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Essential oil composition of *Mentha* spp. extracted seasonally and their effects against *Candida* yeast growth and biofilm formation

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ABSTRACT

Studies with essential oil of *Mentha* spp. in the literature have shown potential antimicrobial activity against *Candida* spp. In this context, the aim of this study was to evaluate the antifungal activity, biofilm inhibition and disruption of *Candida* spp., and cytotoxic activity of essential oils from *Mentha* spp. extracted seasonally. The essential oils of *Mentha* spp. (*Mentha aquatica, Mentha arvensis* and *Mentha piperita*) were extracted monthly over a year, followed by the analysis of gas chromatography. The oils were tested against *Candida* spp. for obtainment of minimal inhibitory concentration determined by the broth microdilution test (MIC). The biofilm formation and mature biofilm were measured by a spectrophotometer of microplates after exposure to XTT. The cytotoxic activity was determined by the antiproliferative activity test of HaCat cells and analyzed by IC 50%. The *Mentha* spp. oils, harvested from April to September, showed growth inhibition in planktonic cells of *Candida* spp. at 2 mg/ml. Compounds of higher production in this period were: dihydrocarveol and carvotanacetone (*Mentha aquatica*); menthol (*Mentha arvensis*); menthofuran and menthyl acetate (*Mentha piperita*). EOs from the three species of *Mentha* were able to inhibit the formation of biofilm by *C. albicans* MYA 2876 and to deconstruct mature biofilm (24 h). All tested concentrations (initial 2mg.ml^{-1}) showed IC 50% below or equal to 50% for HaCat cell lineage. The antifungal activity and detection of essential oil components by *Mentha* spp. were related to seasonal weather conditions. The essential oil of *Mentha* spp. is a potential biofilm inhibitor for *C. albicans,* and exhibits low cytotoxicity in HaCat cells.

Keywords: *Mentha* spp., medicinal plants, antifungal activity.

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INTRODUCTION

Medicinal plants have been used for centuries as an alternative treatment for diseases in different regions of the world, due to their therapeutic value (Cechinel Filho and Yunes, 1998; Santos et al., 2009; Cragg and Newman, 2013). Plants of genus *Mentha* spp. are distributed worldwide, and known for their medicinal properties, producing one of the most used essential oil (EO), a complex mixture of volatile compounds, such as terpenoids. Recent studies have demonstrated that EOs from species of *Mentha* reveal antimicrobial properties, especially antifungal activity against yeasts from genus *Candida*, which make them promising sources of therapeutic compounds for oral infections (Kasrati et al., 2012; Oumzil et al., 2002; Marcos-Arias et al., 2011).

In the last two decades, *Candida* species have become more frequent as agents of fungal infections, particularly associated with immunocompromised patients. They emerged as major agents of hospital acquired infections,

ranked as the third or fourth most commonly isolated bloodstream pathogens, passing gram-negative bacilli in frequency (Jabra-Rizk et al., 2004; Santana et al., 2013; Montero et al., 2012).

At the same time, it has been reported in literature, an increasing occurrence of resistance of these microorganisms to available antifungal drugs, such as polyenes and azoles agents. Therefore, researchers in this field have searched new alternatives compounds for drugs with potential action against these organisms. Although *C. albicans* is the predominant etiologic agent of candidiasis, other *Candida* species tend to be less susceptible to the commonly used antifungal drugs such as *C. krusei, C. glabrata, C. lusitaniae*, and *C. dubliniensis*, emerging as substantial opportunistic pathogens (Ramesh et al., 2011; Pfaller, 2012; Singh-Babak et al., 2012; Rodrigues et al., 2014).

Most manifestations of candidiasis are in fact associated with the formation of C*andida* biofilms on surfaces, and this phenotype is associated with infection at both mucosal and systemic sites (Santana et al., 2013). Superficial infections caused by Candida are frequent in patients with prosthesis, reaching 65% of the cases (Chandra et al., 2001; Jabra-Rizk et al., 2004).

Determining the antimicrobial properties of EOs might help to overcome microorganisms' resistance to antibiotics. The chemical composition of aromatic plants depends largely on the individual genetic variability and different plant parts (Wynn et al., 1999; Harry et al., 2002; White et al., 2002). The presence and concentration of certain chemical constituents of EOs also fluctuate according to the season, climatic condition, and site of plant growth (Zargari, 1990; Burt, 2004; Saharkhiz et al., 2012; Kasrati et al., 2012). Therefore, the aim of this study was to: 1) investigate the chemical composition of essential oils obtained from aerial parts of three *Mentha* species (*M. piperita, M. aquatica* and *M. arvensis*) extracted monthly over one year; 2) evaluate *in vitro* antifungal activity of the EOs extracted; 3) test the action of essential oil on biofilm formation and mature biofilm; 4) investigate the presence of cytotoxicity of the EOs on HaCat cells.

MATERIALS AND METHODS

Selected plants and EO extraction

It was included in this study three species of *Mentha*: *Mentha piperita* (CPMA 560)*, Mentha arvensis* (CM 406), and *Mentha aquatica* (CM 19). Selected plants were collected from the agronomic experimental area of the Research Center for Chemistry, Biology and Agriculture, State University of Campinas (CPQBA/UNICAMP), Brazil.

Exsiccates of selected plants are deposited in Herbarium CPQBA/UNICAMP with the following numbers: *Mentha piperita* (UEC 127.110), *Mentha arvensis* (CPMA 406), and *Mentha aquatica* (CPMA 1916).

The plants were extracted monthly, around 9:30 p.m., over one year. The essential oils were obtained from 100 g of aerial fresh plant parts by water distillation, using a Clevenger-type system during 1.5 h. The aqueous phase was extracted three times with 50 ml of dichloromethane. The pooled organic phases were dried with sodium sulphate, filtered, and the solvent was evaporated until dryness. Oil samples were stored at −25°C in sealed glass vials.

Gas chromatography with mass spectrometry analyses (GC-MS)

The identification of volatile constituents was performed using a Hewlett-Packard 6890 gas chromatograph, equipped with an HP-5975 mass selective detector and HP-5 capillary column (25 m × 0.25 mm × 0.33 µm diameter). GC-MS were performed using split/splitless injection, with the injector set at 220°C, column set at 60°C, with a heating ramp of 3°C.min^{-1} and a final temperature of 240°C, and the MS and FID detector set at 250°C. Helium was used as a carrier gas at 1.0 ml.min^{-1}.

The GC-MS electron ionization system was set at 70 eV. A sample of the essential oil was solubilized in ethyl acetate for the analyses. Retention indices (RIs) were determined by co-injection of hydrocarbon standards. The oil components were identified by comparison with data from the literature, the profiles, the Nist 05 library, and by co-injection of authentic standards, when available.

Yeasts samples

Nine referenced strains *of Candida* spp. were included in this study. They were obtained from Centraalbureau voor Schimmelcultures - Netherlands (CBS) and Instituto Zimotécnico – USP, Brazil (IZ): *Candida albicans* (CBS 562), *Candida parapsilosis* (CBS 604), *Candida krusei* (CBS 573), *Candida guilliermondii* (CBS 566), *Candida tropicalis* (CBS 94), *Candida dubliniensis* (CBS 7987*), Candida utilis* (CBS 5609)*, Candida glabrata* (IZ 07) *and Candida rugosa* (IZ 12).

Eight clinical strains of *C. albicans* from patients with dental prosthesis, isolated from the oral cavity and/or the surface of the prosthesis, were gently provided by Professor Dr. Marcelo Fabiano Gomes Boriollo (Research Ethics Committee protocol number CAAE: 51583115.2.0000.5418).

Antifungal activity assay - Minimal inhibitory concentration (MIC)

Yeasts were grown overnight at 37°C in Sabouraud Dextrose Agar (SDA) plates. Inoculum for the assays were prepared by diluting scraped cell mass into 0.9% NaCl solution, adjusted to McFarland scale 0.5, and confirmed by spectrophotometric reading at 530 nm. Cell suspensions were finally diluted to 10^4 UFC.ml^{-1} in RPMI-1640 medium (Sigma) for assays. MIC tests were carried out according to CLSI 2008, using tissue culture test plate (96 wells), containing 100 µl RPMI-1640 medium. The stock solutions of the essential oil were diluted in DMSO, mixed with RPMI medium and transferred into the first well, and serial dilutions were performed to obtain concentrations ranging from 2 to 0.0312 mg.ml^{-1}. Fluconazol (Sigma) was used as antimycotic control reference ranging from 64 to 0.0625 µg.ml^{-1}. The yeast inoculum was added to all wells, and plates were incubated at 37°C for 48 h. MIC was defined as the lowest concentration of oil that inhibited visible growth (CLSI, 2008).

Biofilm assay

Biofilm formation assay

The wild-type *C. albicans* strain MYA 2876 (ATCC) was used to perform the biofilm assays.

Briefly, the strain grown aerobically in SDA medium for 24 h was inoculated into Yeast Peptone Dextrose (YPD) broth under agitation in an incubator shaker at 30 rpm (Shaker incubator - Solab Instruments). After another 24 h incubation, cells were washed twice with PBS (phosphate buffer) and suspended in RPMI 1640, posteriorly the cells were counted in hemocytometer, acquiring a concentration of 1.0×10^6 cells/ml. Aliquots of 100 µl of cell suspension were transferred into each well of a presterilized flat-bottomed 96-wells microtiter plates of polystyrene (Global Plast.), and the plate was incubated for 90 min at 37°C in an orbital shaker (Nova Instruments thermo Shaker incubator) at 100 rpm for biofilm adhesion. Subsequently, the cell suspension was gently aspirated and the plate washed twice with PBS and 100 µl of essential oil (concentration ranging from 8 to 0.0078 $mg.ml^{-1}$) diluted in RPMI-1640 supplemented with Tween 80 (final concentration of 0.001% *v*/*v*) was added to each well. The plates were incubated for 24 h at 37°C in aerobically incubator (Da Silva et al., 2010 modified).

Mature biofilm assay

The strain grown aerobically in SDA medium for 24 h was inoculated into Yeast Peptone Dextrose (YPD) broth under agitation in an incubator shaker at 30 rpm (Shaker incubator - Solab Instruments). After 24 h incubation, cells were washed twice with PBS (phosphate buffer) and suspended in RPMI 1640, posteriorly the cells were counted in hemocytometer, acquiring a concentration of 1.0×10^6 cells/ml. Aliquots of 100 –µl of cell suspension were transferred into each well of a presterilized flat-bottomed 96-wells microtiter plates of polystyrene (Global Plast.), and the plate was incubated for 24 h at 37°C. After this period of incubation, the biofilm mature was adhered at the plate; and to remove planktonic cells, the cell suspension was gently aspirated, and the plate washed twice with PBS. Then, 100 µl of essential oil (concentration ranging from 4 to 0.00365 $mg.ml^{-1}$), diluted in RPMI-1640 and supplemented with Tween 80 (final concentration of 0.001% *v*/*v*) was added to each well. The plates were incubated for 24 h at 37°C in aerobically incubator (Pierce et al., 2008).

Quantification of biofilm

The biofilms were measured by the addition of 80 µl of 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT- Sigma Chemical Co.), reacting for two hours. Optic density was measured by a microplate reader at 590 nm, which is a semi quantitative method, and measured the metabolic activity of cells in the biofilm (Pierce et al., 2008). All experiments were repeated three times and performed in triplicate.

Cytotoxicity assay- antiproliferative activity – HaCat cells

The cell line of keratinocytes HaCat was used for the test. In a 96-well, cell-culture plates were incubated for 24 h at concentration of 6.5×10^4 $cel.ml^{-1}$ in RPMI supplemented with 10% FBS (Fetal Bovine Serum). After the incubation, the cells were gently aspirated and added 100 µl of EO (concentration ranging from 2 to 0.0625 $mg.ml^{-1}$), diluted in RPMI-1640 and supplemented with Tween 80 (final concentration of 0.001% *v*/*v*). The plate was incubated for 24 h at 37°C and 5% of CO_2. Posteriorly, cultures fixed with 10% of trichloroacetic acid for 1 h at 4°C were stained for 30 min with 0.4% sulforhodamine B (SRB) in 1% acetic acid, and subsequently washed with distilled water. Bound SRB was solubilized with 150 µl 10 mM Tris-base solution. Absorbance was read in an ELISA plate reader at 540 nm. The optic density values were expressed in percentage of cell viability relative to control cells (100%). Data were correlated with the concentration of essential oil used, obtaining cell viability curves. Through this curve, one may find the index cytotoxicity (IC 50%) and the concentration of the extract that induces 50% lysis or cell death (Endo et al., 2010).

Statistical analysis

All statistical tests were performed with one-way ANOVA, and P value of < 0.05 was considered statically significant. The statistical test was run using Bioestat 5.0 software. Tukey's test was used for the statistical tests of essential oil yield and for the antiproliferative activity and biofilm assay, Dunnett's test was carried out.

RESULTS

Composition and yield of essential oil from *Mentha* species

The *Mentha* species tested yielded different quantities of EOs over one year. Oil yields were expressed regarding the fresh weight of the plant material (%w/w). Values between 0.3 (June) to 1.5% (September) were obtained for *M. aquatica*; 0.58 (May) to 1.58% (November) for *M. arvensis*, and 0.3 (May) to 1.4% (April) for *M. piperita*. Larger quantities of EOs were obtained from the three plants species during the months from September to April, and through the period of May to August a low production of Eos was found, except for *M. piperita* that produced similar quantities of EOs in the months of August and September. The monthly values obtained from the EOs are expressed in Figure 1.

Chemical components, present in EOs samples from each month of collection, were obtained through Gas Chromatography. Table 1 shows the major chemicals constituents of the EOs, monthly. Variations were observed in the composition of the EOs for a period of one year. Some compounds were present in greater amounts in some periods as: limonene, cineole, dihydrocarveol, carvone, carvotanacetone for *M. aquatica*; menthone, menthol, pulegone and piperitone for *M. arvensis*; isomenthone, menthone, menthofuran, menthol, pulegone, piperitone and isobornyl acetate for *M. piperita*.

Most of the essential oil composition of *Mentha* spp. is constituted by monoterpenes.

The EOs of the three species of *Mentha* tested showed anti-*Candida* activity, but not for all samples collected. The inhibition of the yeasts was observed in the EOs collected during the months of April to October (Table 2). The MIC values varied from 0.25 to 2.0 $mg.ml^{-1}$ from the oils of November to March, which represent the period of higher temperatures and humidity in Brazil. Most of the strains of *Candida* were susceptible, except *C. glabrata* and *C. tropicalis*, resistant to all samples of EOs tested (Figure 2).

Biofilm assay

EOs from the three species of *Mentha* inhibited the

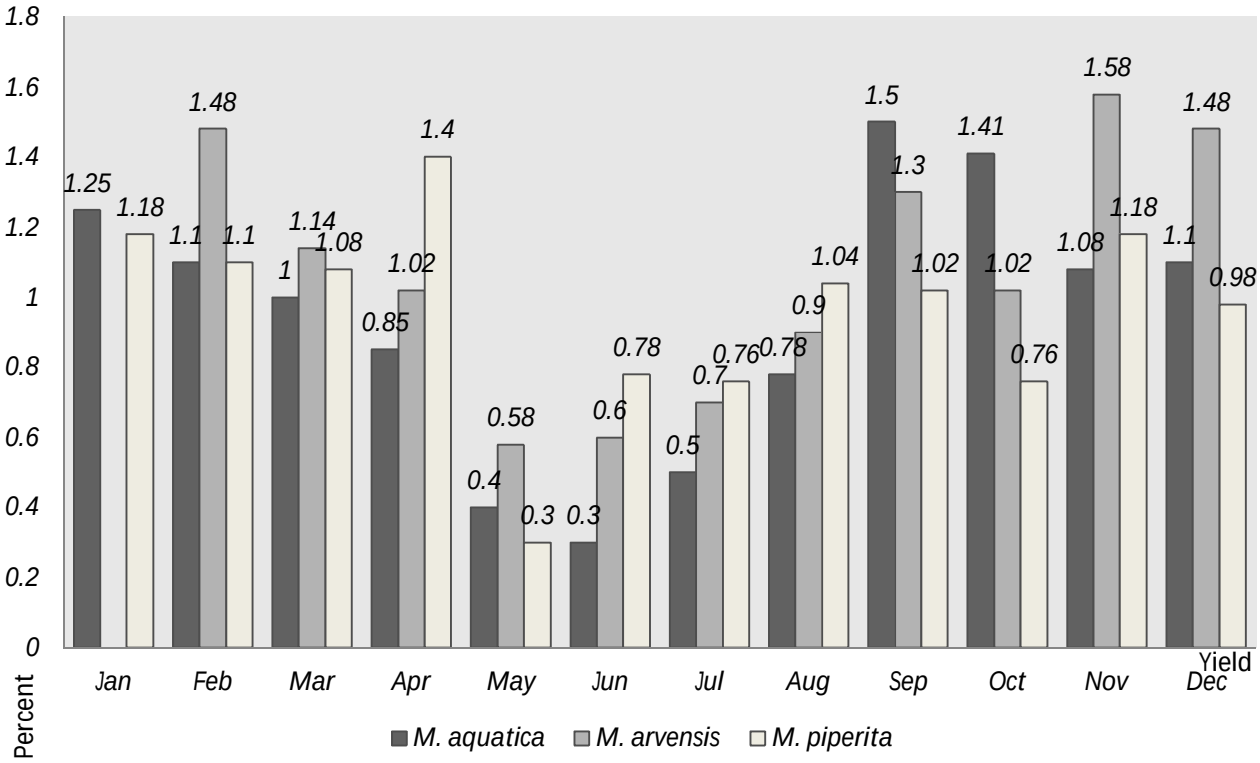

Figure 1. Graphic representation of EOs yield from *Mentha* spp. obtained over one year of analysis (%). The data of january of *Mentha arvensis* was not obtained.

formation of biofilm by *C. albicans* MYA 2876 and deconstructed the mature biofilm (24 h). The oil used was the mixture that exhibited most activity. As shown in Figure 2, the three EOs inhibited up to 50% of the biofilm formation at concentrations from 0.03125 to 4 mg.ml^{-1}. The inhibition of biofilm found in 2 mg.ml^{-1} (MIC value) of EOs was about 10%, compared to the control group without oil.

Figure 3 shows that EOs disrupted up to 50% of the biofilm formation at concentrations from 0.250 to 4 mg.ml^{-1}. The disruption of biofilm found in 2 mg.ml^{-1} (MIC value) of EOs was about 80%, compared to the control group without oil.

Cytotoxicity assay- antiproliferative activity – HaCat cells

All tested concentrations (initial 2 mg.ml^{-1}) showed IC 50% below or equal to 50% for HaCat cell lineage (Figure 4). This shows that the essential oils exhibit low cytotoxicity in HaCat cells.

DISCUSSION

The composition and yield of the EOs might be affected by the developmental stages of the plant and by the seasonal and ambient conditions (Deschamps et al., 2008). Fluctuations in seasonal temperature and precipitation standards affected the composition and the yield of essential oil in the plants (Grulova et al., 2015). We observed that both the production and the composition of the EOs obtained monthly from the three species of *Mentha* suffered variations over the one-year period, corroborating with literature.

The agronomic experimental area of CPQBA/UNICAMP, where the plants were cultivated, is located in the Southeast region of Brazil. In this region, autumn and winter periods include the months from April to September, in which a lower production of EOs was observed. Therefore, it suggests that the production and characteristics of EOs in these three *Mentha* species analyzed (*M. aquatica*, *M. arvensis* and *M. piperita*) were affected by lower temperature (an average of 19°C – data from Center of Meteorological Research – UNICAMP, www.cepagri.com.br). Our results are in accordance with other authors that found lower production of EOs in cold temperatures for different *Mentha* species, including the three tested in this study (Hussain et al., 2010; Deschamps et al., 2008). In addition, during the summer, the higher temperatures and long days induced greater carbon assimilation in photosynthesis, which allows a larger production of biomass by the plant and, consequently an increase of EOs production (Grulova et al., 2015).

Table 1. EOs major chemical constituents from monthly collected plants by gas chromatography analysis (%).

Components	Jan (summer)			Feb (summer)			Mar (autumn)			April (autumn)			May (autumn)			June (winter)		
	Ma	Mar	Mp	Ma	Mar	Mp	Ma	Mar	Mp	Ma	Mar	Mp	Ma	Mar	Mp	Ma	Mar	Mp
Octanol<3->						0.30			7.5	0.4			0.5			8.5		
Limonene	21.8		2.3	0.8	2.9	1.6	15.9	2.6	8.5	12	2.9	1.9	11	2	1.9	2.7	2	1.3
Cineole<1,8->			5.1	20.1		3.5	3		8.6	2.6		3.0	3.7		2.7	0.6		2.2
Terpineol<α->	16			0.7			0.6			0.7			0.7					
Menthone			18.6		24.0	31.0		15.23	13.3		17.7	24.0		5.06			4.4	
Menthofuran			7.3			7.3			13.7			10.6			18.6			5.3
Menthol			40.4		61.1	39.5		53.2	28.7		42.0	36		66.8	33.8		67.6	45.3
Terpinen-4-OL			1.0			1.10			14.3						0.6			0.6
Dihidrocarveol<IS0>													10.6					
Pulegone					4.0			10			31.6	5.3	5.9		2.3			1.4
Piperitone	2.4		0.40	2.21	1.9	0.70	1.6	1.9	17.5	2.0	1.4		1.7	20.6			20.7	
Methyl Acetate			1.2		2.0	0.35		3	18.3		7.2	5.4		0.41	3.2		0.45	4.0
Isobornyl Acetate			12.4			4.0			19.2						23.5			27.0
Dihydrocarveol				0.8			0.5			7.7								
Carvone				57.9			70			68			49					
Carvotanacetone	55.3												0.5					

Components	July (winter)			Aug (winter)			Sept (spring)			Oct (spring)			Nov (spring)			Dec (summer)		
	Ma	Mar	Mp	Ma	Mar	Mp	Ma	Mar	Mp	Ma	Mar	Mp	Ma	Mar	Mp	Ma	Mar	Mp
Octanol<3->																		
Limonene	1.6		30	3.9		0.7	4.5	2.0	2.7	15.0	3.3	1.8	16.0	2.8	1.8	19.0	1.0	1.6
Cineole<1,8->	0.6		0.5	1.6		0.8	1.5		2.45			3.60			4.5			5.5
Terpineol<α->	0.8			0.8			0.9			2.0			1.8					19.2
Menthone			3.0		4.0	8.2		4.5	21.5		22.3	25.5		4.7	28.0		18.4	5.0
Menthofuran			4.7			3.8			18.0			8.7			1.0			49.0
Menthol			7.4		59.3	23.8		65.7	30.0		54.0	41.0		82	40.0		69.0	1.0
Terpinen-4-OL												1.3						
Dihidrocarveol<IS0>																		
Pulegone						1.20			2.1		15.5	1.3		6.8	1.4		5.3	
Piperitone	2.0			2.7	20.0		2.8	20.0		2.0	1.5	0.8	1.8	2.0		2.0	1.3	0.5
Methyl Acetate						3.5			2.0		3.0	1.0		1.5			1.3	0.5
Isobornyl Acetate			7.7			22.2		19.6				12.0			6.0			6.8
Dihydrocarveol	2.4			13.5			2.7									2.8		
Carvone																		
Carvotanacetone	83.4			66.7			77.0			77.7			77.6			65.6		

Ma: *Mentha aquatica*; Mar: *Mentha arvensis*; Mp: *Mentha piperita.*

Table 2. Inhibition of *Candida* strains by *Mentha* spp. essential oils obtained monthly. Minimal inhibitory concentration (MIC).

Candida strains	April (mg/ml)			May (mg/ml)			June (mg/ml)			July (mg/ml)			August (mg/ml)			September (mg/ml)			October (mg/ml)			µg/ml
	Aq	Ar	Pi	Aq	Ar	Pi	Aq	Ar	Pi	Aq	Ar	Pi	Aq	Ar	Pi	Aq	Ar	Pi	Aq	Ar	Pi	Fluco
CBS 562	-	PG	2	-	-	2	-	2	2	PG	-	2	-	PG	2	-	-	-	-	-	-	-
CBS 604	-	2	0.5	-	2	2	-	2	2	PG	-	2	-	-	2	-	-	-	-	-	-	4
IZ 07	-	-	-	-	-	-	-	-	-	PG	-	-	-	-	-	-	-	-	-	-	-	32
CBS 573	-	1	2	PG	2	PG	2	2	2	2	-	2	-	-	2	-	-	-	-	-	-	16
CBS 7987	-	2	1	2	2	2	2	2	2	2	-	2	PG	2	2	-	2	2	-	2	2	1
CBS 566	2	1	1	1	2	2	2	2	2	2	-	2	2	2	2	PG	2	2	-	2	2	1
IZ 12	-	2	PG	-	PG	-	-	PG	-	PG	-	-	-	-	-	-	-	-	-	-	-	64
CBS 5609	1	0.25	0.5	0.5	1	1	1	1	1	0.5	-	1	1	2	0.5	2	2	2	-	2	2	8
CBS 94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MPB 330	-	2	-	-	-	2	2	-	-	1	-	2	2	-	2	2	-	-	-	-	-	-
MPB 335	-	2	2	-	-	-	-	2	-	2	-	2	-	-	2	2	-	-	-	-	-	-
MPB 338	-	2	2	-	-	-	-	2	-	2	-	2	-	-	2	2	-	-	-	-	-	-
MPB 368	-	2	-	-	-	2	2	-	2	1	-	2	2	-	2	2	-	-	-	-	-	-
MPB 378	-	2	-	-	-	2	2	-	2	1	-	2	2	-	2	-	-	-	-	-	-	-
MPB 462	-	2	-	-	-	2	-	-	-	2	-	2	-	-	2	-	-	-	-	-	-	-

PG: Partial Growing, Aq: *Mentha aquatica*; Ar: *Mentha arvensis*; Pi: *Mentha piperita*; Fluco: Reference antifungal Fluconazole; CBS 562: *Candida albicans*; CBS 604: *Candida parapsilosis*; CBS 573: *Candida krusei*; CBS 566: *Candida guiliermondii*; CBS 94: *Candida tropicalis;* CBS 7987: *Candida dubliniensis*; CBS 5609: *Candida utilis*; IZ 07: *Candida glabrata;* IZ 12: *Candida rugosa;* MPB: Clinical Strains of *Candida albicans* from oral cavity and prosthesis; (-):no inhibition observed in the tested concentration range. No inhibition was observed in the months of November to March.

Thus, temperature, genetic and solar radiation incidence may affect the production of EOs. Such factors also suggest an influence to the essential oil's chemical composition. Quantitative changes in production of some important compounds like menthol, menthone and limonene were observed (Gobbo-Neto and Lopes, 2007).

The mostly encountered components (more of them 15% in at least three months) in the EOs of *M. aquatica* were limonene, carvone and carvotanacetone (Figure 1). Limonene is a precursor in the synthesis pathway of carvone, which was abundant since February to May (Autumn season), when temperatures were balmy. Moreover, carvotanacetone is a metabolic of carvone, and it was abundant since July until January. Data were not found in the literature that demonstrate the chemical composition of *M. aquatica* specifically. Therefore, our data will be useful for the comparison with other later studies. Analysis of antifungal activity of *M. aquatica* EOs demonstrates low inhibition of *Candida* strains. Carvone was the major constituent in Spring, Summer, and Autumn samples, while limonene was the major constituent of the Winter sample, followed by carvone. The leading components presented in the essential oil of *Mentha arvensis* were menthol, pulegone and menthone. Through the months of April to October, period of the largest antifungal activity, the most significant compounds found were: menthol and piperitone. The compounds found only in this period were pinene, myrcene, gurjunene and piperitone. The levels of menthol, limonene, menthone and pulegone remained constant year-round. The CG results showed larger amount of menthone, menthofuran, menthol and isobornyl acetate for *Mentha piperita* EO. In the months that essential oil showed antifungal activity, the major compounds were: menthone, menthofuran, menthyl acetate and isobornyl acetate. However, the levels of menthol maintained constant throughout the year. In this period cineole and sabinene maintained low levels when compared to November through March. The compounds presented only in the months from April to October were pulegone and pinene. The compounds that were absent in this period were

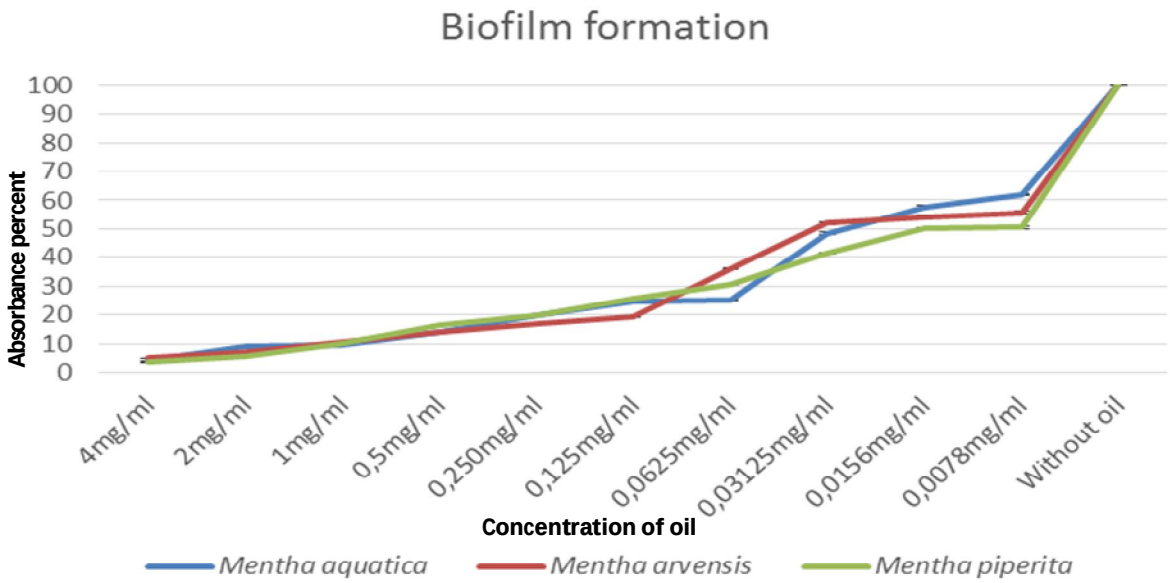

Figure 2. Graphic relative to the biofilm in the formation assay. The values were expressed in absorbance percent × oil concentration. Values present statistical significance, Dunnett Test, Anova, Bioestat Software.

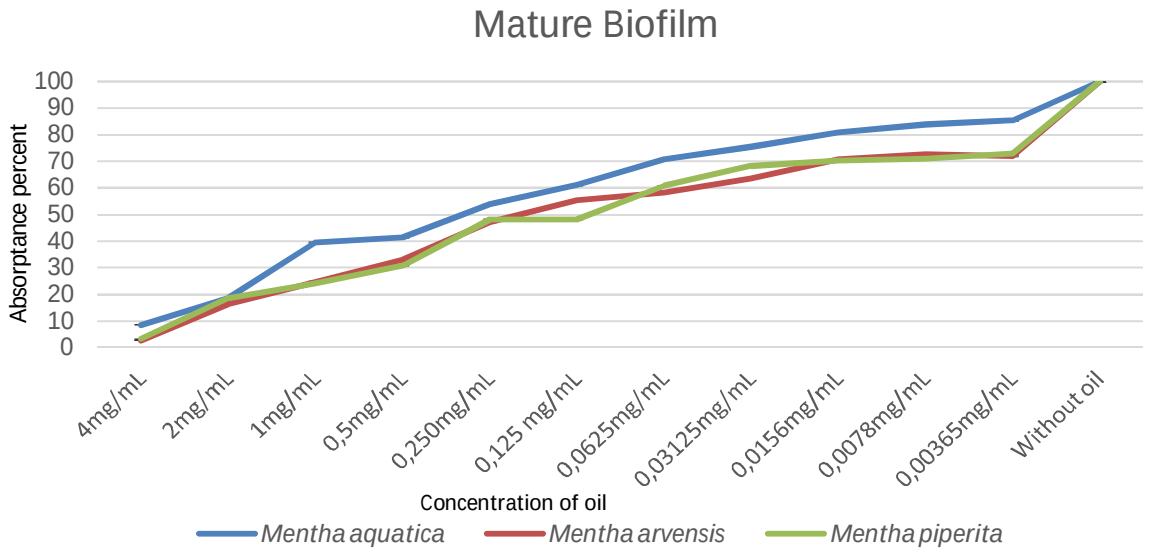

Figure 3. Graphic relative to the biofilm in the formation assay. The values were expressed in absorbance percent × oil concentration. Values present statistical significance, Dunnett Test, Anova, Bioestat Software.

gurjunene and octanol.

In this study, the chemical composition of the EOs was different for each species of *Mentha*. Terpenes group including menthol, carvone, and limonene are volatile secondary metabolites of plants and dominant constituents of essential oils extracted from genus *Mentha* (Lange, 2015). This class of compounds is known for its low molecular weight and hydrophobic characteristics, allowing its incorporation into the cell membrane, breaking it (Schelz et al., 2006; Mkaddem et al., 2009; Zore et al., 2011). The production of chemical compounds may vary depending on the season of the year, as well as the oil activity. Therefore, the period of the year and the climate conditions should be taken into account in researches regarding plant-derived products.

The fact that antifungal activity is observed only in some seasons of the year suggests that this activity is expressed by the presence of higher amounts of certain compounds, such as menthol, pulegone, carvone and menthofuran and/or a combination of their presence. Although antifungal activity has been found, the oils tested show low or weak antimicrobial activity, according

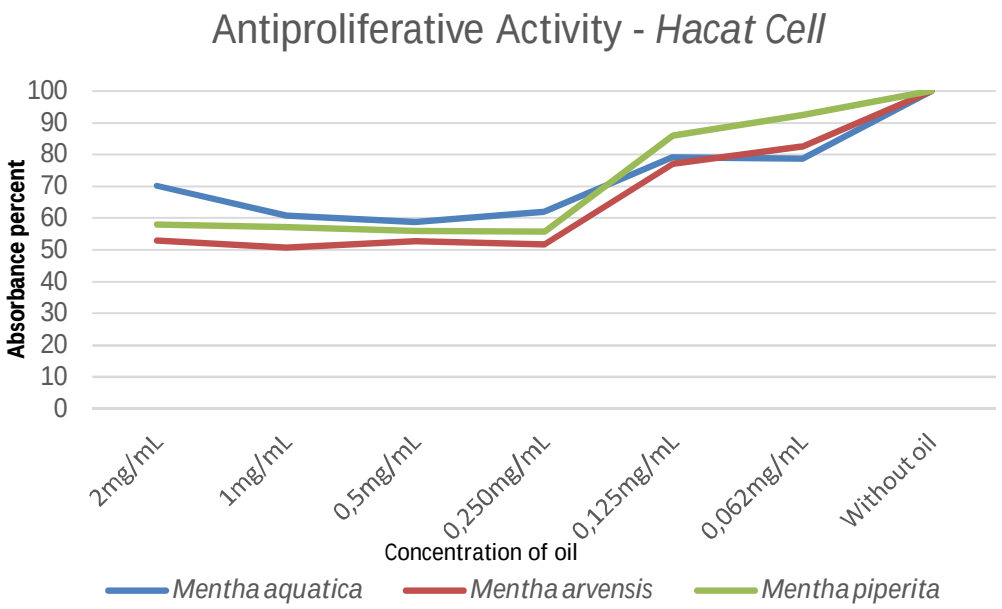

Figure 4. Graphic relative the test to antiproliferative activity at HaCat Cell. The values were expressed in absorbance percent × oil concentration. Values present statistical significance, Dunnett Test, Anova, Bioestat Software.

to Duarte et al. (2005) and Aligiannis et al. (2001).

The ability to form biofilms by grip on biotic and abiotic surfaces is an important virulence factor and an important cause of resistance to antifungal drugs. This has become one of the factors of major clinical relevance (Santana et al., 2013). Our study tested the adherence and metabolic activity of *C. albicans* biofilm by measuring the absorbance in the presence and in the absence of essentials oils. The results clearly showed a decrease of adherence and metabolic activity in the presence of different concentrations of essential oil, with a decrease of biofilm formation up to 95% in the 4 $mg.ml^{-1}$, 90% in the 2 $mg.ml^{-1}$, and 50% in the 0.03125 $mg.ml^{-1}$. The mature biofilms which were treated with oils, showed able to disrupt the biofilm up to 90% at 4 $mg.ml^{-1}$, 80% at 2 $mg.ml^{-1}$, and 50% at 0.250 $mg.ml^{-1}$. All the oil tested showed some activity against *C. albicans* biofilm, being more efficient against the formation of biofilm, than in the disruption of a mature biofilm. However, the value mentioned outcomes in a very efficient destruction of mature biofilm and this result may be related with the oil's nature, promoting interactions with biofilm and becoming agents that remain for a good period in touch with biofilm (Pereira, 2009; Quintas et al., 2015). Moreover, the terpenes compounds also exhibit activity against biofilm growth (Carvalho and Fonseca, 2007). The fact that this virulence factor is one of the causes of resistance to antifungal drugs; our results show a promising natural product, the development of a substance to control clinical biofilms.

Regarding the cytotoxicity, EOs were tested on HaCat cells and did not show IC50 for the concentrations tested, but present indexes upcoming 50%. The essential oil of the tested *Mentha* showed lower toxicity on epithelial cells. Other studies found no cytotoxicity to *Mentha* EOs on different cell lines (Nair, 2001; Kanerva et al., 2001; Peixoto and Hofling, 2010). The activity found to natural products against microorganisms often are not strong, with high values of minimal inhibitory concentration (Duarte, 2005), even so, these products may be useful as adjuvant in the treatment of *Candida* infections by reducing the expression of virulence factors and the resistance to antimicrobials (Ulrich-Merzenich et al., 2010; Wagner, 2011). The presence of synergic effects between natural products and commercial antimicrobial drugs could be an important alternative treatment in cases of resistance. This alternative may reduce a possible toxicity of conventional treatments with antimicrobial products, decreasing adverse effects and enhancing the treatment efficacy, since some natural products exhibit lower cytotoxicity activity, as show in our initials assays with *Mentha* spp. oil.

When it comes to researches involving possible medicinal effects of plants, phytochemical and physiological standards can be a useful tool in the production of chemical compounds to the expected activity.

CONCLUSION

The production and the composition of the essential oil of *Mentha* spp. are dependent of seasonal weather conditions. The oils of the three species of *Mentha*

(*Mentha aquatica, Mentha arvensis,* and *Mentha piperita),* show anti-*Candida* activity at an average temperature of 19°C, in which the greater production of compounds such as dihydrocarveol, dihydrocarvone and carvotanacetone (*M. aquatica*); menthol (*M. arvensis*); menthofuran and menthyl acetate (*M. piperita*) were found. The abundant yield of EOs, are obtained in higher temperatures. The results of this research show that weather conditions influence the composition and performance of *Mentha* spp. essential oils as antifungal agent.

The EOs of the three species of *Mentha* tested are able to inhibit biofilm formation and disrupt the mature biofilm of *C. albicans*. These oils and its components are potential inhibitor factor to *C. albicans* biofilm. The use of these essential oils represents an important alternative in the treatment of infections *by Candida* and in the reduction of its virulence factors of *Candida* spp.

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