doi: 10.5216/rpt.v45i3.43515

ORIGINAL ARTICLE

EFFECTS OF THE ESSENTIAL OIL OF *Thymus vulgaris* L. AGAINST *Cryptococcus neoformans*

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ABSTRACT

Cryptococcus neoformans is an encapsulated yeast found in the environment, responsible for causing of meningoencephalitis in patients with a compromised immune system. In Brazil, cryptococcosis is the second cause of death among systemic mycoses. The limited efficacy of the available antifungal drugs used in its treatment has encouraged the search for therapeutic alternatives, such as medicinal plants. *Thymus vulgaris*, popularly known as thyme, is an aromatic plant whose essential oil (EO) possesses antifungal properties. The aim of this study was to assess the action of *T. vulgaris* EO on *C. neoformans* clinical isolates. This oil was analyzed by gas chromatography/mass spectrometry (GC/MS), which showed that its main components were thymol, ρ-cymene and linalool. Microdilution broth tests showed that this EO was effective against fungal isolates, with minimum inhibitory concentrations (MICs) ranging from 32 to 128 µg/mL. *In vitro* interaction tests between this oil and fluconazole (FCZ) showed no potentiation of the antifungal action of this drug. Its effect on mitochondrial metabolism of fungal cells was also evaluated and results demonstrated alterations on the mitochondrial enzyme activity of fungal cells only at concentrations >1,024 µg/mL. The results of the action of this EO on human erythrocytes indicated that it has low cytotoxic activity at MIC values. This investigation describes the antifungal action of *T. vulgaris,* showing its potential in the development of alternatives in the treatment of *C. neoformans*.

KEY WORDS: *Cryptococcus neoformans; Thymus vulgaris*; antifungal agents.

RESUMO

Efeitos do óleo essencial de *Thymus vulgaris* L. em *Cryptococcus neoformans*

Cryptococcus neoformans é uma levedura encapsulada encontrada no ambiente, responsável por causar meningoencefalite em pacientes com o sistema imune comprometido. No Brasil, a criptococose é a segunda causa de morte por micoses sistêmicas. A eficácia limitada dos fármacos antifúngicos usados no tratamento desta doença tem estimulado a busca por

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Received for publication: 1/7/2016. Reviewed: 11/8/2016. Accepted: 12/8/2016.

terapias alternativas como, por exemplo, o uso de plantas medicinais. *Thymus vulgaris,* popularmente conhecida como tomilho, é uma planta aromática cujo óleo essencial (OE) possui propriedades antifúngicas. Este estudo teve como objetivo avaliar a ação do OE de *T. vulgaris* em isolados clínicos de *C. neoformans*. Este óleo, analisado por cromatografia gasosa acoplada à espectrometria de massa (CG/EM), revelou como seus principais compostos: timol, ρ-cimeno e linalool. Testes de microdiluição em caldo mostraram a eficácia deste OE contra os isolados fúngicos, com concentrações inibitórias mínimas (CIMs) variando de 32 a 128µg/mL. Testes de interação *in vitro* ─ OE de *T. vulgaris* e fluconazol (FCZ) ─ demonstraram não haver potencialização da atividade antifúngica desse fármaco. Avaliou-se também seu efeito no metabolismo celular fúngico e os resultados mostraram alterações na atividade enzimática mitocondrial apenas em concentrações >1.024µg/mL. Os resultados da ação deste OE em eritrócitos humanos indicaram que ele possui baixa atividade citotóxica em valores de MIC. Ao descrever a ação antifúngica de *T. vulgaris*, esta pesquisa mostra seu potencial como alternativa para o tratamento contra *C. neoformans*.

DESCRITORES: *Cryptococcus neoformans; Thymus vulgaris*; antifúngicos.

INTRODUCTION

Cryptococcus neoformans is an encapsulated yeast found in the environment, that initiates infection in humans after inhalation. It is responsible for causing meningoencephalitis in patients with a compromised immune system with approximately 1 million cases worldwide and 650,000 deaths annually (Park et al. 2009). In Brazil, cryptococcosis is the second cause of death among systemic mycoses, having been mentioned in 50.9% of AIDS death certificates (Prado et al. 2009) where 48.4% of the 62 patients in this group died in Goiania, Brazil, in 2009 and 2010 (Souza et al., 2013). The limited efficacy of the available antifungal drugs – due to the high toxicity of amphotericin B (AmB) (Jarvis et al. 2008) and the increasing isolation of fluconazole (FCZ) resistant isolates (Cheong & McCormack 2013) – leads to a growing demand for new antifungals that are more effective and less toxic (Pina-Vaz et al. 2004). Plants have received much attention in recent years and are a promise of natural products with antifungal activity (Suwanmanee et al. 2014). The public acceptance and effectiveness of plant-based alternatives is becoming more evident in recent years (Zuzarte et al. 2013). Aromatic plants and their essential oils (EOs) have long been used as food preservatives and for medicinal purposes with antimicrobial and antioxidant properties (Bakkali et al. 2008, Edris 2007).

Thymus vulgaris L. is an aromatic plant that belongs to the Labiatae family, popularly known as thyme (Khazaie et al. 2008). Its EO has important antioxidative and antimicrobial properties and contains numerous compounds (Baranauskiene et al. 2003, Nikolic et al. 2014). Antifungal activity exhibited by *Thymus* genus EOs has been demonstrated by several researchers on *Candida* spp., *Aspergillus* spp., *Microsporum* spp. and other fungi (Giordani et al. 2004, Soković et al. 2009, Zuzarte et al. 2013).

There is very little information related to the activity of *T. vulgaris* EO against *C. neoformans*, this being the object of this study. In this context, the respective susceptibility profile *in vitro* of this EO was determined alone, as well as combined with FCZ. The effects of this oil on fungal mitochondrial function and its toxicity on human erythrocytes were also evaluated.

MATERIAL AND METHODS

Essential oil

T. vulgaris EO was obtained from Ferquima Industria e Comercio Ltd., Vargem Grande Paulista, São Paulo, Brazil. The solutions used in antifungal assays were prepared when the tests were performed.

Gas chromatograph-Mass selective detector analysis of essential oils

For the analysis of the EO, a gas chromatograph interfaced with a mass selective detector (CG-MS), Shimadzu QP5050A, with a 70 eV ionization voltage was employed, and a fused silica capillary column was used (CBP - 5; 30 m x 0,25mm x 0,25µm). The carrier gas used was Helium at a flow rate of 1mL/min. Temperature program used was: ramp up from 60 to 240°C at 3°C/min, increased to 280°C at 10°C/min, and completed with 10 min at 280°C. The injection volume was 1 µL diluted with CH_2Cl_2 at a ratio of 1:5. For the identification of oil constituents, their mass spectra were compared with those present at the National Institute of Standards and Technology (NIST, 1998) and also by comparing their mass spectra and calculated linear retention indexes (RI) with literature values (Adams 2007). RIs were obtained by co-injection with a mixture of linear hydrocarbons, C_9-C_{22} (Sigma, USA) and then calculated using the equation of Van Den Dool & Kratz (1963). The percentages of components were calculated to normalize for the area in the chromatogram employing a Varian gas chromatograph (FID) equipped with a ZB-5 fused silica capillary column (30m x 0.25nm) with film thickness of 0.25µm (5% phenylmethylpolysiloxane). The temperature program used was: an increase from 60 to 240°C at 3°C/min, an increase to 280°C at 10°C/min, and completion with 10 min at 280°C. The carrier gas employed was N_2 (flow rate of 1.0mL/min). The injector port and detector temperatures were 220°C and 240°C, respectively. The samples were injected by splitting (split ratio of 1:20).

Fungal isolates

C. neoformans ATTC 28957 and seven *C. neoformans* clinical isolates from the culture collection of the Mycology Laboratory of the Tropical Pathology and Public Health Institute of Federal University of Goias were included in this study. The strains were isolated from cerebrospinal fluid collected from HIV positive patients from a reference hospital in Goiania, Brazil (Ethics Committee Protocol no. 004/03). Clinical isolates were maintained on Sabouraud-Dextrose Broth (SDB) with glycerol at -70°C. All isolates were cultured in Sabouraud Dextrose Agar (SDA) at 35°C for 48h before the tests.

Antifungal susceptibility testing

The guidelines of the Clinical and Laboratory Standards Institute M27-A3 (CLSI, 2008) were followed for the performance of microdilution assays in order to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of *T. vulgaris* EO. The antifungal susceptibility tests were executed by serially diluting a two-fold solution of EO on 96-well microtiter plates to concentrations from 1,024 to 2µg/mL. A serial dilution of FCZ was also performed leading to final concentrations ranging from 64 to 0.5µg/mL. *C. neoformans* suspensions were prepared in sterile 0.85% NaCl and adjusted to 0.5 x 10^3 to 2.5 x 10^3 CFU/mL. For *T. vulgaris* EO, the lowest concentrations without any visible growth were defined as MICs and, for FCZ, MICs were defined as the lowest concentration producing a reduction in growth of 50%, compared to the drug-free growth control. Tests were performed in triplicates at each concentration.

For the determination of MFCs, 10µL of each well showing no growth (MIC, 2 x MIC and 4 x MIC) were plated on sterile SDA Petri dishes and incubated at 25-28°C for 72h in order to determine if the inhibition was reversible or permanent. The MFC was defined as the lowest concentrations that showed either no growth or fewer than 3 colonies (approximately 99 to 99.5% killing activity of fungal cells) (Espinel-Ingroff et al. 2002).

In vitro *interaction of* T. vulgaris *EO and fluconazole*

The effect of the combination of *T. vulgaris* EO and FCZ was assayed with *C. neoformans*, according to the checkerboard microdilution method (Scott et al. 1995). The interaction coefficient was determined following the calculation of the fractional inhibitory concentration index (FICI). FICIs were defined as synergistic for values $\leq 0,5$; indifferent for $0,5 < FICI \leq 4,0$, and antagonistic for values $> 4,0$ (LiJuan et al. 2010).

Effect of the Essential Oil on Mitochondrial Function

The effect of *T. vulgaris* EO on *C. neoformans* cell viability was determined by a colorimetric assay, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), as described previously, with modifications (Pinto et al. 2013). *C. neoformans* ATCC 28957 was used for this assay. Fungal cells were cultured overnight in Sabouraud Dextrose Broth (SDB) at 35°C and 10 mL of the suspension were centrifuged (10 minutes at 4,000 rpm) and washed in PBS (2x). 100µL fungal suspension in PBS supplemented with 2% glucose (10^6 cells/mL) was incubated for 1 hour/35°C with 100µL of EO solution (final concentrations 4,096–16 µg/mL). 20 µL of the MTT solution (5mg/mL in PBS) were added to each well and incubated for 4 hours in the dark. Then supernatants were discarded and the resulting purple formazan crystals were solubilized with 100µL dimethylsulfoxide (DMSO). The colorimetric change was measured spectrophotometrically at 550 nm.

Hemolytic Assay

The hemolytic activity assay was performed using human blood samples collected in EDTA tubes following the method of He et al. (2007) with modifications. 50µL whole blood were added to 50µL of a two-fold dilution of *T. vulgaris* EO (4,096µg/mL to 16µg/mL) in PBS (pH 7.4) and then incubated for 30 minutes at 37^0C. Following incubation, tubes were centrifuged at 3,000 rpm for 5 min and 20µL of supernatants were transferred to a flat-bottomed microtiter plate. 80µL of the same buffer were added to the wells for a final volume of 100µL. Triton X-100% was used as control for total hemolysis and PBS was used for the absence of hemolysis. Absorbance was measured spectrophotometrically at 560nm. Hemolysis percentages were calculated as follows: [(A_{560} of EO-treated sample – A_{560} of buffer-treated sample)/(A_{560} of Triton X-100-treated sample – A_{560} of buffer-treated sample)] x 100%. Experiments were performed in duplicate.

RESULTS

Essential Oil Composition

The result obtained by GC-MS chemical analysis of *T. vulgaris* EO is presented in Table 1. In total, twenty-five compounds were identified in the EO, which represent 98.9% of the isolated compounds.

Table 1. Chemical composition of *T. vulgaris* essential oil.

	Constituents	RI	%
1	Tricyclene	926	0.15
2	α-Pinene	938	2.20
3	α-Fenchene	952	0.79
4	Myrcene	990	1.37
5	ρ-Mentha-1(7),8-diene	1004	0.08
6	ρ-Cymene	1024	20.71
7	Limonene	1029	0.14
8	1,8-Cineole	1031	1.62
9	γ-Terpinene	1059	7.08
10	cis-Linalool oxide	1072	0.09
11	Fenchone	1086	0.12
12	Linalool	1096	7.45
13	trans-Pinene hydrate	1122	0.12
14	Camphor	1146	1.18
15	Isoborneol	1160	0.27
16	Borneol	1169	1.36
17	Terpinen-4-ol	1177	1.30
18	Thymol	1290	45.56
19	Carvacrol	1299	3.78
20	α-Ylangene	1375	0.07
21	(E)-Caryophyllene	1419	0.90
22	α-Humulene	1454	0.10
23	Caryophyllene oxide	1583	1.37
24	Humulene epoxide II	1608	0.09
25	(Z,Z)-Geranyl linalool	1961	1.02
	Monoterpenes hydrocarbons		32.52
	Oxygenated monoterpenes		62.85
	Sesquiterpene hydrocarbons		1.07
	Oxygenated sesquiterpenes		1.46
Others			1.02
Total			98.92

RI: Retention Index

Antifungal Activity

T. vulgaris EO was tested on seven clinical isolates of *C. neoformans* and *C. neoformans* ATCC 28957, with MIC values from 32 to 128 µg/mL and MFCs from 64 to 512 µg/mL (Table 2). The MIC at which 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were inhibited was 64 µg/mL and 128 µg/mL, respectively.

Table 2. Antifungal activity and interaction assay of *T. vulgaris* EO and fluconazole against *C. neoformans* clinical isolates.

C. neorformans	*T. vulgaris*		Fluconazole		Interaction
isolates	MIC[a]	MFC[a]	MIC[a]	MFC[a]	FICI[b]
L3	64	128	1	8	3,0
L4	64	64	1	4	1,25
L5	32	64	1	4	3,0
L15	128	512	1	8	1,5
L18	64	128	1	4	3,0
L21	64	128	2	8	2,0
L30	64	64	2	16	3,0
ATCC 28957	32	32	0.5	2	4,0

[a]MIC and MFC values expressed in µg/mL; [b]FICI scores: ≤ 0,5=synergism; ICIF >4,0=antagonism and 0,5 < ICIF ≤ 4,0=indifferent

In vitro *interaction of* T. vulgaris *EO and fluconazole*

The interaction assay was performed in order to assess whether this combination would lead to the potentiation of the antifungal action of FCZ by the EO and the results did not show alteration of its MIC values. The FICI values obtained through the checkerboard assay ranged from 1.25 to 4.0 (Table 2).

Effect of the Essential Oil on Mitochondrial Function

In order to determine the metabolic activity of *C. neoformans* ATCC 28957, the MTT assay was performed. Results demonstrated no alterations on the mitochondrial enzyme activity of fungal cells at concentrations ≤ 1,024 µg/mL (Figure). At higher concentrations, 57.8 and 7.3% of yeast cells remained metabolically active at 2,048 and 4,096 µg/mL, respectively.

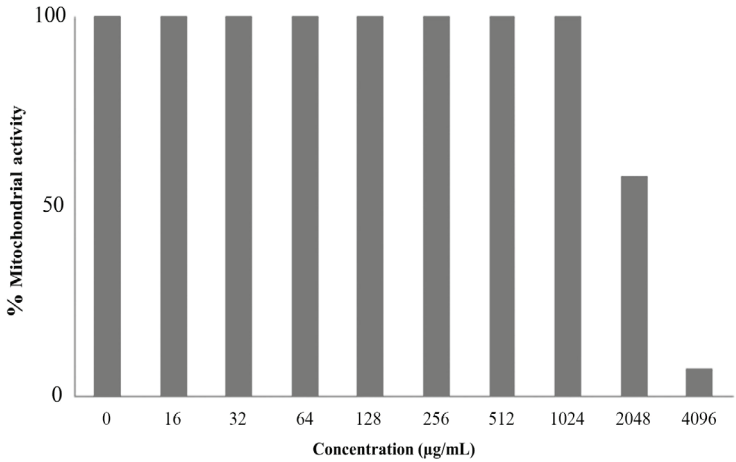

Figure. Mitochondrial activity of *C. neoformans* ATCC 28957 cells treated with different concentrations of *Thymus vulgaris* EO.

Hemolytic Assay

The results of the action of *T. vulgaris* EO on human erythrocytes showed 0.7% hemolysis at the 4,096 µg/mL concentration. At lower concentrations, the hemolysis percentage was inferior to 0.2%.

DISCUSSION

The main complication of cryptococcosis is meningoencephalitis, which is responsible for high mortality rates that affect especially immunocompromised patients (Charlier et al. 2009). AmB is the drug of choice for the treatment of cryptococcosis, followed by maintenance therapy with FCZ for weeks (Perfect et al. 2010, Gullo et al. 2013). Despite the effectiveness of treatment, AmB is responsible for serious side-effects (Guillaume et al. 1996) and long-term maintenance therapies with azoles can result in fungal resistance (Gullo et al. 2013). These difficulties have prompted the search for therapeutic alternatives such as essential oils.

T. vulgaris EO has been described by several researchers for its antimicrobial properties, attributed to the activity of a complex mixture. Twenty-five compounds were identified, representing 98.9% of the total oil weight. Aromatic plants belonging to the same species show variations in the chemical composition of their EOs depending on their chemotype. The most predominant compounds of the EO used in this study were thymol (45.6%),

ρ-cymene (20.71%) and linalool (7.5%), which suggest that it belongs to the thymol chemotype. The analysis showed monoterpenes to be predominant in this oil. Thymol and ρ-cymene have previously been reported to be the major components in *T. vulgaris* EOs (Giordani et al. 2004, Rota et al. 2008, El-Ahmady et al. 2013) and they are described as having prominent antimicrobial activity (Bakkali et al. 2008). Thymol has been shown to have antimicrobial activity against microorganisms such as *Streptococcus* spp., *E. coli* (Inouye et al. 2001) and *Candida* spp. (Pina-Vaz et al. 2004). P-cymene has been reported to have antifungal activity against *Alternaria* spp., *Fusarium* spp. (Kordali et al. 2008) and *Candida* spp. (Pina-Vaz et al. 2004).

The EO of *T. vulgaris* tested in this study presented antifungal activity against *C. neoformans*, with MICs ≤ 128μg/mL. 71.4% of isolates showed growth inhibition at a concentration of 64μg/mL and interestingly, this oil was able to inhibit fungal growth at low concentrations such as 32 μg/mL, showing strong antifungal activity, according to Sartoratto et al. (2004). The high percentages of thymol help to explain the antifungal activity of this EO, since this component is already known for having activity against fungi other than *C. neoformans* (Pina-Vaz et al. 2004, Pinto et al. 2006, Klarić et al. 2007). Antifungal action of this EO has also been described on other fungi, including *C. albicans*, *Aspergillus* spp. and *Trichophyton* spp. (Zollo et al. 1998, Giordani et al. 2004, Klarić et al. 2007, Soković et al. 2009, El-Ahmady et al. 2013), but little about the action of this EO on *C. neoformans* is known. This antifungal action has been attributed to the inhibition of H^+-ATPase, a process that leads to intracellular acidification and cell death (Ahmad et al. 2010). The MFCs for *T. vulgaris* EO ranged from 1 to 4 times the corresponding MICs, and these results suggest that this oil might have fungistatic activity. No reports of MFCs for this EO on *C. neoformans* were found. As previously mentioned, there is very scarce data about the action of *T. vulgaris* EO on *C. neoformans*, which highlights the importance of this investigation. Studies on the interaction of *T. vulgaris* EO and FCZ on *C. neoformans* were not found. Although no synergism was seen from this association, there are reports of synergistic effects from the association of *T. vulgaris* EO with AmB on *C. albicans* and *Aspergillus niger* (Giordani et al. 2004, El-Ahmady et al. 2013). Reports of synergism between this oil and FCZ were not found.

MTT assay measures mitochondrial activity by quantifying the formation of a dark blue formazan product, resulting from the reduction of the tetrazolium ring of MTT. It is thought that MTT reduction mainly occurs in the mitochondria through the action of succinate dehydrogenase, thus providing a measure of mitochondrial activity (Slater et al., 1963). This assay showed that in concentrations ≤ 1,024 μg/mL of *T. vulgaris* EO, 100% of *C. neoformans* cells remained metabolically active (Figure). At 2,048 μg/mL and 4,096 μg/mL this EO caused 42.2% and 92.7% reduction in the number of active cells, respectively. Therefore this assay indicated that the mechanism of inhibition of

T. vulgaris EO at MIC values (32–128 µg/mL) was not due to interferences on mitochondrial function, since it only caused a disruption of the mitochondrial enzyme activity (which impairs the energy production ability of cells) at values ≥ 4 x MIC.

Human erythrocytes are very useful for toxicity studies, since they are easily available, have very well known membrane properties and can be easily monitored concerning their lysis through hemoglobin release monitoring (Situ & Bobek, 2000). The *in vitro* hemolytic assay is a tool that can be used to estimate *in vivo* cell toxicity (Christie et al., 2007). This assay demonstrated that *T. vulgaris* EO led to a very low percentage of hemolysis (inferior to 0.2%) at MIC values. Interestingly, very low hemolysis rates were seen even at concentrations 2 or 4 times the MICs, thus demonstrating that this EO has low cytotoxic effects on erythrocytes.

Data on the action of *T. vulgaris* EO against *C. neoformans* are rare. This shows the importance of the results obtained in this study, which demonstrate the in vitro antifungal activity of this EO on the etiological agent of cryptococcosis. This should encourage further studies on this oil, in the hope that novel antifungals may be developed as alternatives in the treatment against fungal infection.

REFERENCES

1. Adams RP. *Identification of essential oil components by gas chromatography/mass spectrometry.* Allured Publishing Corporation, Carol Stream, Illinois, USA, 2007.
2. Ahmad A, Yousuf S, Khan L, Manzoor N. Proton translocating ATPase mediated fungicidal activity of eugenol and thymol. *Fitoterapia 81*: 1157-1162, 2010.
3. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils – A review. *Food Chem Toxicol 46*: 446-475, 2008.
4. Baranauskiene R, Venskuton SPR, Viskelis P, Dambrauskiene E. Influence of nitrogen fertilizers on the yield and composition of thyme (T*hymus vulgaris*). *J Agric Food Chem 51*: 7751-7758, 2003.
5. Charlier C, Nielsen K, Daou S, Brigitte M, Chretien F, Dromer F. Evidence of a Role for Monocytes in Dissemination and Brain Invasion by *Cryptococcus neoformans*. *Infect Immun 77*: 120-127, 2009.
6. Cheong JWS, McCormack J. Fluconazole resistance in cryptococcal disease: emerging or intrinsic? *Med Mycol 51*: 261-269, 2013.
7. Clinical and Laboratory Standards Institute (CLSI). *Reference method for broth dilution antifungal susceptibility testing of yeast*. Approved standard M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA, 2008.
8. Christie MS, Kenneth LR, David BW. Assessing toxicity of fine and nanoparticles: comparing *in vitro* measurements to *in vivo* pulmonary toxicity profiles. *Toxicol Sci 97*: 163-180, 2007.
9. Edris AE. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. *Phytother Res 21*: 308-323, 2007.
10. El-Ahmady, El-Shazly M, Milad R. The synergetic efficacy of the combination of amphotericin B and certain oils against selected fungal clinical isolates. *J App Pharm Sci 3*: 26-30, 2013.

11. Espinel-Ingroff A, Fothergill A, Peter J, Rinaldi MG, Walsh TJ. Testing conditions for determination of minimum fungicidal concentrations of new and established antifungal agents for *Aspergillus* spp.: NCCLS collaborative study. *J Clin Microbiol 40*: 3204-3208, 2002.
12. Giordani R, Regli P, Kaloustian J, Mikaïl C, Abou L, Portugal H. Antifungal effect of various essential oils against *Candida albicans*. Potentiation of antifungal action of amphotericin B by essential oil from *Thymus vulgaris*. *Phytother Res 18*: 990-995, 2004.
13. Guillaume MP, De Prez C, Cognan E. Subacute mitochondrial liver disease in a patient with AIDS: possible relationship to prolonged fluconazole administration. *Am J Gastroenterol 91*: 165-168, 1996.
14. Gullo FP, Rossi SA, Sardi JCO, Teodoro VLI, Mendes-Giannini MJS, Fusco-Almeida AM. Cryptococcosis: epidemiology, fungal resistance, and new alternatives for treatment. *Eur J Clin Microbiol Infect Dis 32*: 1377-1391, 2013.
15. He M, Du M, Fan M, Bian Z. In vitro activity of eugenol against *Candida albicans* biofilms. *Mycopathologia 163*: 137-143, 2007.
16. Inouye S, Takizawa T, Yamaguchi H. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. *J Antimicrob Chemother 47*: 565-573, 2001.
17. Jarvis JN, Dromer F, Harrison TS, Lortholary O. Managing cryptococcosis in the immunocompromised host. *Curr Opin Infect Dis 21*: 596-603, 2008.
18. Khazaie HR, Nadjafi F, Bannayan M. Effect of irrigation frequency and planting density on herbage biomass and oil production of thyme (*Thymus vulgaris*) and hyssop (*Hyssopus officinalis*). *Ind Crops Prods 27*: 315-321, 2008.
19. Klarić MŠ, Kosalec I, Mastelić, Piecková E, Pepeljnak S. Antifungal activity of thyme (*Thymus vulgaris* L.) essential oil and thymol against moulds from damp dwellings. *Lett Appl Microbiol 44*: 36-42, 2007.
20. Kordali S, Cakir A, Ozer H, Cakmakci R, Kesdek M, Mete E. Antifungal, phytotoxic and insecticidal properties of essential oil isolated from Turkish Origanum acutidens and its three components, carvacrol, thymol and p-cymene. *Bioresour Technol 99*: 8788-8795, 2008.
21. LiJuan L, Wei C, Hui X, Zhe W, Ruo-yu L, Wei L. Antifungal activity of ibuprofen against *Aspergillus species* and its interaction with common antifungal drugs. *J Chin Med Assoc 123*: 2701-2705, 2010.
22. NIST (National Institute of Standards and Technology). *PC version of the NIST/EPA/NIH Mass Spectral Database*. Gaithersburg, MD, U.S. Department of Commerce, 1998.
23. Nikolić M, Glamočlija J, Ferreira ICFR, Calhelha RC, Fernandes Â, Marković T, Marković D, Giweli A, Soković M. Chemical composition, antimicrobial, antioxidant and antitumor activity of *Thymus serpyllum* L., *Thymus algeriensis* Boiss and Reut and *Thymus vulgaris* L. essential oils. *Ind Crops Prods 52*: 183-190, 2014.
24. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS 23*: 525-530, 2009.
25. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS, Larsen RA, Lortholary O, Nguyen MH, Pappas PG, Powderly WG, Singh N, Sobel JD, Sorrel TC. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. *Clin Infect Dis 50*: 291-322, 2010.
26. Pina-Vaz C, Rodrigues AG, Pinto E, Costa-de-Oliveira S, Tavares C, Salgueiro L, Cavaleiro C, Gonçalves MJ, Martinez-de-Oliveira J. Antifungal activity of Thymus and their major compounds. *J Eur Acad Dermatol Venereol 18*: 73-78, 2004.

27. Pinto E, Pina-Vaz C, Salgueiro L, Gonçalves MJ, Costa-de-Oliveira S, Cavaleiro C, Palmeira A, Rodrigues A, Martinez-de-Oliveira J. Antifungal activity of the essential oil of *Thymus pulegioides* on *Candida, Aspergillus* and dermatophyte species. *J Med Microbiol 55*: 1367-1373, 2006.

28. Pinto E, Hrimpeng K, Lopes G, Vaz S, Gonçalves MJ, Cavaleiro C, Salgueiro L. Antifungal activity of Ferulago capillaris essential oil against *Candida, Cryptococcus, Aspergillus* and dermatophyte species. *Eur J Clin Microbiol Infect Dis 32*: 1311-1320, 2013.

29. Prado M, Silva MB, Laurenti R, Travassos LR, Taborda CP. Mortality due to systemic mycoses as a primary cause of death or in association with AIDS in Brazil: a review from 1996 to 2006. *Mem Inst Oswaldo Cruz 104*: 513-521, 2009.

30. Rota MC, Herrera A, Martínez RM, Sotomayor JA, Jordán MJ. Antimicrobial activity and chemical composition of *Thymus vulgaris, Thymus zygis* and *Thymus hyemalis* essential oils. *Food Control 19*: 681-687, 2008.

31. Sartoratto A, Machado ALM, Delarmelina C, Figueira GM, Duarte MCT, Rehder VLG. Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. *Braz J Microbiol 35*: 275-280, 2004.

32. Scott EM, Tariq VN, McCrory RM. Demonstration of synergy with fluconazole and either ibuprofen, sodium salicylate, or propylparaben against *Candida albicans* in vitro. *Antimicrob Agents Chemother 39*: 2610-2614, 1995.

33. Situ H, Bobek LA. *In vitro* assessment of antifungal therapeutic potential of salivary histatin-5, two variants of histatin-5, and salivary mucin (MUC7) domain 1. *Antimicrob Agents Chemother 44*: 1485-1493, 2000.

34. Slater TF, Sawyer B, Sträuli U. Studies on succinate-tetrazolium reductase system III: points of coupling of four different tetrazolilm salts. *Biochem Biophys Acta 77*: 383-393, 1963.

35. Soković MD, Vukojević J, Marin PD, Brkić DD, Vajs V, van Griensven LJLD. Chemical composition of essential oils of *Thymus* and *Mentha* species and their antifungal activities. *Molecules 14*: 238-249, 2009.

36. Souza LKH, Costa CR, Fernandes OFL, Abrão FY, Silva TC, Treméa CM, Silva MRR. Clinical and microbiological features of cryptococcal meningitis. *Rev Soc Bras Med Trop 46*: 343-347, 2013.

37. Suwanmanee S, Kitisin T, Luplertlop N. In vitro screening of 10 edible Thai plants for potential antifungal properties. *Evid Based Complement Alternat Med 2014*: 1-7, 2014.

38. Van Den Dool H, Kratz PDJA. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J Chromatogr 11*: 463-471, 1963.

39. Zollo PHA, Biyiti L, Tchoumbougnang F, Menut C, Lamaty G, Bouchet Ph. Aromatic Plants of Tropical Central Africa. Part XXXII. Chemical composition and antifungal activity of thirteen essential oils from aromatic plants of Cameroon. *Flavour Fragr J 13*: 107-114, 1998.

40. Zuzarte M, Gonçalves MJ, Cavaleiro C, Cruz MT, Benzarti A, Marongiu B, Maxia A, Piras A, Salgueiro L. Antifungal and anti-inflammatory potential of *Lavandula stoechas* and *Thymus herba-barona* essential oils. *Ind Crops Prods 44*: 97-103, 2013.