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Effect of essential oils from *Mangifera indica* L. cultivars on the antifungal susceptibility of *Candida* spp. strains isolated from dogs

Efeito dos óleos essenciais de variedades de "Mangifera indica" L. na susceptibilidade antifúngica de cepas de Candida spp. isoladas de cães

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RESUMO

Este trabalho descreve a composição química e a atividade anti-*Candida* spp. de óleos essenciais de folhas de diversos cultivares de *Mangifera indica*. Os óleos essenciais foram obtidos por meio de hidrodestilação e analisados por cromatografia de gás acoplada à espectrometria de massa. A atividade anti-*Candida* spp. foi avaliada contra cepas isoladas de cães pelo método de difusão em ágar e a concentração inibitória mínima (CIM) pelo método de microdiluição em caldo. O cultivar Tommy Atkins apresentou β–selineno (29.49%), óxido de cariofileno (12.40%) e humuleno II epoxido (8.66%) como seus constituintes principais, enquanto que os principais componentes das variedades Rosa, Moscatel e Jasmim foram óxido de cariofileno (23,62, 48,42 e 30,77%, respectivamente) e humuleno II epoxido (11,56, 23,45 e 16,27%, respectivamente). As médias de zona de inibição foram 11 ± 0,71, 13,5 ± 3,54, 10,5 ± 0,71 e 13,5 ± 0,71mm respectivamente para os cultivares Tommy Atkins, Rosa, Moscatel e Jasmim. Para a variedade Tommy Atkins, a CIM variou de 0,62 a 1,25 mg/mL; para Rosa, 0.31 a 1.25 mg/mL; para Jasmim os valores variaram de 0,31 a 0,62 mg/mL; ao passo que para a variedade Moscatel o valor de CIM foi 1,25 mg/mL para todas as cepas de *Candida* spp. Os óleos essenciais das quatro variedades de *M. indica* foram ativos *in vitro* contra *Candida* spp., demonstrando boa atividade antifúngica, podendo ser uma fonte útil de compostos antifúngicos para uso na medicina veterinária.

Palavras-chave: manga, leveduras, óleos aromáticos, óxido de cariofileno, pequenos carnívoros

SUMMARY

This work reports the chemical study, and anti-*Candida* spp. activity of leaf essential oil from *Mangifera indica* cultivars. The essential oils were obtained by hydro-distillation and analyzed by gas chromatography/mass spectroscopy. The anti-*Candida* spp. activity was evaluated against strains isolated from dogs by the agar-well diffusion method and the minimum inhibitory concentration (MIC) by the broth microdilution method. Tommy Atkins

cultivar presented β–selinene (29.49%), caryophyllene oxide (12.40%) and humulene II epoxide (8.66%) as main constituents, while the main constituents of Rosa, Moscatel and Jasmim varieties were caryophyllene oxide (23.62, 48.42 and 30.77%, respectively) and humulene epoxide II (11.56, 23.45, and16.27%, respectively). The means of inhibition zones were 11 ± 0.71, 13.5 ± 3.54, 10.5 ± 0.71 and 13.5 ± 0.71mm to Tommy Atkins, Rosa, Moscatel and Jasmim varieties, respectively. For Tommy Atkins, the MIC ranged from 0.62 to 1.25 mg/mL; for Rosa, ranged from 0.31 to 1.25 mg/mL; for Jasmim ranged from 0.31 to 0.62 mg/mL; while for the Moscatel variety the MIC value was 1.25 mg/mL for all *Candida* strains. Essential oils of four *M. indica* cultivars were active *in vitro* against *Candida* spp., demonstrating good antifungal activity and can be a useful source of antifungal compounds for veterinary medicine.

Keywords: mango, yeasts, aromatic oils, caryophyllene oxide, small carnivores

INTRODUCTION

Mangifera indica L. (Sapindales: Anacardiaceae), a medicinal and horticultural plant, is among the most popular and best-known tropical fruits, including many cultivars (JAHURUL et al., 2015). Mango occupies the 2nd position as a tropical crop, behind only bananas in terms of production and acreage used (MUCHIRI et al., 2012). Traditionally, the mango plant has medicinal applications as a febrifuge and to treat diarrhea, dysentery, gastrointestinal tract disorders, typhoid fever, sore throat and scurvy. The mango fruits are source of vitamin A and also can be used in treatment of blood disorders (THOMAS et al., 2015).

Studies have reported the activity of several parts of this species, which include anti-inflammatory (SRAVANI et al., 2015), antioxidant (UMAMAHESH et al., 2016; DAS et al., 2015), anti-hyperglycemic (AWASTHI et al., 2016), antiulcerogenic (SEVERI et al., 2009), antihypertensive (RONCHI et al., 2015). The leaf decoction is popularly used as stomachic, anti-diarrheic and against genito-urinary inflammations, bronchitis and asthmas and in external use, in baths or washes against scabies and syphilis (AGRA et al., 2007). Bbosa et al. (2007) reported the antibacterial activity for the leaves and concluded that the use of mango leaf in conjunction with a toothbrush will be a good home care device for maintenance of oral hygiene. The seeds extract demonstrated antibacterial activity that may be due to the presence of tannin and higher amount of total phenol content (VAGHASIYA et al., 2011).

Malassezia sp. and *Candida* sp. are yeasts commonly found in normal flora from small carnivores, as dogs. Nevertheless, despite being saprobes, there have been many reports of infections caused by these microorganisms (BRITO et al., 2009). Infecction which present different clinical manifestations, such as dermatomycosis (YURAYART et al., 2014), systemic infections (SKORIC et al., 2011), urinary infections (ÁLVAREZ-PÉREZ et al., 2016) and otitis externa (EBANI et al., 2017).

Although effective antimicrobials have been developed over the years, there has been increased development of antimicrobial drug resistance to currently available antimicrobials (SANGUINETTI et al., 2015). Due to many activities of *M. indica* leaves, the essential oils from four cultivars found in Brazil, Tommy Atkins, Rosa, Moscatel and Jasmim, were screened for antimicrobial activity against *Candida* spp. strains isolated from symptomatic dogs.

MATERIALS AND METHODS

The leaves of four mango varieties Tomy Atkins, Rosa, Moscatel and Jasmim were collected in the Fortaleza city, State of Ceará situated in northeast

of Brazil (3°33'46'' latitude S, 41°05'42''longitude W). Fresh leaf of mango varieties were subjected to hydrodistillation for 2h in a modified Clevenger type apparatus, as described by CRAVEIRO et al. (1976). The oil was dried over anhydrous Na_2SO_4 (~1 g), filtered and preserved in a sealed vial at 4ºC prior to further analysis, with a yield of 0.85% (w/w). All the essential oils were kept in tightly stoppered bottle in a freezer until used for biological tests.

The chemical analysis of the essential oils constituents were performed on a Shimadzu QP-2010 instrument employing the following conditions: column: DB-5ms (Agilent, part No. 122-5532) coated fused silica capillary column (30m x 0.25mm x 0.25µm); carrier gas: He (1mL/min, in constant linear velocity mode); injector temperature was 250°C, in split mode (1:100), and the detector temperature was 250°C. The column temperature programming was 35 to 180°C at 4°C/min then 180 to 280°C at 17°C/min, and at 280°C for 10 min; mass spectra: electron impact 70 eV. The injected sample volume was 1µL. Compounds were identified by their GC retention times relative to know compounds and by comparison of their mass spectra with those present in the computer data bank (National Institute for Standard Technology – NIST – 147, 198 compounds) and published spectra (ADAMS, 2012; ALENCAR et al., 1984).

A total of three strains of *C. albicans* and two strains of *C. tropicalis* were included in this study. The *Candida* spp. strains were isolated from the preputial, vaginal, oral and perianal mucosae of healthy dogs. The isolates cultured were identified according to their biochemical profile and morphological characteristics (BRITO et al., 2009). The strains were stored in the fungal collection of the Specialized Medical Mycology Center – CEMM (Federal University of Ceará, Brazil), where they were maintained in saline (0.9% NaCl), at 28°C. At the time of the analysis, an aliquot of each suspension was taken and inoculated into potato dextrose agar (Difco, Detroit, USA), and then incubated at 28°C for 2-10 days.

For the agar-well diffusion method, based on Fontenelle et al. (2007), stock inocula was prepared on day 2, grown on potato dextrose agar (Difco, Detroit, USA) at 28°C. Potato dextrose agar was added to the agar slant and the cultures were gently swabbed to dislodge the conidia. The suspension with blastoconidia of *Candida* spp. was transferred to a sterile tube and adjusted by turbidimetry to obtain inocula of approximately 10^6 CFU/mL blastoconidia. The optical densities of the suspensions were spectrophotometrically determined at 530 nm and then adjusted to 95% transmittance.

For the broth microdilution method, standardized inocula (2.5 – 5 x 10^3 CFU/mL for *Candida* spp.) were also prepared by turbidimetry. Stock inocula was prepared on day 2, grown on potato dextrose agar at 28°C. Sterile normal saline solution (0.9%; 3mL) was added to the agar slant and the culture was gently swabbed to dislodge the conidia from the blastoconidia from *Candida* spp. (BRITO et al., 2009). The blastoconidia suspension was transferred to a sterile tube, and the volume of suspension adjusted to 4 mL with sterile saline solution. The resulting suspension was allowed to settle for 5 min at 28°C, and the density was read at 530nm and the adjusted to 95% transmittance. The suspension was diluted to 1:2000 with RPMI 1640 medium (Roswell Park Memorial Institute – 1640) with L-glutamine, without sodium bicarbonate (Sigma Chemical Co., St. Louis, Mo.), buffered to pH 7.0 with 0.165M morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co., St. Louis, Mo.), to

obtain the inoculum size of approximately 2.5 – 5 x 10^3 CFU/mL.

The antifungal activity of essential oils was evaluated against *Candida* spp. by the agar-well diffusion method according to Fontenelle et al. (2007). Petri dishes with 15cm diameter were prepared with potato dextrose agar (Difco, Detroit, USA). The wells (6 mm in diameter) were then cut from the agar and 100µL of essential oil was delivered into them. The oils were weighed and prepared in dimethyl sulfoxide (DMSO) to obtain the test concentrations of 10mg.mL^{-1}. Stock solutions of amphotericin B (0.005 mg.mL^{-1}; Sigma Chemical Co., USA) was prepared in distilled water and tested as positive control for *Candida* spp. Each fungal suspension was inoculated on to the surface of the agar. After incubation, for 3-5 days at 28˚C, all dishes were examined for zones of growth inhibition and the diameters of these zones were measured in millimeters. Each experiment was repeated at least twice.

The minimum inhibitory concentration (MIC) for *Candida* spp. was determined by the broth microdilution method, in accordance to M27-A3 guidelines of Clinical and Laboratory Standards Institute (CLSI, 2008). The minimum fungicidal concentration (MFC) for both *Candida* spp. were determined according Fontenelle et al. (2007). In addition, *C. parapsilosis* (ATCC 22019) and *C. albicans* (ATCC 1023) strains were used as quality controls for broth microdilution method.

The essential oils of *M. indica* varieties were prepared in mineral oil. Amphotericin B (AMB) (Sigma, Chemical Co., USA) was prepared in DMSO. For the susceptibility analysis, the essential oils samples were tested in concentrations ranging from 0.004 to 5mg.mL^{-1}. The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for each isolate tested. The microplates were incubated at 37°C and read visually after 2 days. The assays for all essential oils were run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 100% inhibition of visible fungal growth. The results were read visually as recommended by CLSI. The MFC was determined by subculturing 100µL of solution from wells without turbidity, on potato dextrose agar, at 28˚C. The MFCs were determined as the lowest concentration resulting in no growth on the subculture after 2 days.

Antifungal activity was expressed as mean ± SD of the diameter of the growth inhibition zones (mm). The antifungal activity of the essential oils was analyzed by linear correlation for individual analysis and the two-tailed Student's t-test at 95% confidence intervals was used to evaluate differences between the essential oil and the controls.

RESULTS AND DISCUSSION

The chemical analyses demonstrated that essential oil from leaves of Tommy Atkins cultivar has *β*–selinene (29.49%), caryophyllene oxide (12.40%) and humulene epoxide II (8.66%) as main constituents, while the main constituents of Rosa, Moscatel and Jasmim cultivars are caryophyllene oxide (23.62, 48.42 and 30.77%, respectively) and humulene epoxide II (11.56, 23.45, 16.27%, respectively). The constituents, italicene epoxide, spathulenol, caryophyllene oxide, humulene epoxide II and cyclocolorenone are common to the four essential oils. These results are shown on Table 1. The *M. indica* essential oils are mainly composed of sesquiterpenes.

Table 1. Chemical composition of the essential oils from leaves of *M. indica* cultivars

Constituent	Composition (%*)				
	Tommy Atkins	Rosa	Moscatel	Jasmim	K.I.**
Monoterpenoids					
α-Pinene	-	-	-	3,13	939
2-*δ*-Carene	-	-	-	3,31	1003
Piperitenone	1,41	-	-	3,17	1307
Sesquiterpenoids					
α-Copaene	3,43	-	-	-	1375
β–Elemene	1,45	1,30	-	-	1391
α-Gurjunene	-	5,64	-	4,33	1401
Longifolene	3,52	-	-	-	1406
E-Caryophyllene	-	5,40	-	4,58	1414
Aromadendrene	1,82	-	-	-	1437
α-Humulene	-	4,95	2,68	4,74	1450
Allo-aromadendrene	3,71	2,84	-	-	1459
Drima-7,9(11)-diene	2,49	-	-	-	1469
β –Selinene	29,49	-	-	2,3	1483
Viridiflorene	-	2,06	-	2,2	1485
Valencene	1,15	-		-	1492
Trans-cycloisolongifolol-5-ol	-	2,11	-	-	1512
Silfiperfol-5-en-3-ol-B	-	3,64	-	-	1529
Eremophila ketone	1,44	-	-	-	1534
Italicene epoxide	7,81	2,56	4,42	3,32	1551
β-Germacrene	1,02	-	-	1,19	1562
Espathulenol	1,93	4,32	9,19	5,81	1577
Caryophyllene oxide	12,40	23,62	48,42	30,77	1583
Viridiflorol	1,77	-	-	-	1604
Humulene epoxide II	8,66	11,56	23,45	16,27	1610
Eudesmol(10-epi-gamma)	-	-	-	4,37	1624
Allo-aromadendrene epoxide	2,85	-	-	-	1630
Xanthoirhizol	1,40	-	-	-	1748
Ciclocolorenone	7,26	5,91	4,55	2,68	1757
Aristolone	1,58	-	-	-	1761
Methylated fatty acid					
Methyl linoleate	-	3,04	2,26	-	2092
Total (Contet %)	96,59	78,99	94,27	92,17	-

* % peak area of the compounds in GC-FID chromatograms; ** Retention index. The identified constituents are listed in their order of elution from a non-polar column.
-: Components was not detected

Plant essential oils are a potentially useful source of antimicrobial compounds, and important in the development of new drugs due to have served as models for the synthesis of drugs with diverse pharmacological properties and chemical structures (SOBRINHO et al., 2016). It is often quite difficult to compare the results obtained from different studies, because the compositions of the essential oils can vary greatly depending upon the geographical region, the variety, the age of the plant, the method of drying and the extraction method (FIGUEIREDO et al., 2008). Several previous studies have demonstrated the activity of essential oils isolated from Brazilian biomes plants against *Candida* species (DUARTE et al. 2005). Previous study described anti-*Candida* activity against *Lippia sidoides* Cham. (FONTENELLE et al., 2007) and *Croton* species (FONTENELLE et al., 2008), Brazilian Northeast plants with ethnomedicinal and bioactive properties.

However, there is no specific study of the anti-*Candida* activity of essential oils from leaves of *M. indica* varieties. The antifungal activity of the essential oils was initially tested by the agar-well diffusion assay, at the 10mg/mL concentration, against 2 strains of *Candida* spp. isolated from symptomatic dogs. All of then induced growth inhibition zone, these results are shown in Table 2. The means inhibition zones were 11 ± 0.71, 13.5 ± 3.54, 10.5 ± 0.71 and 13.5 ± 0.71mm to Tommy Atkins, Rosa, Moscatel and Jasmim cultivars, respectively. The positive control amphotericin B induced a significant growth inhibition zone of 9.5 ± 0.71mm.

Based on this initially screen, all of mango essential oils were submitted to the broth microdilution method (Table 3) for *Candida* spp. strains (*n*=5). For Tommy Atkins, the MIC ranged from 0.62 to 1.25 mg/mL and the geometric mean was 0.87 mg/mL; for Rosa, ranged from 0.31 to 1.25mg/mL and the geometric mean was 0.68mg/mL; for Jasmim ranged from 0.31 to 0.62 mg/mL and the geometric mean was 0.49mg/mL; and for Moscatel, the MIC value was the same for all strains of *Candida* spp. The most effective variety was Jasmim, following by Rosa, Tommy Atkins and Moscatel.

Table 2. Antifungal activity of the essential oils of *M. indica* varieties against *Candida spp.* in the agar-well diffusion assay

Strains	Growth inhibition zones (mm)				
	Tommy Atkins 10 mg/mL	Rosa 10 mg/mL	Moscatel 10 mg/mL	Jasmim 10 mg/mL	Amphotericin B 0.005 mg/mL
Candida spp.					
CEMM 01-2-078 (*C. tropicalis*)	10	16	10	13	10
CEMM 01-2-081 (*C. tropicalis*)	12	11	11	14	09
(mean ± SD)	(11 ± 0.71)	(13.5 ± 3.54)	(10.5 ± 0.71)	(13.5 ± 0.71)	(9.5 ± 0.71)

Each experiment was performed in duplicate

Table 3. Minimum inhibitory concentrations of varieties of *M. indica* essential oils against *Candida* spp.

Strains	*M. indica* essential oils			
	Tommy Atkins	Rosa	Moscatel	Jasmim
	MIC (mg/mL)	MIC (mg/mL)	MIC (mg/mL)	MIC (mg/mL)
Candida spp.				
CEMM 01-3-068 (*C. albicans*)	0.62	0.62	1.25	0.62
CEMM 01-3-069 (*C. albicans*)	1.25	0.62	1.25	0.62
CEMM 01-3-077 (*C. albicans*)	1.25	0.62	1.25	0.62
CEMM 01-2-078 (*C. tropicalis*)	0.62	0.31	1.25	0.31
CEMM 01-2-081 (*C. tropicalis*)	0.62	1.25	1.25	0.31
(Geometric mean)	0.87	0.68	1.25	0.49

MIC = minimum inhibitory concentration expressed in mg/mL; CEMM = specialized Medical Mycology Center. Each experiment was repeated at least twice.

The constituents α-pinene and 2-δ-carene, found just in Jasmim variety, have antibacterial against many bacterial strains reported by Dorman & Deans (2000). The α-pinene is a compound of *Mentha arvensis* var. *piperita* L. essential oil, and Duarte et al (2005) shown a MIC value of 1.1mg/mL against *C. albicans* for this oil. These antimicrobial compounds may have increased the antifungal activity of Jasmim essential oil. Therefore, the high content of sesquiterpenes in both mango essential oils may be a factor that influences the antimicrobial activity observed. Caryophyllene oxide, main constituent from essential oils, showed antifungal activity against *C. albicans* by broth microdilution method (SKALTSA et al., 2003).

Probably there is a correlation between the antifungal activity of the studied oils and their main constituents. Corroborating this hypothesis, previous studies have demonstrated that the essential oils in which spathulenol and caryophyllene oxide are the main compounds have inhibitory activity on filamentous fungi species (FARAG et al., 2004; WENQIANG et al., 2006). These compounds must have inhibitory activity against *Candida* species too, because are abundant in all essential oils tested in this study. These results are important because strains of *Candida* spp. isolated from dogs showed high resistance to azole antifungal agents (BRITO et al., 2009).

The results of the present study indicate that the essential oils obtained from leaves of *M. indica* cultivars found in Brazil showed antifungal activity against *Candida* spp. strains. These results corroborate the importance of ethnopharmacological surveys in the selection of plants for bioactivity screening. The results contribute to the characterization of the anti-*Candida* activity of essential oils and plant extracts of traditional medicinal plants from the Brazilian flora. Subsequently, bio-guided fractionation will be conducted on plants showing potential anti-*Candida* activity to identify the active compounds. Evaluations of the toxicological aspects and antimicrobial activity against other important human and especially animal pathogens are also being conducted.

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