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Short Communication

CHARACTERIZATION AND ANTIFUNGAL ACTIVITY OF THE ESSENTIAL OIL OF *TAGETES MINUTA* FRONT OF THE *CRYPTOCOCCUS SPP*. ISOLATES FROM THE ENVIRONMENT

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ABSTRACT

Objective: This study evaluated the chemical composition and antifungal activity of the essential oil of inflorescences of *Tagetes minuta* (EOTM) belonging to the *Asteraceae* family against *Cryptococcus* spp. This microorganism is the encapsulated yeast-like and is recognized as an opportunistic fungal pathogen of great clinical importance.

Methods: The inflorescences of *T. minuta* were collected in Itaara/RS, Brazil, in April 2013, and identification of the components was performed by GC-MS. The species of fungi are environmental isolates of *Cryptococcus* spp. identified by direct examination with India ink, urease test, culture and agar Niger medium canavanine glycine bromothymol blue, and all fungi isolates were confirmed by the use of automated panel MicroScan® Rapid Yeast ID (SIEMENS®). ATCC strains of *C. gattii*, *C. neoformans* and *C. grubii* belonging to the Microbiology Laboratory of the Centro Universitário Franciscano of Santa Maria/RS, Brazil were also used. The antifungal activity of the EOTM was evaluated by microdilution.

Results: Most strains of *Cryptococcus* spp. were sensitive to EOTM even at low concentrations, except when the microorganism in question was *Cryptococcus grubii* which the essential oil showed a weak antifungal action.

Conclusion: The EOTM appears as promising in prospecting for new drugs for the treatment of cryptococcosis.

Keywords: Cryptococcosis, Natural products, Antifungal, Marigold

Cryptococcus spp is an encapsulated yeast fungus widely spread and worldwide recognized as opportunistic pathogenic fungus. *Cryptococcus* genus includes more than 30 species, among which there are the *Cryptococcus neoformans* and *Cryptococcus gattii,* causing more severe clinical manifestations of cryptococcosis [1]. The infection is acquired through inhalation of viable proposals in the environment, affecting the lungs as the primary site of infection, and the spread can lead to meningoencephalitis, a clinical manifestation diagnosed in humans [2]. The detection of the fungus in environmental samples and the antifungal activity of constituents are important towards of decreased resistance of microorganisms over the drugs that are already used for the treatment of cryptococcosis [3]. The therapeutic use of the plant species has been described since the dawn of humanity, even without scientific basis [4]. Among the various plant species of medicinal interest, are included plants of the *Asteraceae* family, one of the families with the highest number of species between dicotyledonous and being also known as compositae [5]. *Tagetes minuta* L. belongs to the *Asteraceae* family and is an aromatic species native of the fields and mountainous regions of South America, although it has spread around the world. This plant species popularly known as marigold are often found growing in areas with loose soil, blooming between October and November. A better quality of the essential oil is obtained when it is produced in temperate regions, where the nights are humid and it's very cold during the growth and flowering plant [6]. Morphologically is an upright plant, sparsely branched, measuring about 1-2 m tall with reproduction by seed only [7]. The plant shows photosensitizers and antiviral activities due to thiophenes. The antimicrobial activity is related to the presence of acyclic monoterpenes and ketones in the constitution. One of the constituents of the plant, *E*-ocimenone, is responsible for the larvicidal activity of the oil against *Aedes aegypti* [8]. The decoction is obtained from the whole plant and this vegetal species has antibacterial activity against Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecium* and in the more moderate way on the Gram-negative bacterium *Salmonella gallinarum* [4]. The essential oil from the aerial parts of *T. minuta* presented antibacterial activity against *Staphylococcus* [9]. This study aims to evaluate the chemical composition and antifungal activity of essential oil of *T. minuta* (EOTM) against the *Cryptococcus* spp.

The inflorescences of *T. minuta* were collected in Itaara (The Rio Grande do Sul, Brazil), in april 2013. The species were deposited in the herbarium of the Department of Biology, UFSM as vouchers specimens (N ° HDCF 6400). After that, the inflorescences were subjected to hydro distillation for 3 h using a Clevenger apparatus for essential oil extraction [10]. The identification of the components of EOTM was performed by gas chromatography system coupled to mass spectrometry detector Agilent 7890A and 5975C masses. Analysis parameters: the split mode (1: 100), carrier gas: Helium (1 ml/min); HP5MS capillary column (Hewlett-Packard, 5% phenyl methyl siloxane, 30 m x 0.25 mm x 0.25 mM), temperature program: 40 °C for 4 min, 40-320 °C, 4 °C/min, injector temperature: 250 °C, the interface temperature: 250 °C, the ionization energy: 70 eV.

The OE components were identified based on retention indices determined by the use of an n-alkane calibration curve injected under the same conditions and the samples based on mass spectrum [11, 12]. The quantification of the components was performed using Agilent 7890A with a flame ionization detector in accordance with the parameters mentioned above, except for the following: the splitless mode; injector and detector temperature: 300 °C. The species of fungi come from environmental isolates of *Cryptococcus* spp. (14.1, 29.1, 15.1, 13.1, 7.1, 15, 44, 45.4, 13.2) and strains of *C.*

gattii (ATCC 56990), *C. grubii* (ATCC 208821) and *C. neoformans* (ATCC 28957) stored at the Microbiology Laboratory of the Centro Universitário Franciscano of Santa Maria/RS, Brazil. These samples were subcultured on Sabouraud dextrose agar for 24 h at 37 °C for the identification and determination of minimum inhibitory concentration through the serial microdilution technique. Direct examination of the colonies was performed using a blade containing a heave of the colony, an India ink drop and overlapping a coverslip, and observed by microscopy in a 40x objective. The presence of the yeast with regular shapes, globular, round, with or without encapsulated bud and the presence of *Cryptococcus* spp, were evaluated [1]. Identification of the isolates was obtained by using physiological methods and biochemical test. The suggestive colonies of *Cryptococcus* spp. were picked in Agar urea and Agar Niger, respectively, to evaluate the action of urease and phenol oxidase, and incubated at 30 °C for 48 h. For biochemical tests the automated MicroScan® Rapid Yeast ID panel (SIEMENS®) was used. The system displays a list of microorganisms and their relative probability of identification based on a number of biotypes formed by nine digits. All the methodology used in this system, with regard to technical and interpretation of results, was made systematically according to the manufacturer's recommendations (SIEMENS®). The microdilution technique was performed as recommended by the CLSI M27-A3 protocol [12]. In 96-well plate were inoculated into each well 100 µl of RPMI-1640 supplemented with MOPS. Then, 100 µl EOTM was pipetted in the first well and a serial dilution to the proportion 1:64 was performed. After dilution, 10 µl of the standardized inoculum over the range of 0.5 McFarland (1.5 X 10^8 CFU/ml) was added to all wells. This test was performed in triplicate. Plates were incubated for 48 h and the reading was made microscopically for the presence or absence of pits in yeast. However, due to the viscosity of the EOTM it was seeded all proportions (1:2 to 1:64) on Sabouraud agar, and the plates incubated at 37 °C for 48 h to confirm the result shown in microscopy. Positive and negative controls were performed in the test. In the chemical composition of the EOTM were qualified and quantified ten constituents, being identified about 91% of the sample as shown in table 1.

Table 1: Chemical identification the essential oil of inflorescences of *Tagetes minuta*

Constituent	Retention time	Calculated kovats index	Tabulated kovats index	Source	Percentage (%)
E-Tagetone	21.67	1159	1152	Adams	40.37
Z-Tagetone	20.91	1138	1138	NIST	29.50
Dihydro Tagetone	17.70	1053	1053	NIST	7.57
Ocimene	17.093	1037	1044	NIST	6.11
Limonene	16.68	1026	1027	NIST	3.99
E-caryophyllene	30.47	1419	1421	Adams	0.86
Octanal	15.65	999	1001	NIST	0.38
α-pinene	13.15	937	939	NIST	0.32
α-humulene	31.59	1455	1451	NIST	0.40

According to the literature, this plant species is rich in essential oil and has with main components: *β*-phellandrene, limonene, *β*-ocimene, dihydro tagetone, tagetone and tagetenone [13, 14] as evidenced on the study results by GC-MS. There are a lot of reports in the literature about *in vitro* evaluation of antimicrobial, acaricide, antioxidant, antiseptic, insect repellent and nematicides activity of *T. minuta*, still showing antimicrobial activity with marked selectivity for Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecium*, and more moderate way on the Gram-negative bacterium *Salmonella gallinarum* [4]. In addition to these aforementioned properties, the EOTM is used in the perfumery and food industry market, including alcoholic beverages, dairy frozen, sweets, cakes, cookies, jellies, puddings, and condiments. In direct examination performed with India ink, all the environmental isolates showed positive results for *Cryptococcus* spp. For urease activity, urea was hydrolyzed after 48 h incubation in urea agar. The pH change was observed when the color of the medium changed from orange to pink. The species differentiation tests observed that in Niger agar the samples showed beige, light colored colonies, and 4% samples showed an activity of phenoloxidase, visualized by the formation of brown pigmentation in the colonies. Environmental isolates 29.1 and 45.4 were identified as *C. neoformans*. In chemical characterization comprising the use of *L*-canavanine medium glycine bromothymol blue, the results were analyzed according to the change of pH of the yellow to blue, showing that all other analyzed environmental isolates were identified as *C. gattii*.

The results obtained for biochemical tests in automated ID Rapid Yeast Micro Scan® panel (SIEMENS®) confirmed the presence of two strains of *C. neoformans* and seven strains characterized as *C. gattii*. In this study, environmental isolates *Cryptococcus* spp. mostly presented susceptible to EOTM, with MIC less than or equal to 3.22±0.18 µg/ml of the value shown by the negative control, Amphotericin B (a widely used drug for the treatment of various fungal infections). Among the nine strains of *Cryptococcus* spp., only the strain ATCC *Cryptococcus grubii* (ATCC 208821) was resistant compared to the other strains and the negative control, with MIC of 51.4±1.89 µg/ml. However, all MIC essential oil compared to the microorganisms tested showed lower results than the MIC presented by the positive control that was carried out with the medium itself (RPMI-1640) in which was inoculated only the fungus as shown in table 2.

Table 2: Minimum inhibitory concentration (MIC) of the essential oil of inflorescences of *Tagetes minuta* against 12 pathogenic fungi estimated by the microdilution method

Microorganism	MIC (µg/ml)*
C. neoformans	6.43±0.08
C. grubii	51.4±1.89
C. gattii	6.43±0.67
15.1 (*C. gattii)*	3.22±0.03
15 (*C. gattii)*	3.22±0.01
44(*C. gattii)*	6.43±0.05
14.1(*C. gattii)*	3.22±0.05
7.1 (*C. gattii)*	1.28±0.06
29.2 (*C. neoformans*)	1.28±0.08
13.1(*C. gattii)*	3.22±0.08
13.2 (*C. gattii)*	3.22±0.07
45.4 (*C. neoformans*)	3.22±0.05
Amphotericin B	3.22±0.18
Positive Control	411.5±0.14

*The results were expressed as mean±SEM; n=3.

The data obtained in this study indicate that EOTM is effective against *Cryptococcus* spp. Essential oils can be a good option to treating since they are renewable and less harmful to humans. Information regarding the interaction and to address the effect of the EOTM in strains of *Cryptococcus* spp. were not found in the literature reviewed. However, a recent study has associated an excellent antifungal property to the EO of the vegetal species *Satureja calamintha nepeta* against the fungi *Fusarium* spp. and *Aspergillus* spp [15], showing that EO obtained from native plants are a promising alternative to combat pathogenic fungi. In this study, it was possible to conclude that most of the environmentally isolated strains of *Cryptococcus* spp. were sensitive to the EOTM even at low concentrations, except against *C. grubii*, where the OE showed a weaker action compared to other strains. Thus, the EOTM is presented as promising in prospecting of new drugs for the treatment of cryptococcosis.

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CONFLICTS OF INTERESTS

Declared none

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