 mm depending on the fungus tested. It was, however, observed that the growth of *Alternaria* sp., isolated from tomato (A_1), was most sensitive with a zone diameter of inhibition of 9 mm at the lowest concentration (0.01 mg/disc) tested.

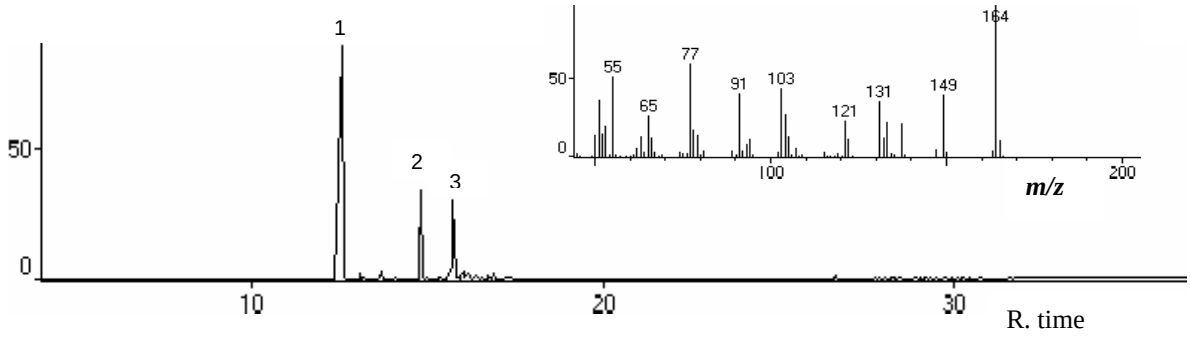

Figure 1 - Chromatogram of essential oil of *O. gratissimum* and mass spectrum of major constituent (peak 1, eugenol).

Table 1 - Antifungal activity of essential oil as determined by agar diffusion method.

Microorganism	Concentrations (mg/disc)						
	0.01	0.02	0.04	0.08	4.12	7.50	Control
	Inhibition zone (mm)						
Alternaria sp (A_1)	9	9	8	12	8	55	0
Alternaria sp (A_2)	0	0	0	0	36	52	0
Botryosphaeria sp	0	0	0	0	23	38	0
Aspergillus niger	0	0	0	10	32	53	0
P. chrysogenum	0	0	0	0	45	48	0
Rhizoctonia sp	0	0	0	10	41	41	0

The essential oil was examined by the TLC bioautography technique (Homans and Fuchs, 1970) using *Cladosporium herbarum* as the test fungus. One antifungal spot was observed at R_f 0.43. The active compound was isolated and identified as eugenol by spectral analysis using ^{1}H- and ^{13}C-NMR, IR and GC/MS, and by comparison with literature values (Saito et al., 2004). Antifungal activity of eugenol was evaluated by the agar diffusion assay against *Alternaria* sp. (A_1) and *P. chrysogenum*. The minimal inhibitory concentrations of eugenol were 0.16 and 0.31 mg for *Alternaria* sp. (A_1) and *P. chrysogenum*, respectively (Table 2).

Table 2 - Antifungal activity of eugenol as determined by agar diffusion method

Microorganism	**Concentrations (mg/disc)**						
	Eugenol						Oil
	0.08	0.16	0.31	0.62	4.40	Control	3.73
	Inhibition zone (mm)						
Alternaria sp (A_1)	0	13	13	16	23	0	25
P. chrysogenum	0	0	7	15	51	0	48

CONCLUSIONS

The constituent present in the essential oil of *O. gratissimum* responsible for antifungal activity was not present in the steam-distillation residue. The essential oil of *O. gratissimum* from the Londrina region obtained with a yield of 1 % (w/w) represented eugenol as the major constituent. Eugenol was the compound responsible for antifungal activity of the essential oil of *O. gratissimum*. Essential oil of *O. gratissimum* showed antifungal activity against phytopathogenic fungi such as *Botryosphaeria rhodina* and *Alternaria* spp. This is the first report on this property of the essential oil of *O. gratissimum*. The results demonstrated the potential application of *O. gratissimum* essential oil in agriculture as first observed by Kéita et al. (2001) with *O. gratissimum* from Africa.

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RESUMO

O óleo essencial resultante da destilação por arraste a vapor das partes aéreas de *Ocimum gratissimum* e o extrato etanólico obtido do resíduo da destilação foram avaliados quanto à atividade antifúngica, utilizando-se o método de difusão em agar. O óleo essencial inibiu o crescimento de todos os fungos testados, incluindo os fitopatogênicos *Botryosphaeria rhodina* e duas espécies de *Alternaria* sp, enquanto que o extrato do resíduo da destilação não apresentou atividade. O óleo essencial foi, então, submetido ao método de bioautografia em TLC para detecção do composto ativo. O componente ativo foi isolado e identificado através da análise por cromatografia

gasosa acoplada à espectrometria de massas como o eugenol, constituinte majoritário do óleo estudado. Ensaios de atividade antifúngica revelaram a atividade do eugenol contra *Alternaria* isolada de tomate (A_1) e *Penicillium chrysogenum*. As concentrações inibitórias mínimas foram 0,16 mg/disco e 0,31 mg/disco para *Arternaria* sp (A_1) e *P. chrysogenum*, respectivamente.

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