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Wood Metabolites of *Myrcia insularis* Gardner (Myrtaceae) have Potential *Anti-Candida Activity*

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Abstract

The present work aimed to isolate secondary metabolites from *Myrcia insularis* Gardner (Myrtaceae) wood and to evaluate the anti-*Candida* activity and further extracts obtained by partition and the respective main isolated compounds. Wood was collected in a Seasonal Semideciduous Forest remnant of the Atlantic Forest of northern Rio de Janeiro State, Brazil. Chromatographic and spectrographic techniques were used to isolate and identify secondary metabolites. Methanol extract inhibited the growth of *Candida buinensis* and *Candida tropicalis,* with inhibition being approximately 82% for the latter. The main compound isolated from the ethyl acetate partitions was arjunolic acid, a triterpene. The antimicrobial activity was first observed with the wood metabolites of *M. insularis* adds to our understanding of the antifungal properties of this species and other species within the Myrtaceae family, including the presence of arjunolic acid, which may play a role in this activity.

Keywords: Atlantic Forest, phytochemistry, triterpene, antifungal activity, arjunolic acid.

1. INTRODUCTION AND OBJECTIVES

Studying the classes of compounds found in plants has contributed to discovering new natural compounds with applications in diverse areas, such as agriculture, medicine, and pharmacology (Vieira et al., 2016). More than 25% of all medicines are of plant origin, making them an important study subject. Besides, it´s necessary to search different sources to obtain active principles responsible for pharmacological actions and/or therapeutics (Ahmed et al., 2016; Gevú et al., 2019).

Plants possess a wide variety of secondary metabolites, alkaloids, terpenes, flavonoids, lignins, tannins, and glycosides (Bisoli et al., 2008; Vieira et al., 2016; Laursen et al., 2015; Knudsen et al., 2018; Li et al., 2020). Many of these compounds possess a pharmacological potential and can be found in all parts of the plant, including roots, leaves, flowers, and stems (Tungmunnithum et al., 2018).

The improper or indiscriminate use of antibiotics has contributed to the emergence of microorganisms with resistant multiple drugs (Chandra et al., 2017). This scenario has discovered new antimicrobial agents needed to combat these microorganisms, increasingly becoming one of the biggest threats to global health (Afroz et al., 2020). Since antiquity, natural products have performed essential roles worldwide in treating diseases of humans and other animals. They have also served as a source of new microbial agents due to their unique and enormous chemical diversity (Torrent et al., 2012; Wong et al., 2015; Ghosh et al., 2019; Afroz et al., 2020).

The occurrence of invasive fungal diseases in humans, such as candidiasis, is a concern for the global population, affecting immunosuppressed patients and elevating the number of deaths from septicemia (Scorzoni et al., 2017). There are about 200 species described for the fungal genus *Candida,* of which 17 have been identified as pathogenic to man. Although *Candida albicans* continues to be the most common clinical isolate, other

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species of *Candida* are being recovered from clinical samples with increasing frequency, such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, among others (Gabaldón et al., 2016; Colombo et al., 2017; Sharma et al., 2019). Infections caused by fungi of the *Candida* are usually treated with synthetic antifungal agents, especially azoles (itraconazole and fluconazole) and opolien (amphotericin B). However, these drugs have disadvantages, such as high toxicity to the host and the emergence of resistant strains (Duraipandiyan & Ignacimuthu, 2011; Spampinato & Leonardi, 2013; Perfect, 2017; Shekhova et al., 2017). The concern and studies of new drugs have grown in recent years in search relates natural products to an inhibitory activity against *Candida* spp. (Cavalcanti et al., 2011; Alexandrino et al., 2016; Gevú et al., 2019; Wang et al., 2021).

Species of the family Myrtaceae are known for producing secondary metabolites, emphasizing phenolic compounds. Investigations of some species have found terpenes, flavonoids, steroids, and tannins (Araújo et al., 2019; Batiha et al., 2020). The genus *Myrcia* includes species with pharmacologic potential, including a hypotensive, diuretic, hypoglycemic, antidiarrheal, antimicrobial, antitumor, and hepatoprotective properties (Silva et al., 2019).

Some studies of metabolite isolation and biological activity with *Myrcia* spp. have revealed hypoglycemic activity for myricetin, compounds known as "insulin plant," anti-inflammatory, antinociceptive, antioxidant, enzyme inhibitor, antifungal, antibacterial, or antimicrobial activities in general and acaricide were also described for Myrcia essential oils (Cerqueira et al., 2007; Cascaes et al., 2015; Ribeiro et al., 2022), mainly extracted from the leaves, flowers and fruits and less frequently on bark and fine stems. Terpenes have diverse biological proprieties, such as anticancer, antifungal, antiparasitic, antibacterial, antiallergic, antihyperglycemic, and immunomodulatory activities (Chan-Bacab & Pena-Rodrigues, 2001; Theis & Lerdau, 2003; Salem & Werbovetz, 2006; Paduch et al., 2007; Masoko et al., 2008, 2010; Liu, 2011; Zacchino et al., 2017; Wang et al., 2021). Studies with similar chemical classes evidenced activities relevant to the central nervous system, such as sedative, anticonvulsant, and pro-convulsive activities, and compounds used in natural insecticides (Passos et al., 2009; Huang & Osbourn, 2019) (Table 1). So, the Myrtaceae family, has great economic potential, with species used for food and pharmaceutical purposes. Even with this representativeness, the species *Myrcia insularis* Gardner (Myrtaceae), has no studies related to its wood, which is essential to contribute to research on anti-candida pharmacological effects.

Table 1. Main terpenoids found in amounts greater in *Myrcia* species and their chemical and biological activities. (Jorge et al., 2000; Limberger et al., 2004; Stefanello et al., 2006; 2010; Andrade et al., 2012; Silva et al., 2013; 2018; Cascaes et al., 2015; 2019; Pereira Junior, 2018; Santana et al., 2018; Silva et al., 2018; Franco et al., 2021; Jerônimo et al., 2021; Ribeiro et al., 2022; Fehlberg et al., 2023; Santana et al., 2023; Santos et al., 2023).

Species	Main Terpenoids	Chemical and biological activities
M. alagoensis O. Berg.	Germacrene B.	Antibacterial (Gram-positive and Gram-negative)
M. bella Cambess.	Guaiol acetate; a-cadinol; Sesquiterpene Hydrocarbons; Oxigenated sesquiterpenes.	Antioxidant, Allelopathic, Hypoglycemic, Antinociceptive
M. bracteata (L.C. Rich.) DC.	(*E*)-nerolidol; (*E*)-β – farnesene, spathulenol	Hypoglycemic, Antinociceptive, Antioxidant.
M. cuprea (O. Berg.) Kiaersk.	Myrcene; *β*-caryophyllene; *α*-pinene; germacrene D; Germacrene B.	Anticholinesterase; Larvicide; Fungicide
M. fallax (L.C. Rich.) DC.	α-pineno; β-pineno; β-elemene.	Antioxidant, Antiproliferative, Antimicrobial Activity, Antifungal
M. fenestrata DC.	(*E*)-Cariofileno; Espatulenol; α-Cadinol; Caryophyllene oxide; β-Copaen-4-α-ol.	Antibacterial, Antimalarial, Antioxidant, Anthelmintic
M. hiemalis Cambess.	2α,3β,21α-Trihydroxy-28,20β-hydroxytaraxastanolide; Sesqui-, di- and tetraterpenoids eudesm-4-(15)-en-7α,11-diol and geranylgeranyl acetate and α-tocopherol.	Antiparasitic, Antibacterial; Antifungal.
M. laruotteana Cambess.	α-bisabolol; 14-hidroxi-α-muuroleno.	Antioxidant, Antiproliferative Activity.
M. multiflora (Lam.) DC.	β-caryophyllene; Selin-11-en-4α-ol.	Antioxidant, Enzyme Inhibitor, Hepatoprotective, Antiobesity, Hypolipidemic, Allelopathic.

Table 1. Continued...

Species	Main Terpenoids	Chemical and biological activities
M. myrtillifolia DC.	Betulinic acid; Betulonic acid; Betulinaldehyde; Betulona; Oleanolic acid; ursolic acid.	Antimicrobial Activity.
M. oblongata DC.	Caryophyllene oxide; Trans-verbenol.	Antioxidant, Antimicrobial, Acaricidal.
M. obtecta Kiaersk.	*Trans*-calameneno; Monoterpeno α-terpineol.	Antioxidant.
M. pubiflora DC.	Cariofileno; Mustakone; 1,8-cineol; Tricicleno.	Antinociceptive, Anti-inflammatory
M. pubipetala Miq.,	Biciclogermacreno; Espatulenol.	Antienzymatic, Antioxidant.
M. rostrata DC.	Carotol; Germacreno B; *E*-cariofileno; Germacreno D; Dauceno.	Antibacterial, Antifungal
M. rufípila McVaugh.	β- and α-amyrin; Desmantin-I; and some flavonoids derived from terpenoids: Dihydromyricetin-4'-*O*-gallate; myricetin-3-*O*-α-L-rhamnopyranoside; Myricetin; Myricitrin; Isovitexin.	Hypoglycemic, Antioxidant.
M. splendens DC.	*E*-caryophyllene	Antinoceptive, Antifungal, Anti-inflammatory, Allelopathic, Insecticide. Antioxidant, Cytotoxic Activity against gastric, melanoma and colon human cancer cells.
M. sylvatica DC.	*E*-caryophyllene; γ-Elemene; Germacrene B.	Antioxidant Capacity, Cytotoxic Activity against melanoma and gastric human cancer cells.
M. tomentosa DC.	γ-elemeno; Germacreno D; (E)-cariofileno.	Antifungal, Antibacterial, Antioxidant, Allelopathic

Myrcia insularis, commonly called vapiranga or guapiranga, is endemic to Brazil, where it occurs in Espírito Santo, Rio de Janeiro, São Paulo, Paraná, Bahia, and Pernambuco (Santos et al., 2020). In Brazil, *M. insularis* is distributed in the Atlantic Forest domain in the vegetation of Dense Ombrophylous, Seasonal Semideciduous forest and Restinga, supposedly presenting fragmented subpopulations; it is found in several conservation units and has a high population density (CNCFlora, 2012). In northern Rio de Janeiro State, this species is known to occur in Seasonal Semideciduous Forest (SSF) in the Environmental Protection area of Morro do Itaoca, Campos dos Goytacazes, RJ, Brazil. As the species currently have little information on research related to the isolation of metabolites and biological activity they can be a potential source of research in the area, considering the family to which it belongs (Cascaes, 2015; 2019; Jeronimo et al., 2021). Thus, investigations of the phytochemistry of the wood of *M. insularis* can be necessary for studies about your biological proprieties, and contributes to knowledge about antifungal activities as a base for other species within Myrtaceae. So, this study aimed to isolate metabolites of *M. insularis* wood and evaluate the anti-*Candida* activity of the partitions. Beyond that, to isolate and identify the main compound of these partitions.

2. MATERIALS AND METHODS

2.1. Plant material and study area

Wood samples of *M. insularis* were collected by non-destructive methods of a remnant of SSF in the Atlantic Forest. The selected individuals (n. 5) without apparent defects, and with DBH above 10 cm had samples of the parts of the branches removed manually using a handsaw (Stanley®) and taken to the laboratory. The SSF remnant is located on a rocky outcrop of the Morro do Itaoca (21°48'0" S - 41°26'0" W) in the municipality of Campos dos Goytacazes, in northern Rio de Janeiro State (RJ), Brazil. The identified material was deposited in the wood collection "Xiloteca Drª Cecília Gonçalves da Costa" (HUENFw) under register nº 565, and herbarium HUENF of Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF) under register nº 10663 (Table 2). The samples were dried through the rotary oven and then ground by the hammer mill at the UENF Chemical Science Laboratory, resulting in approximately 2,0724 Kg.

Table 2. General data regarding of *M. insularis* on the Morro of Itaoca (SSF) and information on registration.

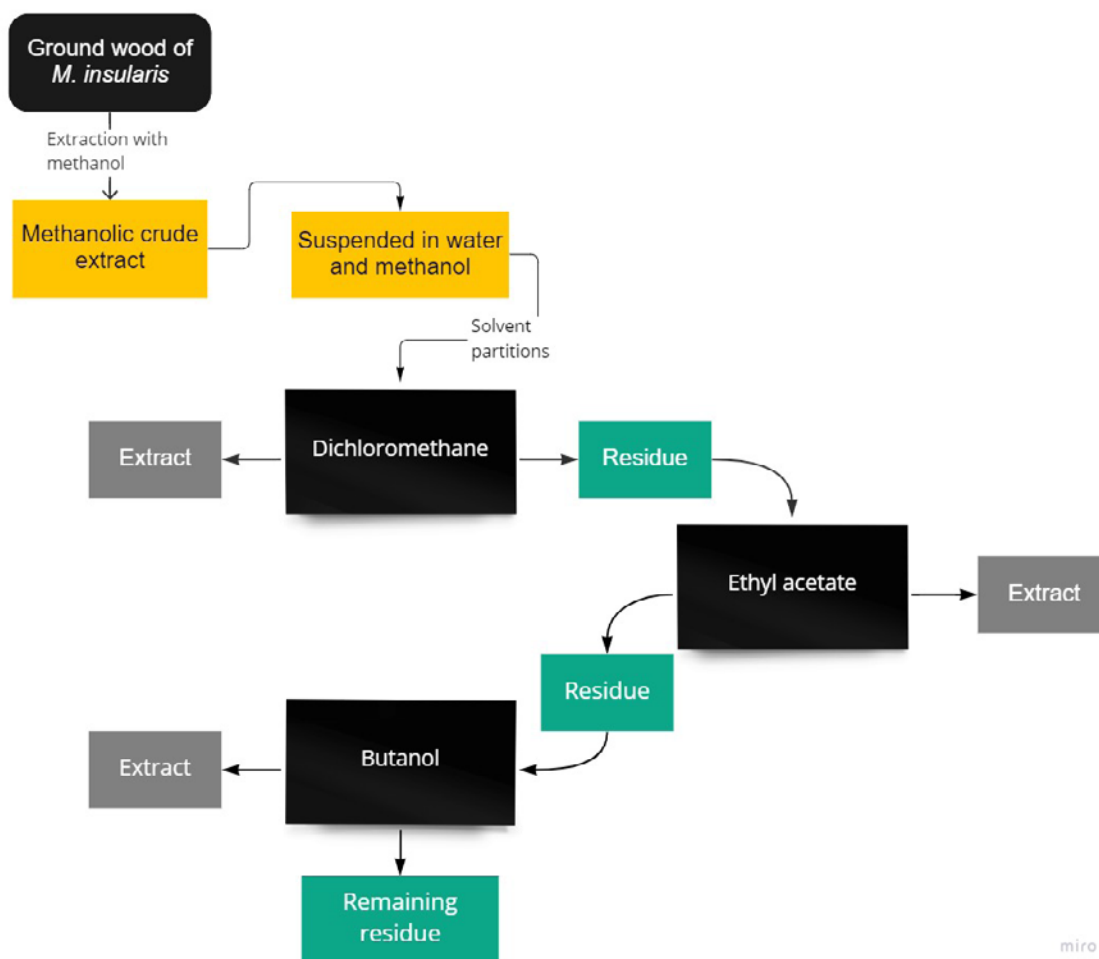
Data	SSF
Xiloteca HUENFw (nº)	565
Exsiccates HUENF (nº)	10663
Average height of individuals (m)	9.71
Average diameter of stalk (cm)	32.88

2.2. Isolation and extraction

The ground wood samples of *M. insularis* were subjected to extraction by cold maceration with methanol for four days. The extracts were concentrated in a rotary evaporator (FISATOM 802) and dried in a fume hood. This procedure was repeated three times, guaranteeing all the crude wood extract. A rotary evaporator was used to concentrate the extract, which was suspended in water and methanol (H_2O:MetOH - 1:3) and partitioned using the solvents dichloromethane (CH_2Cl_2), ethyl acetate (AcOEt), and butanol (ButOH) in ascending order of polarity (Figure 1). The steps were carried out at the UENF Chemical Sciences Laboratory.

The extracts obtained were submitted to anti-*Candida* activity tests, selects the best results of inhibition of yeast growth were selected for chromatographic and spectroscopic analysis.

Figure 1. Fractionation steps of the crude methanolic extract of *M. insularis* wood. collected at remnant of SSF in the Atlantic Forest.



2.3. Yeast growth-inhibition assay

Strains of the species *Candida tropicalis* (CE017) and *Candida buinensis* (3982) were maintained in Sabouraud agar (1 % peptone, 2 % glucose, and agar 1.7 %; Merck). The cell strains were provided by Physiology and Biochemistry of Microorganisms Laboratory at UENF, municipality of Campos dos Goytacazes, RJ, Brazil.

Inoculants of each stock of *C. buinensis* and *C. tropicalis* were transferred to Petri dishes containing Sabouraud agar and left to grow at 30 ºC for 24 h. Each cell aliquot was added to a 10 mL sterile culture medium (Sabouraud broth, 1 % peptone, 2 % glucose; Merck). The cells were counted in a Neubauer chamber (Optik Labor) under light microscopy (Axioplan, ZEISS). Only the CH_2Cl_2 and AcOEt extracts that presented significant antimicrobial activity were analyzed further in the other experiments.

The yeast strains *C. buinensis*, and *C. tropicalis* (1 x 104 cel.mL^{-1}) were incubated in Sabouraud broth containing 200, 100, and 50 µg.mL^{-1} of each fraction of the CH_2Cl_2 and AcOEt extracts (solubilized in 20 % DMSO), with the final volume adjusted to 200 µL. The test was performed in 96-well microplates (NUNC, Nunclein Surface) at 30 ºC for 24 h. The control cells were cultured in the absence of extracts. The procedure followed the method of Broekaert et al. (1990) with adaptations and was performed entirely under aseptic conditions. Cell growth was analyzed after 24 h of incubation by optical density in a microplate reader at a wavelength of 620 nm. The cells were then observed by differential interference contrast (DIC) under a light microscope (Axioplan, A2, Zeiss). The experiments were performed in triplicate.

2.4. Spectrometry analysis

This analysis was performed by uni-two-dimensional Nuclear Magnetic Resonance (NMR) with the operation of 500 MHz for hydrogen (^{1}H NMR) and 125 MHz for carbon-13 (^{13}C NMR) with Ascend 500 (Brüker) spectrophotometer. Spectrometry analysis was performed with a high-resolution mass spectrometer (micrOTOF-Q II Brucker Daltonics) using the negative mode of study. Column chromatography (CC) was performed with a 60G silica gel (Merck), while thin layer chromatographic analysis was done with chrome aluminum foils (CCD Silica gel 60 F254, Merck). The compounds found were observed by ultraviolet irradiation at 254nm or 365nm and/or revealed with the sulfuric vanillin chromogenic reagent by warming.

The AcOEt (1.53g) partition was subjected to CC, eluted with CH_2Cl_2:MeOH of gradually increasing concentration until 100% of MetOH. Seven fractions (MIF1 – MIF7) were obtained. Fraction MIF4 (253mg) was resubmitted to CC, eluted with MetOH and CH_2Cl_2 until 100% of MetOH to obtain nine fractions (MIF4.1 – MIF4.9), with the compound 1 identified in the MIF4.6 (36.8 mg) fraction.

2.5. Statistical analysis

Growth inhibition data were evaluated by One-Way ANOVA, with differences of $p < 0.05$ being considered significant. All statistical analyses were performed using GraphPad Prism Software (6.0, version for Windows).

3. RESULTS

Of the three partitions obtained from the *M. insularis* wood only that of AcOEt and CH_2Cl_2 showed significance in the tests. The antimicrobial activity of the fractions obtained from AcOEt and CH_2Cl_2 was tested on the strains of *C. buinensis* and *C. tropicalis* using the concentrations of 200, 100, and 50 µg.mL^{-1} (Figures 2 and 3).

Figure 2. The growth inhibition of yeast cells of *C. buinensis* (A) and *C. tropicalis* (B). Growth in the absence of extract (control) and the presence of 200, 100, and 50 µg.mL-$_1$ of AcOEt fractions. (*) Indicates significance by One-Way ANOVA ($p < 0.05$) among the treatments and their respective controls.

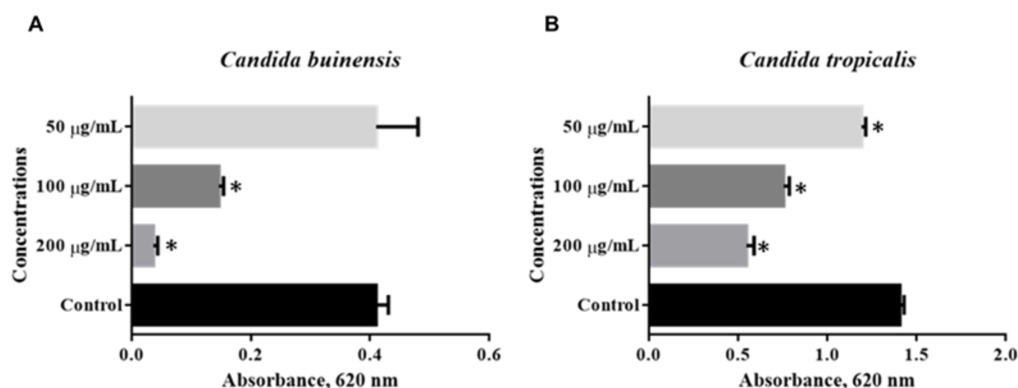
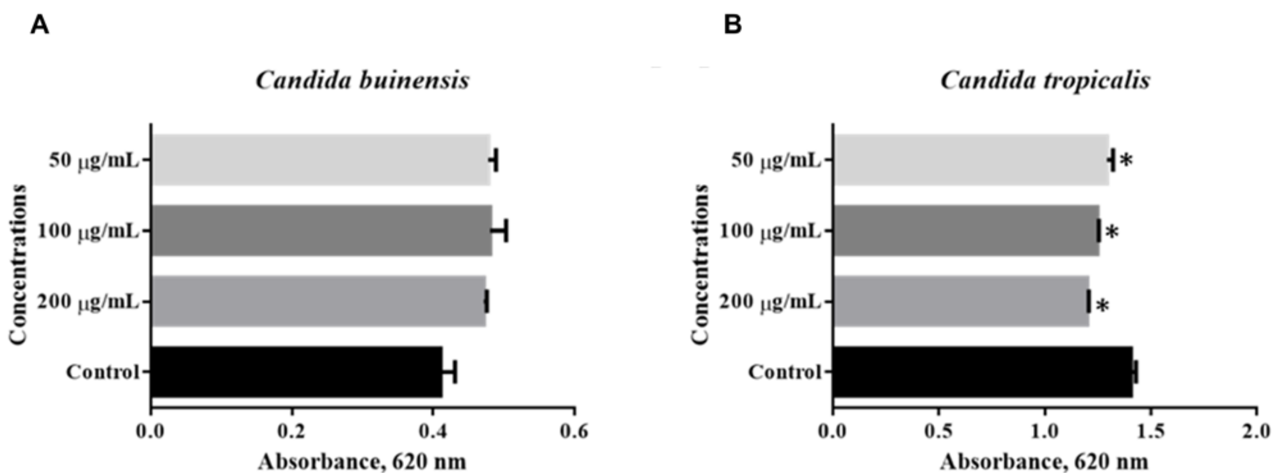


Figure 3. Growth inhibition of yeast cells of *C. buinensis* (A) and *C. tropicalis* (B). Growth in the absence of extract (control) and the presence of 200, 100, and 50 μg.mL^{-1} of CH_2Cl_2 fractions. (*) Indicates significance by One-Way ANOVA ($p < 0.05$) among the treatments and their respective controls.



At concentrations of 200 and 100 μg.mL^{-1}, the AcOEt partition was able to significantly inhibit *C. buinensis* cells (Figure 2A), resulting in approximately 73% and 64% growth inhibition, respectively. The tested concentrations were also able to significantly inhibit *C. tropicalis* cells, with reductions of about 61%, 46%, and 15% in the growth of the strain in the concentrations of 200, 100, and 50 μg.mL^{-1}, respectively (Figure 2B).

The CH_2Cl_2 partition had a lower inhibitory effect against the yeasts than the AcOEt partition (Figures 2 and 3), with none of the three tested concentrations inhibiting the growth of C. *buinensis* cells (Figure 3A). However, growth inhibition against *C. tropicalis* cells was approximately 14%, 11%, and 8% for the concentrations of 200, 100, and 50 μg.mL^{-1}, respectively (Figure 3B). Data obtained through optical density corroborated the images obtained by light microscopy; however, only cells treated with 200 μg.mL^{-1} of both partitions were recorded due to this concentration having the greatest inhibition (Figure 4).

Since the AcOEt partition inhibited more than 60% of yeast growth in the initial tests, it was selected for chromatographic analysis. The fraction MIF4 was set similarly for CC since it had better inhibition against *C. buinensis*. Finally, the fraction MIF4.6 showed growth inhibition of *C. buinensis* and *C. tropicalis* of approximately 72% and 82%, respectively (Figure 5).

The analysis of the MIF4.6 fraction by CCD revealed its degree of purity and, thus, was used to perform the NMR and HRESI-MS experiments. Based on these experiments, together with data from the literature (Table 3) and molecular structure (Figure 6), the substance was identified as arjunolic acid (Mann et al., 2012).

Figure 4. Light microscopy of yeast cells of *C. buinensis* and *C. tropicalis* visualized by DIC after 24 h of incubation with 200 μg.mL^{-1} extracts obtained from AcOEt or CH_2Cl_2. Bars: 20 μm.

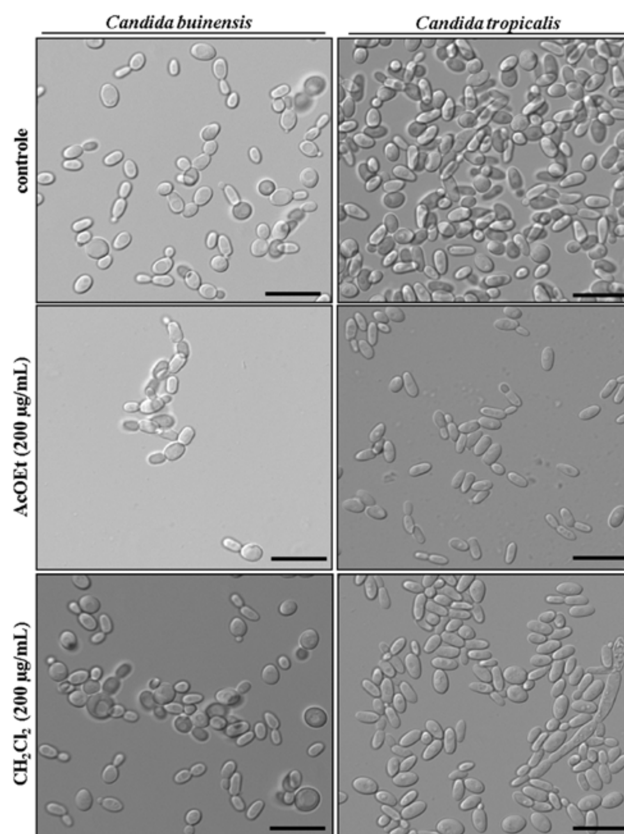


Figure 5. Growth inhibition of yeast cells of *C. buinensis* (A) and *C. tropicalis* (B). Growth in the absence of extract (control) and in the presence of the MIF4.6 fraction.

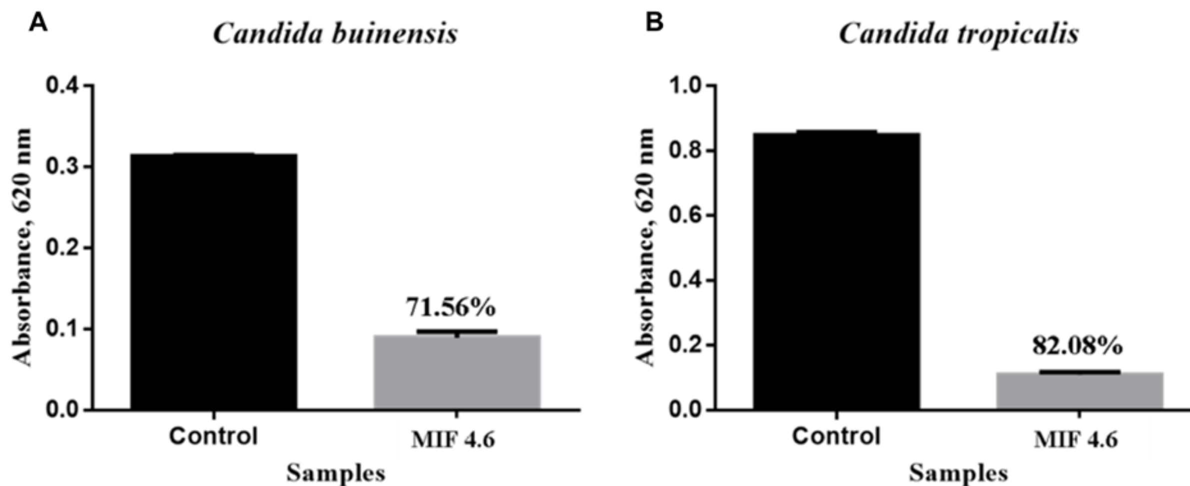


Figure 6. Molecular structure of arjunolic acid extracted from the MIF4.6 fraction.

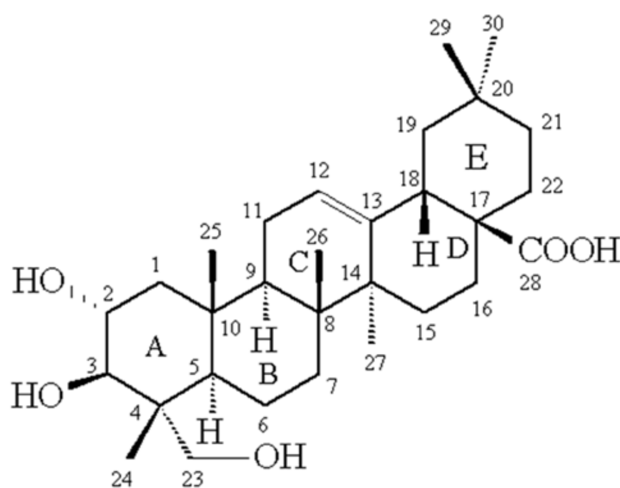


Table 3. ^{1}H (500 MHz) and ^{13}C (125MHz) NMR data for compound 1, and correlation obtained by HSQC and HMBC spectra in CD_3OD as solvent.

C	δ_C	δ_H	HMBC
1	46.5	1.90 (m), 0.90 (m)	2, 25
2	68.3	3.71 (ddd, 11.3, 9.6, 4.5)	1, 3
3	76.8	3.37 (d, 9.6)	1, 2, 23, 24
4	42.7	-	3, 23, 24
5	46.8	1.65 (m)	1, 23, 24, 25
6	17.7	1.40 (m), 1.10 (m)	-
7	31.9	1.70 (m), 1.30 (m)	26
8	39.2	-	26, 27

Table 3. Continued...

C	δ_C	δ_H	HMBC
9	47.5	0.95 (m)	12, 25, 26
10	37.6	-	1, 25
11	23.2	2.02 (m), 1.78 (m)	12
12	122.0	5.27 (t, 3.5)	18
13	144.0	-	12, 18, 27
14	41.6	-	18, 26, 27
15	27.4	1.80 (m), 1.08 (m)	27
16	22.6	2.02 (m), 1.78 (m)	-
17	46.3	-	16
18	41.3	2.84 (dd, 13.7, 4.1)	12
19	45.8	1.78 (m), 1.15 (m)	18, 29, 30
20	30.2	-	29, 30
21	33.5	1.75 (m), 1.40 (m)	-
22	31.4	1.56 (m), 1.25 (m)	-
23	64.9	3.52 (d, 11.1), 3.28 (d, 11.1)	3, 24
24	12.5	0.72 (s)	3, 23
25	16.1	1.05 (s)	-
26	16.4	0.84 (s)	-
27	25.1	1.20 (s)	-
28	180.4	-	16a, 18, 22a
29	32.2	0.93 (s)	30
30	22.6	0.96 (s)	29

4. DISCUSSION

There have been important studies on plant secondary metabolism that produces compounds active in the defensive system. These metabolites have been extracted in different ways and from other plant parts and have served as the raw material for producing essential oils and extracts for different uses (Machado et al., 2006). These secondary metabolites perform various functions, such as protection against herbivores, pathogens, and other external influences, including temperature, humidity, and nutrient deficiency (Batiha et al., 2020).

Initial tests of the CH_2Cl_2, AcOEt, and ButOH partitions in the present study were performed to evaluate antifungal activity. The partitions with greater inhibition were those of CH_2Cl_2 and AcOEt. The AcOEt extract inhibited *C. buinenis* growth by about 73%. Giordani et al. (2015) found that CH_2Cl_2 and ethanol (C_2H_5OH) extracts of leaves and roots of *Eucalyptus* camaldulensis have positive anti-*Candida* actions. Despite the methanol extract from the wood having a lower inhibitory percentage, the data obtained in this study reiterate the presence of bioactive compounds against yeast.

The chromatography and spectrometry analyses of *M. insularis* extract isolated a skeleton triterpene pentacyclic arjunolic acid. This terpene was isolated from the MIF4.6 fraction derived from methanol extract. Arjunolic acid was previously isolated from leaves of other species of Myrtaceae, such as *Melaleuca alternifolia* (Vieira et al., 2004), *Myrcia guianensis* (Fehlberg, 2006), and *Myrcia rotundifolia* (Silva, 2014). Several studies have reported on the pharmacological potential of arjunolic acid. Facundo et al. (2005) considered this triterpene a multifunctional drug with anti-inflammatory, antinociceptive, and anticholinesterase activities. Besides that, this acid showed antifungal activity against *Candida krusei*, *Candida albicans*, and *Cryptococcus neoformans* (Silva et al., 2020).

Investigations of the chemical composition of the essential oil of the stem of *Myrcia alternifolia* identified diverse terpenes, including arjunolic acid, and another oil constituents presents antifungal, antibacterial, antiviral, and analgesic properties (Buck et al., 1994; Hammer et al., 1996; Halcón & Milkus, 2004; Veras et al., 2019). This triterpene isolated has shown a relatively high antimicrobial activity (Moreira, 2010). The author also mentioned that this substance was the major component (or the main component) found in *M. alternifolia*. On the other hand, several essential oil compounds showed that anti-*Candida* effectiveness was lower due to the interaction of other compounds present. The AcOEt and CH2Cl2 partitions of the present study inhibited growth by approximately 73% and 61% at 200 µg.mL-1 for *C. buinensis* and *C. tropicalis,* respectively. So the fraction MIF4.6 inhibited about 72% and 82%, containing the isolated principal substance, arjunolic acid. Some terpenoids compounds function as repellents and/or attractants and are components of the typical aroma of several plant species, such as the high monoterpenes present in some essential oils (Silva et al., 2019; An et al., 2020). Furthermore, terpenes can be toxic at high concentrations and can serve as important combatants against pathogens and herbivory (Theis & Lerdau, 2003; Crowell, 2002).

There have been no published studies on the antimicrobial activity of *M. insularis*. Nevertheless, this study presents positive data regarding the growth inhibition potential of the species with the elimination of approximately 72% and 82% of *C. buinensis* and *C. tropicalis*, respectively, in 200 µg.mL-1 of the fraction MIF 4.6.

5. CONCLUSIONS

A compound belonging to the terpene class, arjunolic acid, was identified for the first time from *M. insularis* wood. The fraction contained this metabolic presented significant anti-*Candida* potential. Further investigations should be undertaken regarding the chemical and biological properties of this species of the family Myrtaceae, aiming for biotechnological applications, and serving as a basis for further studies.

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Ivo José Curcino Vieira: Conceptualization (Lead), Methodology-Supporting, Resources (Supporting), Writing – review & editing (Supporting).

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