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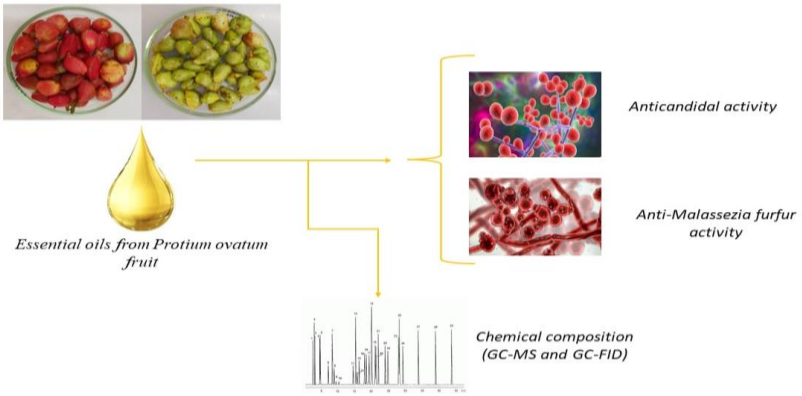
Antifungal Activities of Essential Oils from *Protium ovatum* Engl. Against *Malassezia furfur* and *Candida* Species

Gardênia Barbosa Carrijo Rodrigues [a], Cassia Cristina Fernandes [a], Suzana Maria Loures de Oliveira Marcionilio [a], Vitor de Paula Castro [b], Carmen Magaly Alvarez [b], Regina Helena Pires [b], Mariana Brentini Santiago [c], Carlos Henrique Gomes Martins [c], Reginaldo dos Santos Pedroso [c], and Mayker Lazaro Dantas Miranda* [d]

Fungal opportunistic infections have increased in recent decades due to the increase in the immunosuppressed patients and the indiscriminate use of antifungals. In Brazil, a country with the greatest biodiversity in the world, studies that seek new antifungals from natural sources have been stimulated. *Protium ovatum* Engl., belongs to the Burseraceae family and is a shrub tree found mainly in Brazil, in the Cerrado biome, and has medicinal, food and aromatic uses. This study aims to investigate the chemical composition and the anti-*Malassezia furfur* and anticandidal activities of essential oils (EOs) from *Protium ovatum* ripe fruit (RF-EO) and unripe fruit (UF-EO). The EOs antifungal activities were determined by microdilution broth methodology. GC-FID and GC-MS analyses showed that limonene, α-pinene and myrcene were the major components of both EOs. MIC values of RF-EO and UF-EO against *M. furfur* were 375 and 1500 µg/mL, respectively. RF-EO exhibited MIC values between 62.5 and 250 µg/mL while UF-EO was slightly active (> 1000 µg/mL) against *Candida* species. In addition, RF-EO showed antibiofilm activity against *Candida* species and was not toxic to *C. elegans* larvae. This study suggests that EOs from *P. ovatum* could be an alternative therapeutic option for infectious diseases caused by *M. furfur* and *Candida* species.

Graphical abstract



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[a] Instituto Federal de Educação, Ciência e Tecnologia Goiano, *Campus* Rio Verde. Av. Sul Goiana, 75901-970, Rio Verde, GO, Brazil. [b] Universidade de Franca (UNIFRAN), Franca, São Paulo, Brazil. [c] Universidade Federal de Uberlândia (UFU), Uberlândia, Minas Gerais, Brazil. [d] Instituto Federal de Educação, Ciência e Tecnologia do Triângulo Mineiro, *Campus* Uberlândia Centro, Rua Blanche Galassi, 150, Morada da Colina, 38411-104, Uberlândia, MG, Brazil. *Corresponding author. E-mail: **maykermiranda@iftm.edu.br**

1. Introduction

Pityriasis versicolor is a chronic noncontagious superficial fungal infection caused by yeast belonging to the *Malassezia* genus, and usually is asymptomatic, benign and relapsing since it may develop easily as the result of skin oiliness, humidity and temperature [1-2]. Diseases caused by *Malassezia* species are characterized by erythema and scaly lesions whose color ranges from white to brown and are often found on the upper limbs, upper body and face [3]. Specifically, *M. furfur* may be found in three forms of superficial infections: pityriasis versicolor, folliculitis and seborrheic dermatitis [4].

Other opportunistic fungi include yeasts from the *Candida* genus that cause candidiasis. There are several types of candidiasis as mucosal candidiasis, cutaneous candidiasis, onychomycosis and systemic candidiasis [5]. Although *C. albicans* is still the predominant etiologic agent, species called non-*albicans Candida* have emerged as infectious agents especially in immunocompromised patients, neonates, elderly, and hospitalized patients. Among these agents, *C. glabrata, C. tropicalis, C. parapsilosis* (currently subdivided into *C. parapsilosis* stricto sensu, *C. metapsilosis* and *C. orthopsilosis*) and *C. krusei* prevail [6]. However, many pathogenic fungi have become resistant to available antifungals. So, plants have been deeply studied as alternative sources of bioactive compounds, such as the ones found in essential oils (EOs) [7].

The plant species named *Protium ovatum* (Fig. 1), belongs to the *Burseraceae* family, can be found in the Brazilian Midwest (Cerrado biome) and Bolivia, and yields EOs that may be extracted from its vegetative parts and reproductive organs [8]. Previously, Estevam et al. [9-10] studied the biological potential of EOs from unripe fruit and leaves of *P. ovatum* against *Trypanosoma cruzi* and *Leishmaniasis amazonensis* as well as the cytotoxicity in $LLCMK_2$ adherent epithelial cells. Currently, the use of plant-based products such as essential oils and extracts is widely disseminated. However, the possible toxic effects of these products must be evaluated. The nematode *Caenorhabditis elegans*, due to the ease of handling and storage, short life cycle and low costs, has recognized as model organism for biological research [11].

Nevertheless, few studies have evaluated the biological potential of EOs from *Protium* and the antifungal activity of the EOs fruits against *M. furfur* and *Candida* species has not been investigated. Therefore, this study aimed to (i) identify the volatile constituents of EOs extracted from *P. ovatum* ripe and unripe fruit, and (ii) evaluate *in vitro* anti-*Malassezia furfur* and anticandidal activities of these oils.

Fig. 1. *Protium ovatum* ripe fruit (left) and unripe fruit (right)

2. Material and Methods

Plant material and extraction of EOs

P. ovatum unripe and ripe fruit were collected in the Cerrado biome at the University of Rio Verde (UniRV) in Rio Verde, Goiás, Brazil (17º47'53"S and 50º 55'41"W) in September 2021 (9:00 A.M.). The plant was identified by the botanist Erika Amaral and a sample was deposited at the Herbarium Jataiense Professor Germano Guarim Neto with exsiccate number HJ 7420. Access to the botanical material was approved by the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN) under the code AEACDCA.

EOs were extracted from *P. ovatum* unripe and ripe fruit (100 g), reduced by a knife mill and their EOs were extracted by the hydrodistillation method carried out by a Clevenger-type apparatus at 100 °C for 3 h. The remaining water was removed with anhydrous sodium sulfate. After filtration, the EOs were stored in amber bottles and kept in a refrigerator at 4°C until analysis.

Chemical analyses

UF-EO and RF-EO were dissolved in ethyl ether and analyzed by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC–MS) with the use of Shimadzu QP5000 Plus and GCMS2010 Plus (Shimadzu Corporation, Kyoto, Japan) systems. The temperature of the column in GC-FID was programmed to rise from 60 to 240°C at 3°C/min and was held at 240°C for 5 min; the carrier gas was H_2 at a flow rate of 1.0 mL/min. The equipment was set to operate in the injection mode with the injection volume of 0.1 µL (split ratio of 1:10) while injector and detector temperatures were 240 and 280°C, respectively [12]. Relative concentrations of components were obtained by normalizing peak areas (%). Relative areas consisted of the average of triplicate GC-FID analyses. Volatile components identification (Table 1) was based on their retention indices on an Rtx-5MS (30 m X 0.25 mm; 0.250 µm) capillary column under the same operating conditions used for GC relative to a homologous series of *n*-alkanes (C_8-C_{20}). The identification of compounds was performed by comparing the mass spectra obtained with NIST database, Wiley Library, FFNSC1.2, and Adams Book [13]. In addition to comparing the mass spectra, the Kovats index was also used to propose the chemical composition of the oils.

Anti-Malassezia furfur assay

To evaluate anti-*Malassezia furfur* activity, the broth microdilution test with modifications was carried out as proposed by Leong et al. (2017) [14]. The *Malassezia furfur* ATCC 14521 was used. Concentrations of EOs ranged from 1.46 to 3000 µg/mL. The culture medium Roswell Park Memorial Institute - RPMI-1640 (Sigma, St. Louis, MO, USA), buffered with morpholino propanesulfonic (MOPS, Sigma), pH 7.0, supplemented up to 2% glucose, 1% olive oil (Native, Itapemirim, ES, Brazil), 1% tween 80 (Neon Comercial Reagentes Analíticos Ltda, Suzano, SP, Brazil) and 0.5% dry bovine bile (Sigma) was used in these assays. Yeast suspension was adjusted by a spectrophotometer (530 nm) to reach a final concentration from 0.5 to 2.5 × 10^3 cells/mL. *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 strains and ketoconazole (Pfizer Inc, NY, USA), with concentrations ranging from 0.031 to 16 µg/mL were employed as quality controls. The 96-well flat-bottom plate

was incubated in a microbiological incubator at 30ºC for 96 h. The yeast growth was revealed by 30 µL 0.01% resazurin (Sigma) aqueous solution added to the wells [15]. Plates were re-incubated for 24h and the analysis was based on color variations between blue (without yeast growth) and pink (with yeast growth). The lowest concentration of the test sample in which the well remained blue was considered the MIC.

Anticandidal assay

Candida species reference strains, namely *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001, *C. krusei* ATCC 6258, *C. metapsilosis* ATCC 96143, *C. orthopsilosis* ATCC 96141, *C. parapsilosis* ATCC 22019, *C. parapsilosis* ATCC 90018, *C. rugosa* ATCC 10571 and *C. tropicalis* ATCC 13903 were used to evaluate the oils anti-*Candida* activity. Strains were maintained at -80 °C in sterile distilled water and 50% glycerol and subcultured in Sabouraud dextrose agar (SDA, Difco, Detroit, MI) and CHROMagar Candida medium (Becton Dickinson and Company, Sparks, MD) at 37 °C for 24 h to ensure purity and viability. *In vitro* antifungal susceptibility assays were performed by the broth microdilution method in agreement with protocol M27-S4 from the Clinical and Laboratory Standards Institute (CLSI, 2012) [16]. Sterile 96-wells microtiter plates (Corning Inc., NY, USA) were used. Final inoculum size was 2.5 × 10^3 cells/mL. Amphotericin B (AMB) and EOs ranging from 0.03 to 16 µg/mL and 3.90 to 2.000 µg/mL, respectively, were used. AMB and UF-EO and RF-EO were solubilized in dimethyl sulfoxide (DMSO, 2%) and diluted in RPMI 1640 (Sigma) medium with 0.2% glucose. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 strains, and AMB were included as quality controls (CLSI, 2012) [16]. Minimum inhibitory concentration (MIC) was determined by the fluorometric indicator resazurin at 0.01% (w/v) [17]. MIC was defined as the lowest antifungal/UF-EO and RF-EO concentration that maintained the blue hue. Wells in which microorganism growth occurred got pink. All tests were conducted in triplicate. In addition, the activity of plant products against *Candida* biofilms were evaluated. The minimum biofilm inhibitory concentration (MBIC) was determined. Biofilms were formed on sterile 96-wells flat-bottom microtiter plates (Corning) according to Pierce et al. (2008) [18]. EOs (final concentration at 1.95 to 2000 µg/mL) were dissolved in DMSO and diluted in RPMI 1640 medium. *C. albicans* SC 5314 and AMB (final range concentrations 1-16 µg/mL) were used as quality control. Biofilm viability was measured by the tetrazolium salt (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide-XTT) reduction assay. Wells containing culture medium/biofilms/XTT/menadione (control wells) and wells containing culture medium/XTT/menadione (background wells) were included. Optical density (OD) was read by a microtiter plate reader (Asys - Eugendorf, Salzburg, Austria) at the wavelength of 492 nm. Effective concentration of EOs capable of reducing ≥ 80% in the absorbance compared to that of control wells was considered the MBIC.

Nematicidal activity

The *C. elegans* AU37 [glp-4(bn2) I; sek-1(km4) X] mutant strain was cultivated and maintained at 16 °C on nematode growth medium (NGM) agar plates seeded with an auxotroph strain of *E. coli* (OP50) [19]. To synchronize the worms, they were washed from the plates with M9 buffer (22 mM KH_2PO_4; 42 mM Na_2HPO_4; 85,5 mM NaCl; 1 mM $MgSO_4$), removing residual bacteria by washing with M9 buffer before bleaching pregnant adults using 1% (v/v) alkaline hypochlorite solution (2 mL 5% sodium hypochlorite, 1 mL 4 N NaOH, 7 mL dH_2O) to obtain the eggs. After 10 minutes, the hypochlorite was removed by washing the eggs three times with M9 buffer. The eggs were allowed to hatch overnight at 16°C to give rise to a population of synchronized L1 larvae. After 48h in NGM medium with *E. coli* OP50, the larvae in the L4 stage were washed again with buffer M9, and approximately 20 worms were added to wells of 96-well microplates containing culture broth (60% 50mM NaCl, 40% BHI (brain heart infusion broth), cholesterol (10 µg/mL), kanamycin (90 µg/mL), and ampicillin (200 µg/mL). RF-EO at 62.5 to 1,000 µg/mL were added to wells. The microplates were maintained at 25 ºC, and individual worm survival was assessed after 24 h. Nematodes were considered dead when they were rod-shaped and did not respond to touching [20]. AMB (2 µg/mL) and DMSO (2%) were used a control in this assay.

3. Results and Discussion

Volatile constituents of EOs from *P. ovatum* unripe fruit (UF-EO) and ripe fruit (RF-EO) were identified by GC-FID and GC–MS being highlighted that both EOs exhibited the same major chemical constituents, but at different concentrations (Table 1).

Table 1. Chemical composition of EOs from *Protium ovatum* unripe fruit (**UF-EO**) and ripe fruit (**RF-EO**).

Compounds			%RA	
	RI_{lit}	**RI_{exp}**	**UF-EO**	**RF-EO**
α-Thujene	924	922	0.5	-
α-Pinene	932	930	**16.7**	**16.5**
Sabinene	969	968	0.5	0.3
β-Pinene	974	974	5.1	2.0
Myrcene	988	987	**30.1**	**20.7**
δ-3-Carene	1008	1007	9.1	-
o-Cymene	1022	1020	-	0.4
Limonene	1024	1024	**36.2**	**60.1**
β-Ocimene	1032	1030	1.8	-
Hydrocarbon monoterpenes			100.0	100.0
Total			100.0	100.0

RI_{exp} = Retention index relative to *n*-alkanes (C_8–C_{20}) on the Rtx-5MS column; **RI_{lit}** = Kovats retention index (values found in the literature[13]). **%RA** = Relative abundance

Hydrocarbon monoterpenes comprised the only class of compounds found in both EOs. The limonene, myrcene and α-pinene concentrations in UF-EO were 36.2%, 30.1% and 16.7% whist in RF-EO, the concentrations were 60.1%, 20.7% and 16.5%, respectively. A large difference was observed in the limonene concentration found in the EOs. The EOS chemical composition was similar to those related by Sousa et al. (2021) [8] and Estevam et al. (2017) [9] although Estevam et al. (2018) [10] have reported higher myrcene content in UF-EO.

The UF-EO and RF-EO exhibited anti-*Malassezia furfur* activity with MIC values at 1500 µg/mL and 375 µg/mL (Table 2), respectively.

The RF-EO showed MIC values similar to others EOs tested against *Malassezia* species such as the extracted oils from *Salvia Rosmarinus* and *Mentha spicata* [21]. EOs from *Origanum vulgare* L. and *Thymus vulgaris* L. showed MIC values at 780 µg/mL and 920 µg/mL, respectively, as reported by Vinciguerra and co-workers (2018) [22]. Donato et al. (2020) [23] considered a promising activity when MIC values ranged between 450 and 900 µg/mL and attributed potential antimicrobial activity against *M. furfur* to the EOs under study. Based on these findings, the activity exhibited by RF-EO against *M. furfur* may be considered satisfactory.

Table 2. Minimum inhibitory concentration (MIC = µg/mL) results obtained with the essential oils from *Protium ovatum* against *Malassezia furfur*

	UF-EO	RF-EO	Ketoconazole*
Malassezia furfur ATCC 14521	1500	375	0.0625
Candida parapsilosis ATCC 22019	-	-	1
Candida krusei ATCC 6258	-	-	2

UF-EO: unripe fruit essential oil; RF-EO: ripe fruit essential oil; -: not tested; * : reference drug.

Concerning anticandidal activity, notable results were also found. RF-EO was the oil that exhibited the highest activity. Table 3 shows the MIC values exhibited by RF-EO against *Candida* species highlighting the results obtained against C. *albicans* (62.5 µg/mL), *C. glabrata* (62.5 µg/mL), *C. metapsilosis* (250 µg/mL) and *C. parapsilosis* (250 µg/mL). Nonetheless, UF-EO exhibited low activity against all *Candida* strains under investigation (MIC > 1000 µg/mL) (Table 3).

Table 3. Minimal inhibitory concentrations (MIC = µg/mL) results obtained with the essential oils from *Protium ovatum* against *Candida* species.

***Candida* species**	**UF-EO**	**RF-EO**	**Amphotericin B***
C. albicans ATCC 90028	>1000	**62.5**	-
C. glabrata ATCC 2001	>1000	**62.5**	-
C. krusei ATCC 6258	>1000	1000	1.00
C. metapsilosis ATCC 96143	>1000	**250**	-
C. orthopsilosis ATCC 96141	>1000	1000	-
C. parapsilosis ATCC 22019	>1000	**250**	0.25
C. parapsilosis ATCC 90018	>1000	1000	-
C. rugosa ATCC 10571	>1000	1000	-
C. tropicalis ATCC 13903	>1000	1000	-

UF-EO: unripe fruit essential oil; **RF-EO:** ripe fruit essential oil; -: not tested; *Reference drug.

In view of the good results obtained against four *Candida* species, the minimum biofilm inhibitory concentration (MBIC) was evaluated. The MIC values obtained against *C. albicans*, *C. glabrata* and *C. parapsilosis* were 250 µg/mL while against *C. metapsilosis*, 1000 µg/mL (Table 4). Studies with plants products against *Candida* species with MIC ≤ 1000 µg/mL are considered relevant while the ones in which MIC ≤ 250 µg/mL are highly interesting [12, 24]. This study suggests that good activities exhibited by RF-EO may be related to their high limonene concentrations (60.1%) since a well-known constituent antifungal activity [25-26].

Table 4. Minimal inhibitory concentrations (MIC) results obtained with the ripe fruit essential oil (RF-EO) from *Protium ovatum* against *Candida* species.

***Candida* species**	**RF-EO MBIC (µg/mL)**	**AMB* MBIC (µg/mL)**
C. albicans ATCC 90028	250	-
C. glabrata ATCC 2001	250	-
C. metapsilosis ATCC 96143	1000	-
C. parapsilosis ATCC 90018	250	-
C. albicans SC 5314	-	2

-: not tested; *Reference drug

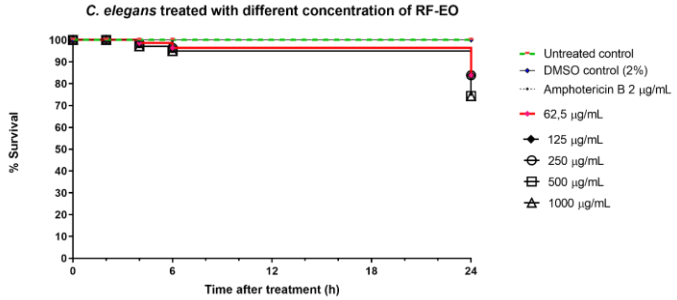

Fig. 2. Percentage of *C. elegans* L4 larvae survival when exposed to *P. ovatum* essential oil (ripe fruits) at different concentrations (62.5 - 1000 µg/mL) compared to control (untreated larvae).

L4 larvae of the nematode *C. elegans* AU 37 strain were subjected to different concentrations (62.5 - 1000 ug/mL) of RF-EO and monitored for 24 h (Figure 2). Larvae survival was evaluated after 6, 12, 18 and 24h by the Log-rank test (Mantel-Cox) which showed that there was no significant difference ($p>0.05$) between the curves in the 24h period, demonstrating absence of RF-EO oil toxicity to *C. elegans* (Fig. 2). Lately, *C. elegans* has gained notoriety in toxicity studies including volatile compounds or compounds with low solubility. In conclusion, a great agreement has been observed between the data obtained with the nematode and data obtained with other study models such as zebrafish, rats or rabbits [27].

4. Conclusions

The present work reported for the first time the *in vitro* anti-Candidal and anti-*Malazessia furfur* activities of essential oils extracted from *P. ovatum* ripe and unripe fruit. Among these, the most effective against *Candida albicans*, *C. glabrata*, *C. metapsilosis* and *C. parapsilosis*. The bioactivity presented by these oils may be related to their prevalent constituents, which are known for their proven antifungal properties. The main components of essential oils were limonene, α-pinene and myrcene. Therefore, based on our encouraging results, these oils are promising phytochemicals for the development of new formulations of therapeutic agents for the treatment of fungal diseases caused by *Candida* species and *M. furfur*. Nonetheless, this present research requires further studies, such as toxicological tests and others that are capable of evaluating their *in vivo* biological potential.

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Author Contributions

G.B.C.R., C.C.F. and S.M.L.O.M.: colleted fruits of *P. ovatum* and extracted both essential oils. V.P.C., C.M.A. and R.H.P.: carried out anticandidal and nematicidal activities of essential oils. M.B.S., C.H.G.M. and R.S.P.: carried out anti-*Malassezia furfur* activity of essential oils. M.L.D.M.: contributed for the written, review and editing of the manuscript.

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