

Antifungal activity from *Ocimum gratissimum* L. towards *Cryptococcus neoformans*

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Cryptococcal infection had an increased incidence in last years due to the explosion of acquired immune deficiency syndrome epidemic and by using new and effective immunosuppressive agents. The currently antifungal therapies used such as amphotericin B, fluconazole, and itraconazole have certain limitations due to side effects and emergence of resistant strains. So, a permanent search to find new drugs for cryptococcosis treatment is essential. Ocimum gratissimum, plant known as alfavaca (Labiatae family), has been reported earlier with in vitro activity against some bacteria and dermatophytes. In our work, we study the in vitro activity of the ethanolic crude extract, ethyl acetate, hexane, and chloroformic fractions, essential oil, and eugenol of O. gratissimum using an agar dilution susceptibility method towards 25 isolates of Cryptococcus neoformans. All the extracts of O. gratissimum studied showed activity in vitro towards C. neoformans. Based on the minimal inhibitory concentration values the most significant results were obtained with chloroformic fraction and eugenol. It was observed that chloroformic fraction inhibited 23 isolates (92%) of C. neoformans at a concentration of 62.5 µg/ml and eugenol inhibited 4 isolates (16%) at a concentration of 0.9 µg/ml. This screening may be the basis for the study of O. gratissimum as a possible antifungal agent.

Key words: *Ocimum gratissimum* - essential oil - eugenol - *Cryptococcus neoformans*

Cryptococcal infections have increased dramatically over the last years. This high incidence can be due in large part to the explosion of acquired immune deficiency syndrome (AIDS) epidemic around the world and the use of more potent immunosuppressive agents by increasing numbers of solid organ transplant recipients (Mitchell & Perfect 1995, Dromer et al. 1996).

Cryptococcal meningitis, the most common infection of cryptococcosis is usually chronic and uniformly fatal if untreated (Collazos 2003). Some antifungal drugs, such as polyene macrolides (amphotericin B) and azoles (itraconazole and fluconazole) are currently used in antifungal therapies with certain limitations due to side effects as toxicity and emergence of resistant strains (Terrel 1999, Saag et al. 2000). The lack of response to treatment and resistance in vitro to fluconazole, the drug that now commands maintenance treatment protocols for AIDS patients has began to emerge with *Cryptococcus neoformans* var. *neoformans* in immunocompromised patients undergoing prolonged azole treatment (Alves et al. 1997, Momoff & Parrish 2003). Besides, the development of amphotericin B resistant of isolates *C. neoformans* var. *neoformans* has been related by De Bedout et al. (1999), indicating the need for a permanent search and development of new drugs.

The medicinal plants have been used for several purposes including antimicrobial effects and have showed inhibition of growth to fungi. *Caryocar brasiliensis*, plant widely distributed in Brazil, has in vitro activity against *Paracoccidioides brasiliensis* and *C. neoformans* (Passos et al. 2003) while *Hyptis ovalifolia*, plant native from Brazilian cerrado, has inhibitory effect on dermatophytes (Souza et al. 2002). We are interested on antifungal activity of *Ocimum gratissimum*, plant known as alfavaca cravo, which has presented in vitro inhibitory effect against bacteria as *Staphylococcus aureus*, *Escherichia coli*, and some fungi as dermatophytes (Lima et al. 1993, Nakamura et al. 1999).

The occurrence of cryptococcosis in Goiânia (Fernandes et al. 2003) and its importance in immunocompromised patients justify in studying *Cryptococcus* species in this city localized in the Brazilian midwest region. In this paper the antifungal activity of ethanolic crude extract, ethyl acetate, hexane and chloroformic fractions, essential oils and eugenol from *O. gratissimum* leaves towards 25 isolates of *C. neoformans*, important pathogen commonly found in our geographic area (Fernandes et al. 2003), was investigated.

MATERIALS AND METHODS

Plant material - *O. gratissimum* was collected in Goiânia city, state of Goiás, Brazil (768 m, 33.3°S/ 16°40'39.5"W 49°14') in June 2001, at 11:00 am. Voucher specimens (number 27157) were deposited at the herbarium of the Universidade Federal de Goiás (UFG).

Powdered air-dried leaves of *O. gratissimum* were successively extracted in cold with 96% ethanol as solvent at

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room temperature. Ethanolic extract was concentrated and dried under reduced pressure at 40°C in a vapor motor and dispersed in methanol/water 7:3 mixture. The resultant dispersion was filtered in Celite, to remove chlorophyll (Fernandes et al. 1997) and lately extracted with hexane, chloroform, and ethyl acetate to obtain the hexane, chloroformic, and ethyl acetate fractions free of chlorophylls and all solvents. The essential oil of *O. gratissimum* leaves was isolated by hydrodistillation in a Clevenger type apparatus for 4/5 h, yield the pure oil which was dried by treating with anhydrous Na₂SO₄.

Eugenol (Sigma), main constituent of the essential oil of *O. gratissimum* was commercially obtained.

Chemical analysis of essential oil - Oil sample analysis was performed on a Shimadzu QP5050A gas chromatograph interfaced to a mass spectrometer (GC/MS) instrument employing the following conditions: fused silica capillary column (CBP-5; 30 m x 0.25 µm x 0.25 µm) which was programmed as follows: 60°C for 2 min and then up to 240°C at 3°C/min, then to 270°C at 10°C/min ending with a 10 min at 270°C. The carrier gas was He at a flow rate of 1 ml/min, split mode, with ratio of 1:5, and injection volume of 1 µl in CH₂Cl₂ and the ionization voltage, 70 EV. The calculation of the retention indexes was made through co-injection with C₈-C₃₂ n alkanes series (Van Den Doll & Kratz 1963). Identification of the oil constituents was made based on the retentions indexes (Adams 1995) and by comparison of mass spectra with the computer search using NIST libraries of mass spectral data. Percentage composition of the oil sample was calculated from GC peak areas.

Microorganisms - All the 25 strains of *C. neoformans* used in this study were clinical isolates from human immunodeficiency virus-infected patients with cryptococcal meningitis, obtained at Laboratório Central de Saúde Pública Dr Giovanni Cysneiros, Goiânia, GO, Brazil. *Candida albicans* ATCC 10231, was used as reference strains for susceptibility tests and was included whenever a test was run. The stock cultures were maintained on Sabouraud dextrose agar medium (SDA, Difco) at 25°C. Each isolate was subcultured at least twice on Sabouraud dextrose agar to ensure purity and optimal growth before testing.

Antifungal assay - The dilution in agar technique was used to test the plant extracts against *C. neoformans* (Alves & Cury 1992, Passos et al. 2003). Plant extracts, essential oil, and eugenol (dry and solvent free) were solubilized in 1 ml of dimethyl sulfoxide (DMSO) and serially two-fold diluted in RPMI-1640 broth with L-glutamine and without sodium bicarbonate (Sigma), buffered to pH 7.0 with 0.165M MOPS (morpholinepropanesulfonic acid) to obtain a concentration range of 10-10,000 µg/ml. Of this solution, 2 ml were transferred into sterile Petri dishes and 18 ml of liquefied RPMI agar at 45°C were added and immediately mixed, obtaining a concentration range of 1.0-1000 µg/ml.

Cell suspensions were prepared in 0.85% saline and adjusted to an OD (530 nm) equivalent to 1x 10⁶ UFC/ml. These suspensions (3 µl) were inoculated on agar plates

incubated at 37°C for 48-72 h. Cultures containing only RPMI 1640 medium diluted in the same conditions, were used as controls. The minimum inhibitory concentration (MIC) was defined as the lowest concentration able to inhibit any visible fungal growth compared with that of plant free growth control. Cultures containing only DMSO diluted in the same conditions, which did not influence fungal growth, were used as controls. The sensitivity of all *C. neoformans* strains to itraconazole was performed using the same technique. Itraconazole (Janssen) was diluted in polyethylene glycol (PEG-Fisher Scientific). The MIC for itraconazole was defined as the lowest concentration of drug that produced an 80% reduction in fungal growth compared with that of drug-free growth control. All antifungal assays were tested in duplicated.

RESULTS

Sample hydrodistillation of *O. gratissimum* leaves produced a clear, colorless to pale yellow oil, with a yield of 0.69%. The essential oil was a mixture with 21 compounds. The main compounds of the *O. gratissimum* leaves oil were eugenol (57.82%), followed by α-bisabolene (17.19%), and thymol (9.8%). The estragole (methylchavicol) was not found in the essential oil from *O. gratissimum*. The compounds, Kovat's index and calculated retention index of the essential oil are showed in Table I.

The MIC values of *O. gratissimum* against *C. neoformans* clinical isolates observed in this study showed that the ethanolic extract and all fractions of this plant have antifungal activity. The chloroformic fraction of this plant at concentration of 62.5 µg/ml inhibited 92%

TABLE I
Percentage composition of the essential oil from *Ocimum gratissimum* leaves

Compounds ^a	KI ^b	RI ^c	%
Myrcene	991	996	0.34
α-Terpinene	1018	1017	0.30
p-Cymene	1026	1054	2.11
(E)-β-Ocimene	1050	1052	0.49
γ-Terpinene	1062	1061	3.06
p-Cymenene	1089	1087	0.26
Terpin-4-ol	1177	1175	0.24
Thymol	1290	1288	9.80
Carvacrol	1298	1295	0.28
Eugenol	1356	1357	57.82
α-Copaene	1376	1375	0.11
β-Caryophyllene	1418	1417	3.03
α-Humulene	1454	1454	0.31
(E)-β-Farnesene	1458	1457	0.39
Germacrene D	1480	1482	0.79
cis-β-Guaieno	1490	1491	1.06
α-Selinene	1494	1493	0.45
(Z)-α-Bisabolene	1504	1502	17.19
β-Sesquiphellandrene	1524	1521	0.24
Spathulenol	1576	1575	0.18
Caryophyllene oxide	1581	1580	0.82

a: compounds are listed in order of their elution from a CBP-5 column; b: Kovat's Index (Adams 1995); c: Calculated retention index (Van Den Doll & Kratz 1963)

of *Cryptococcus* isolates and the hexanoate fraction inhibited 8 (40%) of these same isolates in a concentration of 250 µg/ml. The natural product (eugenol) from *O. gratissimum* showed higher inhibition than essential oil. It was observed that the eugenol inhibited the growth of 16% of isolates of *C. neoformans* at concentration of 0.9 µg/ml while the essential oil showed an inhibition of 8% of isolates at concentration of 125 µg/ml. The sensitivity of the same fungal isolates to itraconazole showed a range MIC of 15.6 to 0.03 µg/ml. It was verified that 16% were inhibited of growth by eugenol at concentrations below than 1 µg/ml while 24% of isolates were inhibited by itraconazole at concentrations below than 1 µg/ml. The MIC ranges, MIC₅₀ and MIC₉₀ values of isolates of *C. neoformans* against plant extracts and itraconazole are showed in Table II. The MIC of 0.25 µg/ml was found to reference strain *C. albicans* ATCC 10231 for itraconazole. This value was within the standard acceptable range of MICs.

DISCUSSION

The chemical composition of the essential oil of *O. gratissimum* can change according with geographical distribution and daytime of collect. The chemical test result revealed that the constituents of *O. gratissimum* essential oil collected in Goiânia city, in this study, included eugenol, terpenoids, and thymol (Table I). Similar results were observed by Dubey et al. (2000), who verified citral, ethyl cinnamate chemotype, eugenol, linalol as the dominant constituents of *O. gratissimum* essential oil. On the other hand, Martins et al. (1999) did not find eugenol as constituent of the essential oil in *O. gratissimum* from Portugal Island.

The high concentration of eugenol, as main constituent of essential oil of *O. gratissimum* verified in our plant, confirm data shown by Vasconcelos Silva et al. (1999) that showed a higher content of eugenol in the collection at 12 h (98%) than in the collection at 17 h (11.3%).

Vincenzi et al. (2000) reported that estragole a naturally occurring genotoxic carcinogen in experimental animals after chronic exposure or after a few repeated doses can be present in *O. gratissimum*. The absence of estragole in our *O. gratissimum* oil permits its usage in phytomedicines.

All the extracts of *O. gratissimum* studied in our work showed activity in vitro towards *C. neoformans* by using agar dilution method. This method has been used for several searchers to determine the activity of plant against fungi (Steinmetz et al. 1995, Larshini et al. 1996). Investigation earlier has showed that *O. gratissimum* has activity against other fungi. Lima et al. (1993) and Nwosu and Okafor (1995) verified inhibition of growth of *Trichophyton rubrum* and *T. mentagrophytes*. Dubey et al. (2000) had already established that thymol a constituent of *O. gratissimum* essential oil was highly active against *T. rubrum*, *T. mentagrophytes*, *C. neoformans*, *C. albicans*, and *Malassezia pachydermatis*. In our work is important to note that 92% *C. neoformans* isolates were inhibited of growth at concentrations of 62.5 µg/ml by chloroformic fraction and 100% of these isolates were inhibited at 250 µg/ml by essential oil and eugenol as verified in Table II.

TABLE II

In vitro antifungal activity of *Ocimum gratissimum* and itraconazole against 25 clinical isolates of *Cryptococcus neoformans*

<i>O. gratissimum</i>	Minimal inhibition concentration (MIC) (mg/µl)		
	Range	MIC ₅₀	MIC ₉₀
Ethanol extract	250 > 1000	1000	>1000
Hexane fraction	62.5 – 500	250	500
Chloroform fraction	62.5 – 125	62.5	62.5
Ethyl acetate fraction	250- > 1000	>1000	>1000
Essential oil	125 – 250	250	250
Eugenol	0.9 – 250	250	250
Itraconazole	0.03 – 15.6	3.9	15.6

The results in vitro susceptibility tests of the fungal isolates to eugenol, main constituent of essential oil of *O. gratissimum* obtained in our work presented MIC values near to itraconazole for 16% of the isolates. However prior investigations are need for establishing comparison between plants and itraconazole activity.

The results suggest that the chloroformic fraction, essential oil and eugenol, constituents of *O. gratissimum* leaves, are important sources of new antifungal agents. This study open perspectives to find more effective drugs of vegetal origin in the treatment of fungal infections.

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