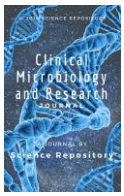

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Research Article

Chemical and antifungal analysis of essential oils and phytochemicals against *Candida albicans* and *Candida tropicalis*

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ABSTRACT

The aim of this study was to evaluate the chemical composition and antifungal activity of four essential oils (EO) and four phytochemicals against *Candida albicans* and *Candida tropicalis*. The chemical analysis of EO was carried out using a gas chromatograph coupled with a mass spectrometer. The antifungal evaluation was determined by means of the disc-diffusion in agar technique. The major components found were: eucalyptol (41.06%) and limonene (28.08%) in the EO of *Cinnamomum camphora*, geranial (51.17%) and neral (36.40%) in the EO of *Melissa officinalis*, (E)-anethole (77.14%) in the EO of *Ocinum basilicum* and citronellal (37.75%), citronellol (14.27%), citronellal (37.75%) and geraniol (18.84%) in the EO of *Cymbopogon nardus*. Against *C. albicans* and *C. tropicalis* strains, essential oils and phytochemicals produced, respectively, the following growth inhibition halos: *C. camphora* (11 mm; 14 mm), *M. officinalis* (32 mm; 30 mm), *O. basilicum* (32 mm; 30 mm), *C. nardus* (15 mm; 30 mm), alpha pinene (35 mm; 30 mm), citral (32 mm; 25 mm), citronellal (30 mm; 30 mm) and carvacrol (30 mm; 30 mm). Monoterpenes constitute the majority of the chemical components of essential oils tested. Phytochemicals have antifungal activity, inhibiting the growth of *C. albicans* and *C. tropicalis*.

Introduction

Despite the variety of antifungal agents for prophylaxis and treatment of candidiasis, there has been a significant increase in the number of cases of infection associated with the expansion of *Candida* species resistant to available drugs [45]. In this context, *Candida albicans* and *Candida tropicalis*, species commonly involved with oral infection, stand out because they have significant morphological plasticity and expression of virulence factors associated with biofilm formation, invasion of the host tissue and adaptation to the environment [16, 37, 43].

The molecular mechanisms involved with virulence are related to the activation of the MAP signal transduction pathway (mitogen-activated protein kinase), where cellular responses involved in invasive growth, cell wall formation, osmotic stress adaptation and reproduction occur through intracellular signaling pathways such as MKc1, Cek1/2 and HOG1 MAP Kinase [28]. Other intracellular signaling pathways such as p38 MAPK, also relate to the pathogenicity of *C. albicans* [30]. Once infection is installed, pro-inflammatory mediators such as TNF-α, IL-1α and IL-2α are synthesized and induce inflammatory response [33]. Furthermore, the production of extracellular enzymes such as phospholipases and proteases contribute to the virulence of *C. albicans* for promoting destruction of host tissues [25]. Candidiasis is usually associated with immunosuppression and may represent a major cause of mortality in individuals with acquired immunodeficiency syndrome

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(AIDS) [40]. This is also quite frequent in patients with malignancies, especially hematologic [42].

Given the increased fungal resistance to available drugs as well as the high cost for treatment of serious infections caused by these microorganisms, alternative drugs should be developed, boosting investigations that consider the pharmacological activities of natural products, especially those derived from plants. In this sense, essential oils and their phytochemicals stand out due to their antimicrobial potential [2, 4,19,21, 24, 27,44,48, 49]. Terpenes, especially monoterpenes are characterized by the chemical formula $C_{10}H_{16}$ with biosynthetic origin derived from isoprene units, which may be cyclic or branched. The chemical configuration of these molecules gives them hydrophobic property, allowing deposition on the lipophilic structures of microorganisms such as the plasma membrane, leading to increased permeability with consequent loss of essential electrolytes for cell survival [4]. It was observed that terpene alcohols such as citronellal, citronellol, geraniol and linallol act as desiccants and solvents that cause protein denaturation [11].

This study aimed to evaluate the chemical composition of essential oils from *Cinnamomum camphora* (white camphor), *Melissa officinalis* (lemongrass), *Ocimum basilicum* (basil) and *Cymbopogon nardus* (citronella), as well as the antifungal activity of these compounds and four phytochemicals: alpha pinene, citral, citronellal and carvacrol against *C. albicans* and *C. tropicalis*.

Material and Methods

Essential oils and phytochemicals used were obtained from Quinari Fragrâncias e Cosméticos Ltda.®, Paraná, Brazil and Sigma-Aldrich®, São Paulo, Brazil, respectively, as described in (Table 1). *Candida albicans* (ATCC 76645) and *Candida tropicalis* (ATCC 13803) strains were used. Samples were subcultured in Sabouraud Dextrose Agar (SDA) (DIFCO®) and incubated at 35°C for 24 hours. Then, fungal suspensions were made in sterile 0.9% saline, equivalent to barium sulfate suspension of tube No. 0.5 of the McFarland scale and adjusted to 90% transmittance in spectrophotometer (Leitz-photometer 340-800), corresponding to approximately 10^6 CFU / mL [10, 13].

Table 1. Essential oils and phytochemicals.

Essential oils and phytochemicals	Part of the plant	Manufacturers
Cinnamomum camphora	Stem bark	QUINARI®
Ocinum basilicum	Leaves	QUINARI®
Cymbopogon nardus	Leaves	QUINARI®
Melissa officinalis	Leaves	QUINARI®
α-pinene	-	SIGMA-ALDRICH®
Citral (mixture of geranial and neral)	-	SIGMA-ALDRICH®
Citronellal	-	SIGMA-ALDRICH®
Carvacrol	-	SIGMA-ALDRICH®

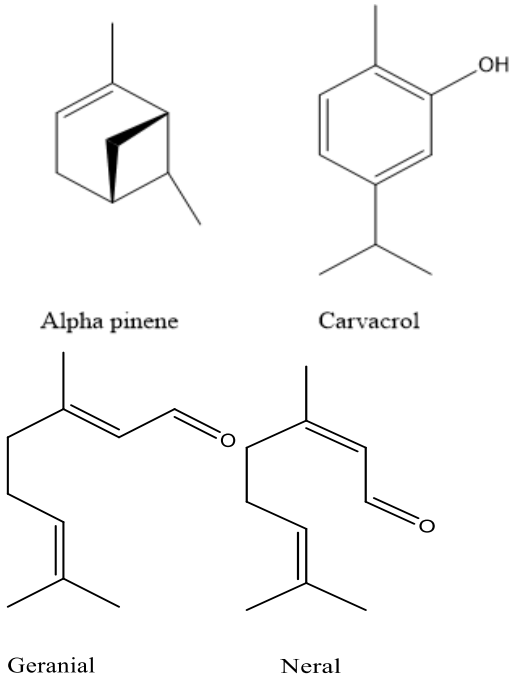

Figure 1: Chemical structures of phytochemicals evaluated.

Chemical characterization of essential oils

Chromatographic analysis was performed using a gas chromatograph coupled to mass spectrometer (Shimadzu GC-MS-QP5050A) with capillary column (J & W SCIENTIFIC®), stationary phase of 5% phenyl and 95% dimethylpolysiloxane with 30 m in length, 0.25 mm of internal diameter and 0.25 mm of film thickness. Initial temperature programming ranged from 60°C to 240°C (3°C / min), while the run programming time was 60 minutes, and the oven injector temperature was 250°C. Helium was used as carrier gas (mobile phase) at flow rate of 1.0 mL / min with 1:20 split ratio and injection volume of 1 µL. The identification of compounds was performed by comparing their mass spectra with those in the database of the equipment (MIST Library, 2008). Samples of essential oils were injected at concentration of 2 ppm and hexane was used as solvent and the integration parameters used were Width: 3 and Slope: 2000.

Antifungal evaluation

The antifungal evaluation was determined by disc-diffusion in agar in ASD [7]. Petri dishes containing ASD were cultivated by the exhaustion technique. Subsequently, filter paper discs with 6.0 mm in diameter containing 20 µL of essential oils and phytochemicals were placed in the culture medium and plates were incubated in an incubator at 35°C for 24 hours. Nystatin (50 mg / mL) was used as positive control and the experiment was performed in triplicate. The results of the susceptibility of products were expressed by the diameter of the fungal growth inhibition halo, considering the following parameters: less than 9 mm, not active (NA); 9-14 mm, partly active (PA); greater than 14-17 mm, active (A) and greater than 17 mm, very active (VA) [3].

Results

The analyses for chemical characterization of essential oils are described in (Tables 2, 3, 4 and 5), highlighting the presence of eucalyptol

(41.06%) and limonene (28.08%) in the EO of *C. camphora*, geranial (51.17%) and neral (36.40%) in the EO of *M. officinalis*, (E)-anethole (77.14%) in the EO of *O. basilicum* and citronellal (37.75%), citronellol (14.27%) and geraniol (18.84%) in the EO of *C. nardus*. The chemical structures of the main phytochemicals identified in the essential oils tested are represented in (Figure 2).

Table 2: Chemical characterization of essential oil of *Cinnamomum camphor*.

Peak	Retention time (min)	Component	Concentration (%)
Peak 1	5.765	α-thujene	0.11
Peak 2	5.979	α-pinene	11.41
Peak 3	6.207	-	0.09
Peak 4	6.402	Camphene	0.24
Peak 5	7.093	Sabinene	1.39
Peak 6	7.222	β-pinene	1.33
Peak 7	7.439	6-methyl-5-hepten-2-one	0.29
Peak 8	7.573	Myrcene	1.83
Peak 9	8.071	α-phellandrene	0.64
Peak 10	8.487	α-terpinene	1.17
Peak 11	8.779	ρ-cymene	10.40
Peak 12	8.953	Limonene	28.08
Peak 13	9.057	Eucalyptol	41.06
Peak 14	9.580	(E)-β-Ocimene	0.13
Peak 15	10.032	ɣ-terpinene	0.79
Peak 16	13.579	Camphor	0.26
Peak 17	15.563	α-terpineol	0.78
TOTAL		-	100.00

Table 3: Chemical characterization of essential oil of *Melissa officinalis*.

Peak	Retention time (min)	Component	Concentration (%)
Peak 1	6.397	Camphene	0.38
Peak 2	7.429	6-methyl-5-hepten-2-one	1.16
Peak 3	10.496	Dipentyl ketone	0.76
Peak 4	13.520	-	0.18
Peak 5	13.766	trans-chrysanthemol	0.14
Peak 6	13.865	Citronellal	0.42
Peak 7	14.370	Isogeranial	1.80
Peak 8	17.193	Citronellol	0.86
Peak 9	17.841	Neral	36.40
Peak 10	18.372	Nerol	1.07
Peak 11	19.189	Geranial	51.17
Peak 12	20.082	2-Undecanone	0.35
Peak 13	24.067	Neryl acetate	1.03
Peak 14	25.707	(E)-Caryophyllene	2.15
Peak 15	29.712	ɣ-candinene	1.37
Peak 16	32.558	Caryophyllene oxide	0.78
TOTAL		-	100.00

Table 4: Chemical characterization of essential oil of *Ocinum basilicum*.

Peak	Retention time (min)	Component	Concentration (%)
Peak 1	8.907	Limonene	0.23
Peak 2	9.022	Eucalyptol	0.17
Peak 3	11.639	Linalool	18.34
Peak 4	13.948	Isomenthone	0.17
Peak 5	14.423	Menthol	1.11
Peak 6	16.010	(E)-anethole	77.14
Peak 7	19.101	Geranial	0.39
Peak 8	25.712	(E)-caryophyllene	0.47
Peak 9	26.352	cis-α-bergamotene	0.62
Peak 10	30.822	(Z)-α-bisabolene	1.28
Peak 11	31.903	ρ-methoxy-cinnamaldehyde	0.09
TOTAL		-	100,00

Table 5: Chemical characterization of essential oil of *Cymbopogon nardus*.

Peak	Retention time (min)	Component	Concentration (%)
Peak 1	8.892	Limonene	3.17
Peak 2	9.226	1-methyl-piperidine	7.78
Peak 3	11.581	Linalool	0.48
Peak 4	13.554	Isoisopulegol	0.87
Peak 5	13.889	Citronellal	37.75
Peak 6	17.183	Citronellol	14.27
Peak 7	18.380	Geraniol	18.84
Peak 8	22.698	Geranial	0.41
Peak 9	22.698	Citronellyl acetate	3.31
Peak 10	22.973	Eugenol	0.64
Peak 11	24.047	Neryl acetate	2.46
Peak 12	24.468	β-elemene	1.93
Peak 13	28.311	α-cadinene	1.04
Peak 14	29.111	α-muurolene	0.48
Peak 15	29.690	ɣ-cadinene	0.54
Peak 16	30.065	Δ-cadinene	1.71
Peak 17	31.107	α-elemol	2.59
Peak 18	32.194	ɣ-muurolene	1.10
Peak 19	35.328	Cadin-4-en-10-ol	0.63
TOTAL		-	100,00

Regarding antifungal activity, all products showed anti-candida activity (Table 6), highlighting essential oils of *M. officinalis* and *O. basilicum*, which promoted fungal growth inhibition halos of 32 and 30 mm for *C. albicans* and *C. tropicalis* strains, respectively. Among phytochemicals, α-pinene inhibited the growth of these organisms, leading to the formation of halos of 35 and 30 mm for *C. albicans* and *C. tropicalis*, respectively.

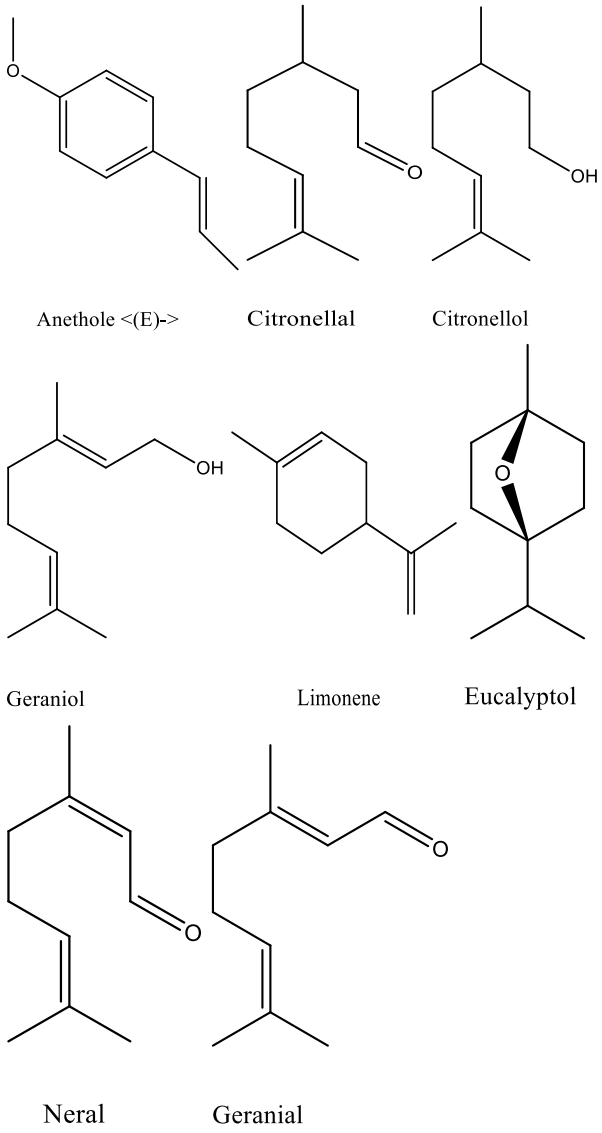

Figure 2: Chemical structures of the main phytochemicals identified in the essential oils tested.

Table 6: Inhibition hales (mm) of essential oils and phytochemicals against *Candida albicans* and *Candida tropicalis* strains.

Essential oils and phytochemicals	***C. albicans***	***C. tropicalis***
Cinnamomum camphora	11	14
Melissa officinalis	32	30
Ocimum basilicum	32	30
Cymbopogon nardus	15	30
Alfa pineno	35	30
Citral	32	25
Citronelal	30	30
Carvacrol	30	30
Nistatina (control)	40	41

Discussion

The knowledge of the chemical composition of natural products allows understanding, from the molecular point of view, the action of phytochemicals, as well as the relationship between chemical structure and pharmacological action, considering pharmacodynamic aspects. This information indicates useful pharmacotherapeutic properties of a particular substance, so it can be an effective new drug candidate [6]. It is in this context that natural products have gained prominence within the pharmaceutical industry as they inspire new bioactive molecular patterns [46]. Camphor is the phytochemical most isolated from EO obtained from leaves and bark of *C. camphora* [35, 36]. In this study, there was a significant presence of limonene and eucalyptol in EO extracted from the bark of this plant. This diversity in the chemical composition of EO can be explained from the growth conditions (incubation time, temperature, oxygen content), collection and extraction technique [38]. Similarly, to findings described in literature, the most abundant components present in the EO of *M. officinalis* were monoterpenes geraniol and neral [26, 41].

Linalool and (E)-anethole stood out as the major components present in the EO of *O. basilicum*. Regardless of the period of collection of the botanical material, considering the seasonal variation, linalool was the most isolated compound (56.7 - 60.6%) of EO obtained from the shoots of this plant [17]. Citronellal, citronellol and geraniol were the components most isolated from EO from C. *nardus* leaves. Similar result was observed, indicating significant presence of monoterpenes: citronellal (36.67%), citronellol (11.40%) and geraniol (25.05%) [8]. Also, we identified the presence of citronellal (29.7%) and geraniol (24.2%) in the EO extracted from the shoots of *C. nardus* [23].

Agar diffusion test was used in this study to identify the activity of EO and phytochemicals in inhibiting the growth of *C. albicans* and *C. tropicalis*. This method of simple execution and low cost represents a qualitative assessment of the pharmacological action, since factors related to the products under study such as lipid solubility, influence their diffusion in the culture medium and hence determine the results obtained [34]. Therefore, comparisons between the products evaluated are not recommended, although the agar diffusion test is recognized as an important method for pharmacological screening of products with possible antimicrobial activity [31, 39]. The microorganisms selected for this study are considered to be clinically relevant etiological agents, which makes it important to control. Epidemiological data indicate a high prevalence of these infections in hospital, especially in immunosuppressed individuals [29, 33].

Essential oils of *M. officinalis*, and *O. basilicum* and phytochemicals α-pinene, citral, citronellal and carvacrol were considered very active against *C. albicans* strain [3]. Essential oils of *C. camphora* and *C. nardus* were considered active and partially active, respectively. With respect to *C. tropicalis*, essential oils and phytochemicals have been recognized as very active, with the exception of essential oil of *C. camphora*, which was classified as partially active. The antifungal activity of the essential oil of C. camphora was evaluated against C. albicans and a growth inhibition halo of 10.6 mm was observed [12]. Likewise, evaluated the antifungal activity of *O. basilicum* and observed growth inhibition halo ranging from 27 to 35 mm for *C. albicans* [1, 5, 9]. In another study, analyzed the antifungal activity of *M. officinalis* and a growth inhibition halo of up to 16 mm was observed [20]. The scientific literature also shows antifungal activity of the essential oil of *C. nardus* [22, 50]. This research demonstrated that phytochemicals α-pinene, citral, citronellal and carvacrol showed antifungal activity

against *Candida* strains, which is consistent with studies reported in literature [22, 50]. These phytochemicals are small and generally hydrophobic organic biomolecules, designated as natural antibiotics, which exert their antimicrobial action possibly by disrupting the cytoplasmic membrane [15]. The data analysis of this research, which considers the relationship between chemical structure and biological activity, contributes to the understanding of the anti-Candida activity evidenced by essential oils and phytochemicals assessed in this study, although minor components present in essential oils can assist in the expression of the biological activity through synergism mechanism [47]. Regarding the antifungal action, mechanisms of action of essential oils seem to be predominantly on the fungal cell membrane, increasing its permeability and causing cell death; blocking the synthesis of membrane; inhibiting spore germination, proliferation of fungi and cellular respiration [14]. Due to the high volatility and lipophilicity of essential oils, they readily penetrate the cell membrane and exert their biological effects [18].

The results of this study suggest the conduction of tests to quantitatively assess the anti-*Candida* activity of essential oils and their phytochemicals, increasing the number of strains, either in the platonic form or biofilms, and investigations to elucidate their possible mechanism of action and toxicological standard for human cells.

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