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Antifungal activities of the essential oil and its fractions rich in sesquiterpenes from leaves of *Casearia sylvestris* Sw.

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ABSTRACT

Casearia genus (Salicaceae) is found in sub-tropical and tropical regions of the world and comprises about 160-200 species. It is a medicinal plant used in South America, also known as “guaçatonga”, “erva-de-tiú”, “cafezinho-do-mato”. In Brazil, there are about 48 species and 12 are registered in the State of Rio de Janeiro, including *Casearia sylvestris* Sw. There are many studies related to the chemical profile and cytotoxic activities of extracts from these plants, although few studies about the antifungal potential of the essential oil have been reported. In this work, we have studied the antifungal properties of the essential oil of *C. sylvestris* leaves, as well as of their fractions, against four yeasts (*Saccharomyces cerevisiae*, *Candida albicans*, *C. glabrata* and *C. krusei*) for the first time. The chemical analysis of the essential oil revealed a very diversified (n = 21 compounds) volatile fraction composed mainly of non-oxygenated sesquiterpenes (72.1%). These sesquiterpenes included α -humulene (17.8%) and α -copaene (8.5%) and the oxygenated sesquiterpene spathulenol (11.8%) were also identified. Monoterpenes were not identified. The fractions are mainly composed of oxygenated sesquiterpenes, and the most active fraction is rich in the sesquiterpene 14-hydroxy -9-*epi*- β -caryophyllene. This fraction was the most effective in inhibiting the growth of three yeast strains.

Key words: *Candida* sp., *Casearia* genus, essential oil, medicinal plant, *Saccharomyces cerevisiae*.

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INTRODUCTION

Casearia genus belongs to Salicaceae family, and it is distributed in sub-tropical and tropical regions of the world, comprising 200 species. The genus is found in the Americas, Africa (rarely), Asia and Australia (Sleumer 1980, Mosaddik et al. 2004, Breteler 2008). In Brazil, there are about 48 species, 24 of which are endemic, and 12 species are registered in the State of Rio de Janeiro (Marquete and Vaz 2007, Marquete and Mansano 2010).

Casearia sylvestris Sw. can be found throughout the Brazilian territory. It is popularly known as “guaçatonga”, “tiú” or “erva-de-lagarto”, and has medicinal properties according to ethnobotanical surveys (Bratti et al. 2013, Pinto et al. 2013, Tomazi et al. 2014). This plant is also included in the public health system (SUS) of Brazil and widely used to treat different diseases. According to Sato et al. (1996), the chemical components found in essential oils and extracts from leaves of *C. sylvestris* can be related to their uses in folk medicine as an antiseptic, antimicrobial and antiviral (Esteves et al. 2005, Pereira et al. 2016).

The secondary metabolites of *Casearia* are mainly diterpenes, specially the clerodane type (Kanokmedhakul et al. 2007, Carvalho et al. 2009, Ferreira et al. 2010). The essential oils of this genus are rich in monoterpenes and sesquiterpenes (Tininis et al. 2006, Sousa et al. 2007, Silva et al. 2008, Pereira et al. 2017), and some of the main compounds already identified are α -zingiberene, α -humulene, caryophyllene, and bicyclogermacrene (Esteves et al. 2005, Bou et al. 2013).

In spite of the several phytochemical studies on *C. sylvestris*, the chemical profile and the antifungal activity of essential oils have not been studied so far. It seems sesquiterpenes may play an important role as antifungal agents, and considering the possibility of further studies, non-oxygenated sesquiterpenes such α -humulene found in *Casearia* species can be investigated (Pereira et al. 2017).

Fungal diseases are a menacing problem in the world, affecting mainly immunocompromised patients (Cannon et al. 1995, 2009). The genus *Candida* is the most common agent diagnosed in invasive fungal infections, with high rates of morbidity and mortality. This pathogen is frequently isolated from patients in critical health condition, and is among leading causes of bloodstream and catheter-associated urinary tract infections (Lockhart 2014). *Candida albicans* strains are found in healthy individuals, including *C. glabrata*, *C. tropicalis* and *C. krusei*, albeit they are also opportunistic pathogens (Procop and Roberts 2004, Pfaller et al. 2008). Resistance of *Candida* yeast to current medications, including the class of azoles, has been reported (Pfaller and Diekema 2004, Pfaller 2012), and one of the causes of this resistance is the overexpression of plasma efflux pumps (Holmes et al. 2008, Sanglard and Bille 2002, Rogers and Barker 2003, Cannon et al. 2009).

In view of these facts, studies of biologically active molecules from plants which can play an important role as an alternative antifungal compound or in association with antifungal drugs, are highly recommended. It is important to note that both extracts and essential oils of several species have been shown to possess potent antifungal activity (Cruz et al. 2007, Kalidindi et al. 2015).

Development of new therapeutic approaches may contribute for the treatment of diseases caused by fungi because the resistance to existing drugs is a serious complication for patients (Pfaller et al. 2005, Pfaller and Diekema 2007). This study shows that sesquiterpenes with basic caryophyllene skeleton derived from *C. sylvestris* might be used in association with the current antifungal drugs to treat *Candida* infections.

MATERIALS AND METHODS

Casearia sylvestris Sw. (Salicaceae) was collected in Tijuca National Park (S22°57'05.04" W43°17'10.09"), Rio de Janeiro, Brazil (SISBIO license n. 38765-1/CGEN license n. 010105/2014-0). Plant identification was performed by Dr. Ronaldo Marquete, and the herbarium voucher was deposited in the Botanical Garden Herbarium of Rio de Janeiro with registration number RB 570651.

Fresh leaves of *C. sylvestris* (1.5 kg) were cut to small pieces and submitted to hydrodistillation in a modified Clevenger-type apparatus for two hours. Essential oil was directly separated from the aqueous phase yielding 1.2% (w/v), transferred to amber flasks and kept at low temperature (-20°C) until analysis. The sample was subjected to analysis by gas chromatography coupled to flame ionization detector (HP-Agilent 6890 GC-FID) and by gas chromatography coupled to mass spectrometry (HP Agilent GC 6890 – MS 5973), at the Analytical Platform of Institute of Pharmaceutical Technology (Farmanguinhos) Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), as described previously (Pereira et al. 2016). Briefly, the essential oil was diluted in dichloromethane (1.0 mg/ml) and analyzed by GC-MS to obtain the mass spectra and to perform chemical characterization. Concomitantly, another sample of essential oil (0.5 mg/ml) was analyzed by GC-FID for quantification of chemical constituents and to determine the retention indices (RI). The relative abundance of each essential oil component in the sample was quantified based in the individual relative peak area in the chromatogram. The substances in the essential oil were identified by comparing their mass spectra with database registration (WILEY7n) and by comparison of calculated Retention Indices (RI) with those from the literature (Adams 2001). RI were calculated from GC data of a homologous series of saturated aliphatic hydrocarbons within C8 to C20 (Sigma-

Aldrich), performed using the same column and conditions adopted in the GC analysis for the essential oils, and with the equation proposed by Vandendool and Kratz (1963).

The parameters adopted for HP-5MS were (5% diphenyl and 95% dimethylpolysiloxane), column (30 m × 0.32 mm i.d. × 0.25 µm particle size), temperature programming from 60 to 240°C, with increase of 3°C/min, using synthetic air and hydrogen as the carrier gases, with a flow rate of 1 ml/min and injection volume of 1 µl. For GC-MS, the parameters used for HP-5MS were (5% diphenyl and 95% dimethylpolysiloxane), column (30 m × 0.32 mm i.d. × 0.25 µm particle size), temperature programming from 60 to 240°C, with increase of 3°C/min, using helium as the carrier gas, with a flow rate of 1 ml/min and injection volume of 1 µl (Pereira et al. 2017).

For the phytochemical analysis of the major essential oil compound, the extraction of essential oil of *C. sylvestris* fresh leaves yielded around 20 mL of a pure, clear, fluid and yellowish colored oil. Part of the oil (2 mL) was submitted to a silica gel chromatographic column, which was eluted by n-hexane, ethyl acetate and methanol and their mixtures, using an increasing polarity gradient. The chromatographic running was very slow as well as the increasing polarity of solvent systems, and 99 fractions were obtained. After analysis by TLC plates under UV wavelength, the fractions were reunited according to their similarity, resulting into 11 fractions (1-6; 7-10; 11-13; 14-28; 29-56; 57-62; 63-80; 87-88; 91-93; 94-96; 97-98). The 11 fractions were analyzed by GC-MS under the same conditions used for the pure essential oil sample.

The MIC of total oil and fractions was determined by a microdilution method in 96-well microplates. Yeasts were inoculated in YPD or RPMI medium (200 µl) at a final concentration of 1×10^4 or 2.5×10^3 cells per well for *Saccharomyces cerevisiae* and *Candida* sp., respectively, in the presence of a series of twofold dilutions of each

compound starting at 250 to 0.9 µg/ml. Microplates were incubated at 30 or 37°C for 48h using an orbital shaker (100 rpm). Cell growth was measured at 600 nm with a microplate reader FLUOstar Optima (BMG Labtech, Germany) relative to the control.

RESULTS

GC-MS analysis allowed the characterization of 21 compounds, comprising 98.2% of the pure essential oil from leaves of *C. sylvestris*. The main compounds identified were α -humulene (17.8%), spathulenol (11.8%), and α -copaene (8.5%). Monoterpenes were not found. The essential oil is richer in non-oxygenated sesquiterpenes 72.1% and 25.6% of oxygenated sesquiterpenes. The fraction 1-6 presented mainly oxygenated sesquiterpenes, and 15 compounds could be identified, comprising 83.7% of the essential oil. The main compounds found were α -copaene (8.52%), caryophyllene oxide (11.60%), and 14-hydroxy-9-*epi*- β -caryophyllene (18.09%). The fractions 7-10, 11-13, 57-62 and 91-93 were less rich in non-oxygenated sesquiterpenes. With respect to fractions 7-10, 11-13, 29-56, 57-62 and 91-93 the main compounds were oxygenated sesquiterpenes, comprising more than 40% of the essential oil (Table SI – Supplementary Material).

The antifungal activity of the pure essential oil and their fractions was evaluated using

different concentrations (5, 25, 50, 100 and 250 µg/mL), according to the zone of inhibition of microorganisms (Tables II and III). According to IC₅₀ values, the most sensitive strains were *C. glabrata* and *S. cerevisiae*. However, it was observed that the pure essential oil (*C. krusei* IC₅₀ 74.3 µg/mL) and fraction 1-6 (*S. cerevisiae* IC₅₀ 23.3 µg/mL) were more effective in inhibiting the growth of these strains (Tables II and III).

DISCUSSION

Studies on the pharmacological potential of *C. sylvestris* are mainly related to antitumor, anti-inflammatory and antiviral activities (Esteves et al. 2005, Ferreira et al. 2016, Pereira et al. 2016), on the other hand antifungal activities were comparatively less investigated. In addition, there are no studies on the potential of the essential oil and its fractions.

The essential oil of *C. sylvestris* is rich in sesquiterpenes, regardless of the region in which the plants are found (Tininis et al. 2006, Silva et al. 2008, Esteves et al. 2005, Bou et al. 2013). Monoterpenes can also be identified, but not in significant amounts (Sousa et al. 2007, Stefanello et al. 2010). The potential of terpenes for the treatment of candidiasis has been reported previously (Dalleau et al. 2008, Martínez et al. 2014)

TABLE II
Antifungal activities of the essential oil and its fractions from fresh leaves of *Casearia sylvestris* (IC₅₀ values).

Samples	<i>Saccharomyces cerevisiae</i> (Wild type)	<i>Candida albicans</i> (ATCC 10231D-5)	<i>Candida glabrata</i> (ATCC 2001D-5)	<i>Candida krusei</i> (ATCC 20298)
Pure oil	88.5	>250	83.1	74.3
Fraction 1-6	23.3	97.2	73.5	160.9
Fraction 7-10	166.3	>250	>250	158.9
Fraction 11-13	>250	>250	>250	173.3
Fraction 14-28	93.1	>250	110	149.5
Fraction 29-56	182.9	>250	123.5	196
Fraction 57-62	226.5	>250	>250	>250
Fraction 87-88	85.6	169.5	160.2	165.1
Fraction 91-93	151.2	>250	145.4	>250

TABLE III
Antifungal activities of the essential oil and its fractions from fresh leaves of *Casearia sylvestris* (MIC values).

Samples	<i>Saccharomyces cerevisiae</i> (Wild type)	<i>Candida albicans</i> (ATCC 10231D-5)	<i>Candida glabrata</i> (ATCC 2001D-5)	<i>Candida krusei</i> (ATCC 20298)
Pure oil	125	>250	125	125
Fraction 1-6	62.5	>250	125	250
Fraction 7-10	250	>250	>250	250
Fraction 11-13	>250	>250	>250	250
Fraction 14-28	125	>250	250	250
Fraction 29-56	250	>250	>250	>250
Fraction 57-62	>250	>250	>250	>250
Fraction 87-88	125	250	250	250
Fraction 91-93	250	>250	>250	>250

In the present work it was observed that both pure essential oil and fraction 1-6 are rich in non-oxygenated and oxygenated sesquiterpenes. The major component of the pure essential oil is the non-oxygenated sesquiterpene α -humulene, which is not present in fraction 1-6, in which the major component is the oxygenated sesquiterpene 14-hydroxy-9-*epi*- β -caryophyllene.

It is relevant to note that sesquiterpenes are difficult to be separated into each component and they are usually co-eluted with another sesquiterpenes in the mixture, for instance the non-oxygenated sesquiterpene aromadendrene is present in large quantities in fraction 29-56, but it is absent in other five fractions. In addition, there are many similar components in the essential oil and the same component has several matches in the mass spectral database. Another important remark is related to the frequency of co-eluting components which are identified in the GC column and consequently their spectra are combined.

Both pure essential oil and fraction 1-6 were active against *S. cerevisiae* and *C. glabrata*. This activity may be represented by the group of three substances identified in both samples, such as α -copaene, spathulenol and caryophyllene oxide. Possibly these three substances act synergistically. The other fractions, whose major components

were not the sesquiterpenes α -humulene and 14-hydroxy-9-*epi*- β -caryophyllene did not display relevant inhibitory activity.

The strains *S. cerevisiae*, *C. glabrata* and *C. krusei* were the most susceptible to pure essential oil whose major compound is α -humulene; on the other hand, the fraction 1-6 rich in the oxygenated sesquiterpene 14-hydroxy-9-*epi*- β -caryophyllene was more active against the strains *S. cerevisiae* and *C. glabrata*. Both compounds present the basic skeleton of the bicyclic sesquiterpene caryophyllene, and there are studies which identified essential oils containing sesquiterpenes β -caryophyllene and β -caryophyllene oxide as an alternative therapy for candidiasis (Asdadi et al. 2015). Moreover, sesquiterpenes may act inhibiting the formation of hyphae of *C. albicans* (Xie et al. 2015). In fact, the essential oils rich in caryophyllene and its isomers (β -caryophyllene and α -humulene) have antimicrobial and antifungal activities (Sabulal et al. 2006). In addition, sesquiterpenes β -caryophyllene and caryophyllene oxide may be absorbed by the fungal cell membrane and act as antifungal agent by releasing lipophilic drugs (Sarpietro et al. 2015). According to the work of Khan et al. (2014), essential oils of *Ocimum sanctum* play a crucial role in the

secretion of hydrolytic enzymes responsible for the virulence of *C. albicans*.

Oxygenated sesquiterpenes might not represent an important role as a potent antifungal in *Saccharomyces* and *Candida* strains, because even high concentrations of caryophyllene oxide (fractions 7-10 and 11-13), humulene epoxide and spathulenol (fraction 57-62), viridiflorol and spathulenol (fraction 87-88), viridiflorol and α -muurolol (fraction 91-93) have not achieved significant activity against these strains. On the other hand, studies conferred the importance of polar compounds such as β -caryophyllene oxide, in terms of disrupting the fungal cell membrane (Sarpietro et al. 2015).

There are many studies over the last 20 years that demonstrated the relevance of terpenes in fungal cell permeability associated with changes in the properties of fungal membranes and their functions (Sikkema et al. 1995, Lambert et al. 2001, Grande-Tovar et al. 2016, Kumar et al. 2016). Terpenes are also related to disorders of mitochondrial membrane in *Candida* species (Bakkali et al. 2008, Zuzarte et al. 2011).

Furthermore, fungal lipid bilayers are related to resistance, such as reduction of the amount of plasma membrane ergosterol, biofilm resistance and also release of extracellular vesicles. Hence, molecules that cause conformational changes in the integrity of fungal membranes may help in rational drug design (Cannon et al. 2009, Rella et al. 2016).

Considering the number of antifungal agents, only few classes of new drugs are available for the treatment of mucosal and systemic infections by *Candida* species. For instance, echinocandins, polyenes (amphotericin B), nucleoside analogs and other agents responsible for the inhibition of ergosterol biosynthesis, such as imidazoles and allylamines (Cannon et al. 2009). However, fungal resistance to current drugs over the last 30 years have led to a significant morbidity and mortality of the population, and drugs such fluconazol

and itraconazole have been widely used for the treatment of systemic disease (Pfaller and Diekema 2007). Therefore, research based on new molecules from natural source, including terpenes, can be an alternative to design multifunctional drugs for the treatment of fungal infections.

We can conclude that pure essential oil rich in non-oxygenated sesquiterpene α -humulene as well as its fraction rich in the oxygenated sesquiterpene 14-hydroxy-9-*epi*- β -caryophyllene presented a good action against fungi, and further studies are necessary to investigate how sesquiterpenes may act as important antifungal agents.

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SUPPLEMENTARY MATERIAL

Table SI - Chemical Composition of the Essential Oil from Fresh Leaves of *Casearia sylvestris* (Jun /2014) and their respectively fractions.