Hindawi

Research Article

Identification of Secondary Metabolites from Mexican Plants with Antifungal Activity against Pathogenic *Candida* Species

Nancy A. Vázquez-López [iD],[1] **Gustavo Cruz-Jiménez** [iD],[2] **Armando Obregón-Herrera,**[1] **Estela Ruiz-Baca** [iD],[3] **Mario Pedraza-Reyes** [iD],[1] **Everardo López-Romero** [iD],[1] **and Mayra Cuéllar-Cruz** [iD][1]

[1]*Departamento de Biología, División de Ciencias Naturales y Exactas, Campus Guanajuato, Universidad de Guanajuato, Noria Alta S/N, Col. Noria Alta, C.P, Guanajuato, 36050 Guanajuato, Mexico*
[2]*Departamento de Farmacia, División de Ciencias Naturales y Exactas, Campus Guanajuato, Universidad de Guanajuato, Noria Alta S/N, Col. Noria Alta, C.P, Guanajuato, 36050 Guanajuato, Mexico*
[3]*Facultad de Ciencias Químicas, Universidad Juárez del Estado de Durango, Av. Veterinaria S/N, 34120 Durango, Mexico*

Correspondence should be addressed to Mayra Cuéllar-Cruz; mcuellar@ugto.mx

In the last three decades, invasive fungal infections caused by *Candida* species have become an important public health problem, because they are associated with high rates of morbidity and mortality in immunocompromised and hospitalized patients. The diagnosis and treatment of candidiasis are difficult and usually inefficient. Accordingly, a diversity of available drugs, currently employed to attack candidiasis, frequently induce resistance in patients promoting toxicity due to long-term treatments. Therefore, development of accurate diagnoses and novel antifungals is of high priority to improve life's quality and expectancy of individuals infected with this pathogen. Plants are invaluable sources of new biologically active compounds. Among the plants used in Mexico in traditional herbolary medicine which have empirically been demonstrated to have antifungal activity are *Pedilanthus tithymaloides*, *Thymus vulgaris*, and *Ocimum basilicum*. In the present study, we analyzed whether these plants contain metabolites with antifungal activity against five *Candida* species. The extracts from the different plant organs were obtained by macerating them in ethyl alcohol or hexane and filtering. The obtained extracts were preserved in amber flasks at 4°C until used. The minimum inhibitory concentrations (MICs) of the active compound were determined by a microdilution assay. In addition, the following secondary metabolites were identified: linalool (3,7-dimethylocta-1,6-dien-3-ol), eugenol (4-allyl-2-methoxyphenol), limonene (1-methyl-4-(1-methylethenyl)-cyclohexene), and borneol ([[(2R)-1,7,7-trimethyl-2-bicyclo[2.2.1]heptanyl] formate). All these compounds were found in the three plants, traditionally used in everyday life, and proved to be effective against *Candida* species and therefore a viable alternative to conventional antifungals.

1. Introduction

It has been estimated that there are over 5 million fungal species worldwide, and approximately 300 out of these are known to cause diseases in humans, while 20-25% do it with relative frequency. *Candida* species are among these patho-gens. Most of the fungal infections are nontransmissible among people and routinely do not affect healthy individuals [1]. Several species from the *Candida* genus that can be clas-sified as part of this group of pathogens are widely distrib-uted in nature and are part of the normal microbiota in the oral cavity, the gastrointestinal tract, and the urogenital

system in human host. These organisms do not trigger infections in healthy hosts; however, some of these fungi can behave as opportunistic pathogens when the immune system of the host is compromised, causing infections called superficial candidiasis (cutaneous and mucous infections) and systemic or invasive candidiasis (infection of the bloodstream and invasive candidiasis of organs) [2, 3]. These infections amount, as a whole, to approximately 40 million per year worldwide [4]. Invasive candidiasis (IC) represents one of the most common nosocomial infections due to fungi, particularly, in cancer patients and in individuals under immunosuppression regimes [5, 6]. Candidemia can reach a mortality rate from 30 to 60% in hospitalized immunocompromised patients [7, 8]. In more than half of these mycosis cases, death occurs in the first week after the diagnosis of infection due to Candida [9]. Candida albicans has been identified as the most prevalent and pathogenic species; it is responsible for most oral and systemic candidiasis cases, as well as for community and nosocomial origin candidemia [10]. In the last decades, Candida non-C. albicans species like Candida glabrata, Candida tropicalis, Candida parapsilosis, Candida dubliniensis, and, recently, Candida auris have been increasing [11–15]. Invasive candidiasis causes significant morbidity and mortality despite intensive treatments with antifungal agents [16, 17]. The mortality attributed to this disease has not diminished significantly despite the new diagnostic methods, the new antimycotic treatment options, and the better control of the infection [18]. Starting at the second half of the last century, commercialization of amphotericin B, although toxic, helped importantly in the treatment of systemic mycoses [19]. The advent of topic azoles like miconazole and clotrimazole, and later of systemic azoles like ketoconazole, itraconazole, fluconazole, and voriconazole, has simplified the treatment of superficial and systemic mycoses, improving the healing expectancy of these infections. However, with the increase in the HIV and cancer incidences and the indiscriminate use of steroids, surgical procedures, and transplants, failed cases have been reported with the antimycotic therapy using diverse compounds like ketoconazole, fluconazole, and even amphotericin B [20]. Hence, the mortality attributed to fungi is still too high even with the current antifungal agents; thus, a greater emphasis must be placed in improving the time used for the fungicide activity of the new antifungal agents. Currently, the general treatment course with common antimycotics is too long and, hence, presents the potential of a deficient short-term fungicide effect, a diminution in compliancy and/or tolerability by patients, or even the appearance of direct resistance to the antifungal drugs [21]. A complication that arises with the treatment of mycoses is the resistance to the antifungal agents; this is defined as the capacity acquired by an organism to resist the effects of a chemotherapeutic agent to which it is habitually sensitive [22]. Plants are a viable option to obtain a wide variety of pharmaceuticals because they are easily accessible and can be applied to diverse pathologies [23]. In this way, plants constitute an excellent source of substances that can be used in the formulation of new antifungal agents [24]. However, the development of the pharmaceutical industry and the synthesis of

molecules with diverse activities in the clinical field displaced the use of medicinal plants in many regions of the world, mostly in large cities [25]. Although the current available drugs are usually efficacious, the therapeutic failures and the toxicity after a long treatment are common; thus, plants are a good alternative to be explored to improve treatments of severe infections [26, 27]. It has been calculated that there are from 200 to 500 thousand species of higher plants worldwide; in Mexico, the diversity is estimated from 23 to 30 thousand species [28]. Knowledge on the chemical diversity of plants is still limited worldwide; it is calculated that the chemical structure of around 100 thousand secondary metabolites is known, and there could be at least one million of them in all the species that have not been studied yet [29]. Likewise, the biological properties of secondary metabolites are unknown; in most cases, research is centered on priority problems, like the search of anticancer agents, and they do not attempt to perform integrated studies on all the biological activities. Regarding candidiasis treatment, even though there are currently a large variety of available antifungal agents, it is increasingly more frequent that patients do not respond to treatment, causing toxicity after a long treatment. For this reason, it is necessary to identify new alternatives for the treatment of candidiasis. Plants are an invaluable source of new biological active compounds. Among the reported secondary metabolites in plants with antifungal activity are flavonoids, phenols, glycosides of phenols, and saponins. Among the plants used in traditional medicine that have empirically shown to possess antifungal activity are Pedilanthus tithymaloides, Thymus vulgaris, and Ocimum basilicum. In the present study, we analyzed the secondary metabolites from these plants as well as their antifungal activity against five Candida species.

2. Materials and Methods

2.1. Plant Material.
Pedilanthus tithymaloides was donated and collected from a private orchard in the municipality of Leon (state of Guanajuato, Mexico). Lateral stems of the shrub, detached from the nodes, were collected. Thymus vulgaris and Ocimum basilicum plants were acquired from a nursery in Guanajuato, Mexico.

2.2. Sampling of Plants.
The leaves and stems of each plant were separated, placed in individual recipients, and washed with tap water to eliminate any residues; then, they were washed again with sterile deionized water. The leaves and stems were placed separately on a grid that allowed dripping the excess water to dry the plant material. This was carried out at room temperature and away from light until use.

2.3. Extract Preparation.
The plant material (leaves or stems) was placed on an analytical balance on top of a polyethylene tray until a mass of 40 g was reached and further sterilized in 2% sodium hypochlorite for 10 min. Keeping sterile conditions, this plant material was placed in a mortar, adding 100 mL of 99.5% ethyl alcohol or hexane (which was previously cooled in an ice bath), and crushed to homogeneity. The obtained macerate was filtered with a vacuum

filtration system (Kitasato flask-Büchner funnel-TYPE HVLP, 0.45 μm membrane). The obtained extracts were kept in amber flasks at 4°C until their use.

2.4. Fungal Strains and Culture Media.
The strains of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis* species were obtained from the strain collection of the mycology laboratory, which is part of the Department of Biology of the Natural and Exact Sciences Division, at the *Universidad de Guanajuato*. Strains were grown in YPD (1% yeast extract, 2% Bacto-Peptone, and 2% dextrose) medium. The solid medium was supplemented with 2% agar [30].

2.5. Susceptibility Assays to the Different Extracts by Means of the Disk Diffusion Method.
The minimal inhibitory concentrations (MICs) of the active compounds were determined through a microdilution assay as previously described [31]. The effect of the different extracts on the five *Candida* species was determined with yeasts in the stationary growth phase. For the latter, the five *Candida* species were grown during 48 h at 28°C under constant agitation at 120 rpm. Once the incubation period had ended, the optical density was measured at 600 nm (OD_{600nm}) and adjusted to 0.5 and 0.1 in 1 mL of sterile deionized water. The cells adjusted to the different ODs were streaked on YPD plates with the help of a sterile Digralsky loop, adding 75 μL of the suspension to each plate. Afterward, the plate was divided in sections, and with previously sterilized curved point steel tweezers, sterile filter paper disks (Whatman 40) of ca. 6 mm in diameter were placed in each section. Of each extract to be evaluated, 50 μL was placed on top of each disk (ethanol extract of stem or leaf of *P. tithymaloides*, *T. vulgaris*, or *O. basilicum*. Hexane extracts of leaf or stem of *P. tithymaloides*, *T. vulgaris*, or *O. basilicum* were also used. To assess whether the solvents used for the extracts affected or not the cell growth, ethanol and hexane were used as negative controls. Additionally, another solvent, reported as relatively innocuous, that is, dimethyl sulfoxide (DMSO), was used. As positive control, phenol at different proportions (1 : 10, 1 : 100, 1 : 1000, and 1 : 10000) and absolute phenol were used. Plates were incubated at 28°C for 36-48 h. After the incubation period, photographs were taken using the gel documentation system (GeneGenius Bio Imaging System, Artisan Technology Group, Champaign, IL, USA, from Syngene). Experiments were performed in triplicate.

2.6. Analysis of Extracts

2.6.1. Gas Chromatography Coupled to Mass Spectrometry (GC/MS).
The secondary metabolites contained in the hexane extract of leaves and stems of *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* were identified by means of GC [32] coupled to a Clarus SQ8 MS (Perkin Elmer, Inc., Waltham, MA, USA) equipped with an ion deflector that allowed identifying low volatility compounds and those thermally labile. This equipment is provided with a capillary DB-5 column of phenyl methyl silicone (30 m length, 0.25 mm in diameter, and 0.25 μm of phase thickness). From each sample, 1 μL was taken and injected in splitless

mode, with a total execution time of 30 min. The chromatogram was interpreted with the aid of the AMDIS software. As a previous step to the GC/MS analysis, a derivatization reaction was performed to increase the volatility and thermal stability of the polar compounds which implies to derivatize one or more polar groups of one compound to a less polar group. In 2 mL Eppendorf tubes, 2 mL of the ethanol extract was deposited and lyophilized in the Speed-vac equipment to evaporate the solvent during 3 to 5 h. The lyophilized extract was resuspended in 350 μL of pyridine, then, 50 μL of the derivatizing agent, bis-(trimethylsilyl) trifluoroacetamide (BSTFA), which contains 1% chlorotrimethylsilane, was added, and the tubes were vortexed. Afterward, the mixture was heated in a thermoblock at a constant temperature of 50°C for 1 h. Once the thermal exposure time was concluded, it was centrifuged at 1200 rpm for 4 min. From the derivatized samples, 200 μL was taken, and the metabolites were identified by means of GC/MS. The analysis was performed in triplicate.

2.6.2. High Performance Liquid Chromatography (HPLC).
To obtain the pure fraction of the ethanol or hexane extracts, these were fractionated by means of HPLC (Model Altus A30, Perkin Elmer, Inc., Waltham, MA, USA) [33] with a quaternary pump and a diode detector arrangement (DDA). To inject the sample, the air sampler of the same equipment was used. Separation was performed with a *C18* column in isocratic mode with a mobile 45% acetonitrile phase and 55% water. The flow of the mobile phase was of $1.00 \, mL \times min^{-1}$, performing detection at 275 nm. A 1.5 mL volume was taken from the extracts and filtered through a 0.2 μm pore membrane; the filtered extracts were recovered in 1.5 mL Eppendorf tubes and then lyophilized. The lyophilized extract was resuspended in 1.5 mL of HPLC-grade methanol and sonicated for 30 min. The process was repeated twice. To eliminate the solvent of the collected fractions from HPLC, these were lyophilized. The obtained lyophilized samples were resuspended in 2 mL of the initial solvent of each extract, ethanol or hexane, to perform the susceptibility assays against the five *Candida* species according to the previously described protocol. Experiments were performed in triplicate.

2.6.3. Prediction of Targets for the Identified Metabolites.
To evaluate whether the metabolites identified in *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* with possible antifungal activity did not have targets in human cells, the SwissTargetPrediction (http://www.swisstargetprediction.ch.) free-access server was used. This server predicts accurately the targets of the bioactive molecules based on 2D and 3D similitude measurements with known ligands [34].

2.6.4. Statistical Analysis.
In order to determine which of the *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* extracts showed the highest antifungal effect against the five *Candida* species, growth inhibition halos' diameters in three independent experiments were measured and reported as a mean ± standard error of the mean (SEM). Statistical differences between means were determined using a one-way ANOVA test followed by Tukey's posttest for multiple comparisons.
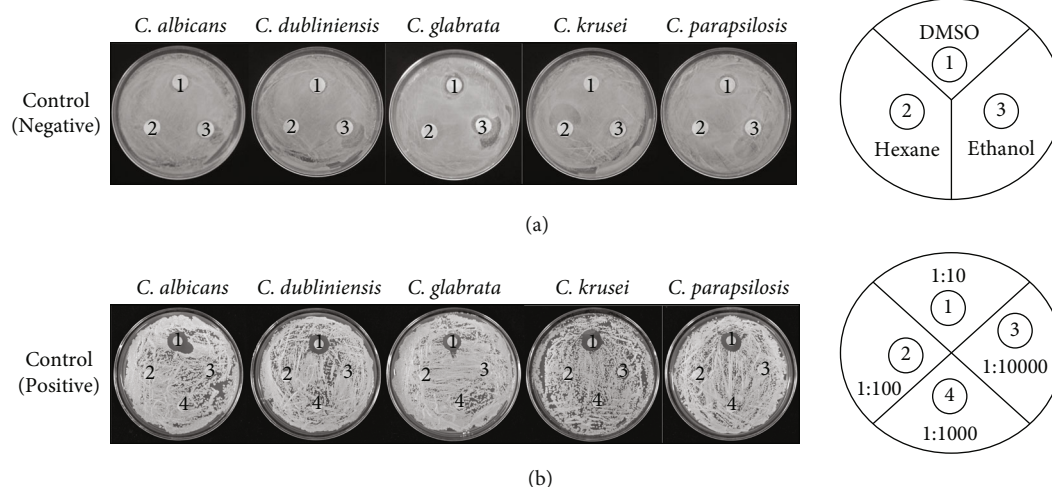
(a)

(b)

FIGURE 1: Susceptibility assays with the solvents used to obtain the *P. tithymaloides*, *T. vulgaris*, or *O. basilicum* extracts.

Normality and homogeneity of data were evaluated using both graphic evaluation and analytical tests such as Shapiro Wilk and Bartlett's test. Minitab 21.1 Software (State Collage PA, USA) was utilized to perform the statistical analysis. Statistical significance was considered when $p \leq 0.05$.

3. Results

3.1. Susceptibility Assays to the Different Plant Extracts with Possible Antifungal Activity. To rule out effects of the solvents employed to prepare the plant extracts, the susceptibility of the five *Candida* species to hexane and ethanol treatment was tested. Additionally, DMSO and phenol were evaluated as a negative and positive controls, respectively. Assays revealed that non-*Candida* species exhibited susceptibility to hexane, ethanol, or DMSO (Figure 1(a)). In contrast, phenol treatment, at the highest dilution (1 : 10) affected the growth of all the *Candida* strains tested (Figure 1(b)). In conclusion, ethanol and hexane were adequate to obtain the extracts from the studied plants to investigate the presence of metabolites with possible antifungal activity.

We investigated whether *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* possessed antifungal activity against five *Candida* species; to this end, ethanolic and hexane extracts prepared with stem and leaves from these plants were tested in disk diffusion assays as described in Materials and Methods. To investigate if the cell concentration impacts the antifungal efficiency of the plant extracts, the cultured *Candida* strains were tested at OD_{600nm} of 0.5 and 1.0, respectively, considering that for *Candida* species, an OD_{600nm} of 1.0 corresponds to 1×10^6 cells. It was decided to work with this amount of cells because susceptibility must be evaluated with a number of cells that will allow observing the antifungal effect of the compounds to be analyzed, which is not possible if working with a large amount of cells, where the antifungal effect is masked by the high cell density.

As shown in Figure 2(a), at both ODs, the ethanol extract of the stems presented a higher inhibition of the five

Candida species, as compared to the leaf extracts. In contrast, except for leaf extract of *T. vulgaris*, the hexane extracts of *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* did not present a significant antifungal activity against the *Candida* species, except the leaf extract of *T. vulgaris* (Figure 2(b)). These results indicate that the metabolites with possible antifungal activity are found mainly in ethanol extracts. Furthermore, they indicate that the antifungal effect is independent from the number of cells and may correspond to the intrinsic effect of the extracts (Figures 2(a) and 2(b)).

To determine which of the *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* extracts showed the highest antifungal effect against the five *Candida* species, we measured the growth inhibition halos' diameters in three independent experiments at both an OD of 0.5 (Table S1) and an OD of 0.1 (Table S2), and the means of all experiments were taken.

For the ethanol extract of the *P. tithymaloides* stem, at both ODs, the *C. dubliniensis* species presented the highest susceptibility, followed by *C. parapsilosis*, *C. glabrata*, *C. krusei*, and *C. albicans* (Figures 3 and 4). For the leaf extract of *P. tithymaloides*, a nil antifungal activity was observed. Assessment of the stem extract of *T. vulgaris* revealed that *C. parapsilosis* and *C. krusei* were the most susceptible species at an OD of 0.5 and 0.1, respectively (Figures 3 and 4).

With the ethanol extract of the *O. basilicum* stem, *C. glabrata* was more susceptible at OD_{600nm} 0.5 and *C. dubliniensis* at OD_{600nm} 0.1 (Figures 3 and 4), whereas *C. albicans* and *C. parapsilosis* were the species with the highest resistance to this extract (Figures 3 and 4). With the ethanol extracts of *T. vulgaris* leaves, at an OD_{600nm} of 0.5, *C. albicans* presented the highest susceptibility (Figure 3), whereas at an OD_{600nm} of 0.1, it was *C. dubliniensis* (Figure 4); the resistance to this extract by *C. parapsilosis*, *C. glabrata*, and *C. krusei* at both ODs was different (Figures 3 and 4). With the stem extract of *O. basilicum*, at an OD_{600nm} of 0.5, *C. albicans* was the species with the highest resistance, followed by *C. parapsilosis*, *C. krusei*, *C. dubliniensis*, and *C. glabrata* (Figure 3). In contrast, at an OD_{600nm} of 0.1, *C. glabrata*, *C. krusei*, and *C. parapsilosis* were the species with the highest
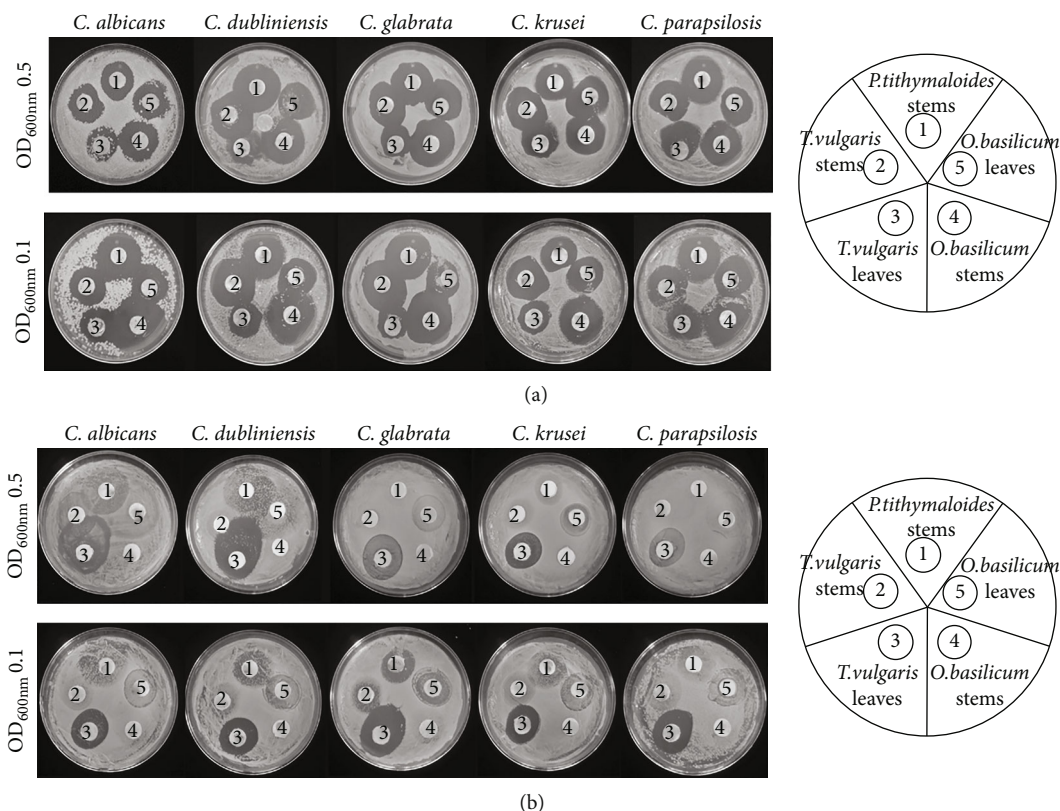
FIGURE 2: Susceptibility tests of stem or leaf extracts, obtained in (a) ethanol or (b) hexane, of *P. tithymaloides*, *O. Basilicum*, and *T. vulgaris* against *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*.
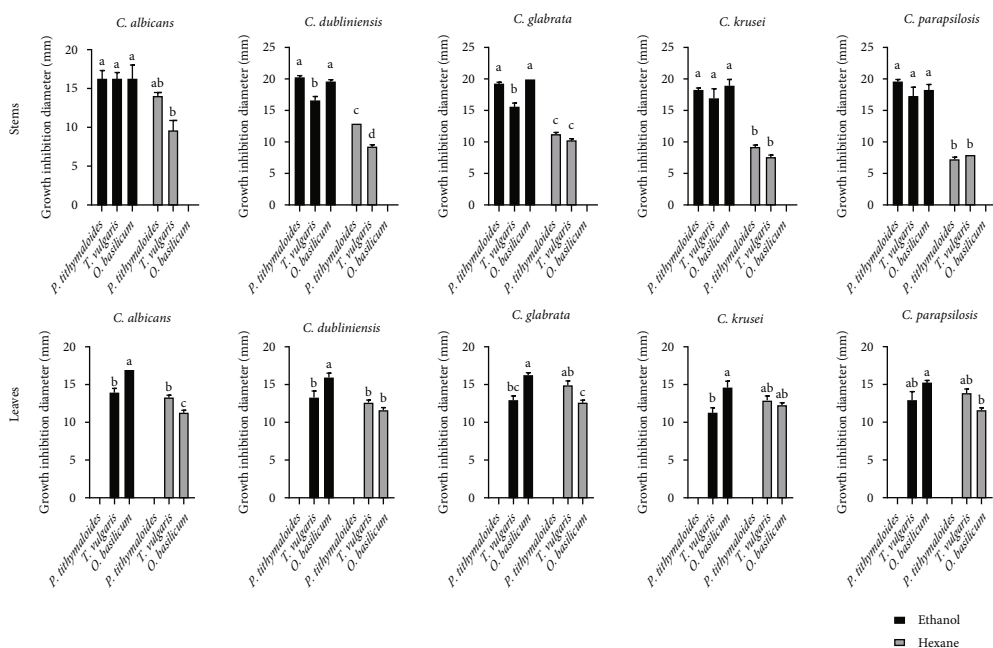

FIGURE 3: Diameters of the inhibition halos of the *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* extracts from stems and leaves (horizontal layout) against the five *Candida* species at an OD_{600nm} 0.5 (vertical layout). Black and dark gray bars represent ethanolic and hexanoic extracts, respectively. Different letters indicate significant differences between treatments (Tukey's test $p \leq 0.05$). Error bars indicate the standard error of the mean (SEM, $n = 3$).

FIGURE 4: Diameters of the inhibition halos of the *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* extracts from stems and leaves (horizontal layout) against the five *Candida* species at an OD_{600nm} 0.1 (vertical layout). Black and dark gray bars represent ethanolic and hexanoic extracts, respectively. Different letters indicate significant differences between treatments (Tukey's test $p \leq 0.05$). Error bars indicate the standard error of the mean (SEM, $n = 3$).
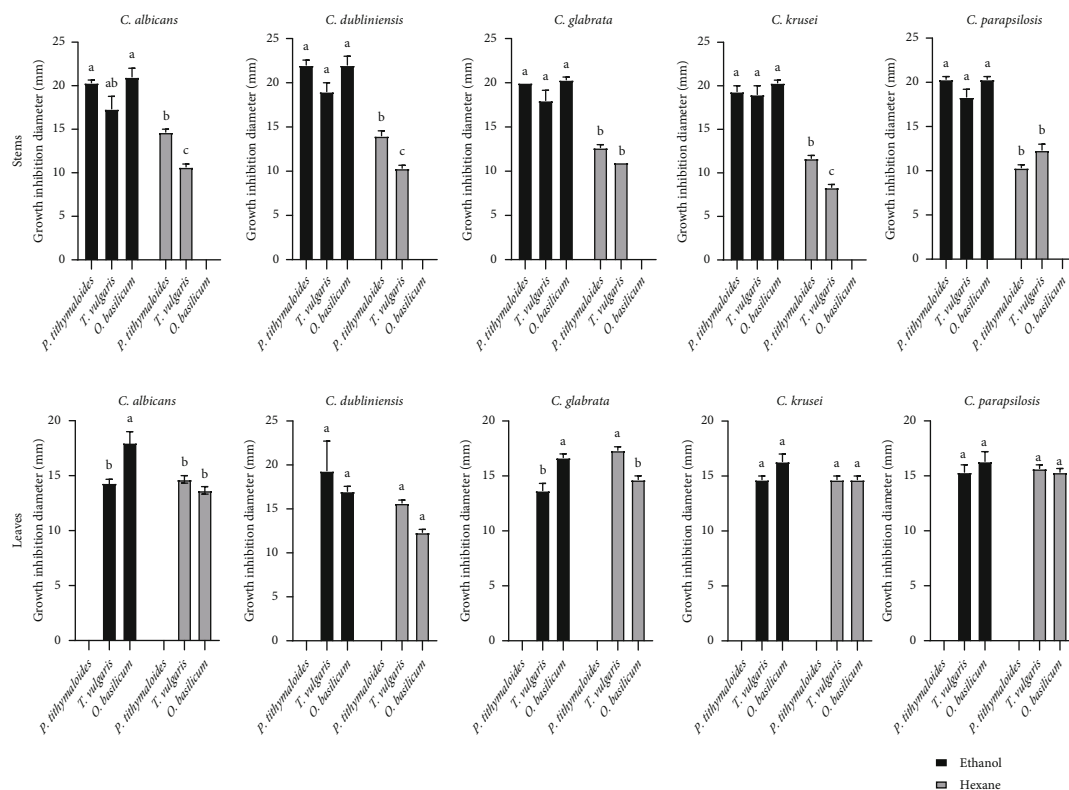
resistance to this extract (Figure 4). The highest susceptibility to the ethanol extract of *O. basilicum* leaves was shown by *C. albicans* at both ODs tested (Figures 3 and 4), whereas *C. parapsilosis* and *C. krusei* were the species with the highest resistance to this extract (Figures 3 and 4). Interestingly, the *T. vulgaris* leaf extracts prepared with hexane but not those from stems affected the growth of the five *Candida* species, when these were tested at both cell's concentrations (Figures 3 and 4).Overall, our results revealed that the solvents employed to prepare the extracts impacted the antifungal activity exhibited by the leaves and stems of the plants tested and that such effects may obey to the existence of a differential profile of metabolites with various degrees of antifungal activity.

3.2. Analysis of the Extracts Obtained with Ethanol and Hexane. The stems and leaf extracts obtained with hexane were analyzed without any GC-MS treatment, whereas the ethanol extracts were subjected to a derivatization reaction to be analyzed with this technique. The chromatograms obtained of each extract were analyzed with the AMDIS software; this approach allowed us to identify a set of secondary plant metabolites (Fig. S1). Table 1 depicts the compounds identified through GC/MS analysis of the extracts that presented antifungal activity against the five *Candida* species.

The analysis of the *T. vulgaris* extracts revealed 15 potential compounds with antifungal activity; the leaf was the

plant tissue with the highest number of chemical compounds with 15 compounds, and ethanol was the extraction agent with the most extracted metabolites. Compounds like carvacrol, eugenol, carveol, and p-cymene were identified only in ethanol extracts of both leaves and stems. The thymol and thymol-methyl-ether were identified in all plant extracts. In *O. basilicum*, 11 compounds were identified in ethanol extracts (Table 1). The compounds were identified in both the leaves and stems, revealing that metabolites are present in both plant organs, but at different concentrations, this would explain why the best antifungal effect against *Candida* species is observed in stems (Figure 2(a)). Regarding *P. tithymaloides* stem extracts, eight compounds were identified. In this plant, no metabolites were identified in the leaf extracts; i.e., no antifungal activity was recorded (Figure 2). Eugenol, linalool, limonene, and borneol were found in more than one of the plants (Table 1); however, linalool was the only compound identified in the extracts of the three plants (Table 1). This finding indicates that possibly, this compound is the main one implicated in the antifungal effect against *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*. Once the metabolites had been identified in the different extracts of *P. tithymaloides*, *T. vulgaris*, and *O. basilicum*, it was decided to purify the different metabolites, to assess the antifungal activity of each identified metabolite and, in this way, know which metabolite is responsible for the antifungal activity. For this, the plant

TABLE 1: Chemical compounds potentially responsible for the antifungal activity present in the plant extracts.

| Plant extract	Plant part	Extraction agent	Identified compound	Chemical formula
T. vulgaris	Leaf Stem	Hex EtOH	Thymol methyl ether	$C_{11}H_{16}O$
	Leaf Stem	Hex EtOH	Thymol	$C_{10}H_{14}O$
	Leaf Stem	EtOH	Carvacrol	$C_{10}H_{14}O$
	Leaf Stem	EtOH	Carvacrol methyl ether	$C_{11}H_{16}O$
	Leaf	Hex	2-Chloropropionyl chloride	$C_3H_4C_{l2}O$
	Leaf	Hex	2-Bromo-2-methylbutane	$C_5H_{11}Br$
	Leaf	Hex	2,4-Dimethylhexane	C_8H_{18}
	Leaf Stem	EtOH	Eugenol	$C_{10}H_{12}O_2$
	Leaf Stem	EtOH	Methyl eugenol	$C_{11}H_{14}O_2$
	Leaf	Hex EtOH	Linalool	$C_{10}H_{18}O$
	Leaf	EtOH	Limonene	$C_{10}H_{16}$
	Leaf	EtOH	Borneol	$C_{10}H_{18}O$
	Leaf Stem	EtOH	α-Terpineol	$C_{10}H_{18}O$
	Leaf Stem	EtOH	Carveol	$C_{10}H_{16}O$
	Leaf Stem	EtOH	p-Cymene	$C_{10}H_{14}$
O. basilicum	Leaf	EtOH	Linalool	$C_{10}H_{18}O$
	Leaf	EtOH	Limonene	$C_{10}H_{16}$
	Leaf	EtOH	Borneol	$C_{10}H_{18}O$
	Leaf	EtOH	Eugenol	$C_{10}H_{12}O_2$
	Leaf Stem	EtOH	α-Bergamotene	$C_{21}H_{22}O_4$
	Leaf Stem	EtOH	Sabinene	$C_{10}H_{16}$
	Leaf Stem	EtOH	α-Pinene	$C_{10}H_{16}$
	Leaf Stem	EtOH	Germacrene D	$C_{15}H_{24}$
	Leaf Stem	EtOH	Bornyl acetate	$C_{12}H_{20}O_2$
	Leaf Stem	EtOH	α-Amorphene	$C_{15}H_{24}$
	Leaf Stem	EtOH	α-Caryophyllene	$C_{15}H_{24}$
P. tithymaloides	Stem	EtOH	Palmitic acid	$C_{16}H_{32}O_2$
	Stem	EtOH	Retinol	$C_{20}H_{28}O$
	Stem	EtOH	Myristic acid	$C_{14}H_{28}O_2$
	Stem	EtOH	Stearic acid	$C_{18}H_{36}O_2$
	Stem	EtOH	Tauric acid	$C_{12}H_{24}O_2$
	Stem	EtOH	Citronellol	$C_{10}H_{20}O$
	Stem	EtOH	Stigmasterol	$C_{29}H_{48}O$
	Stem	EtOH	Linalool	$C_{10}H_{18}O$
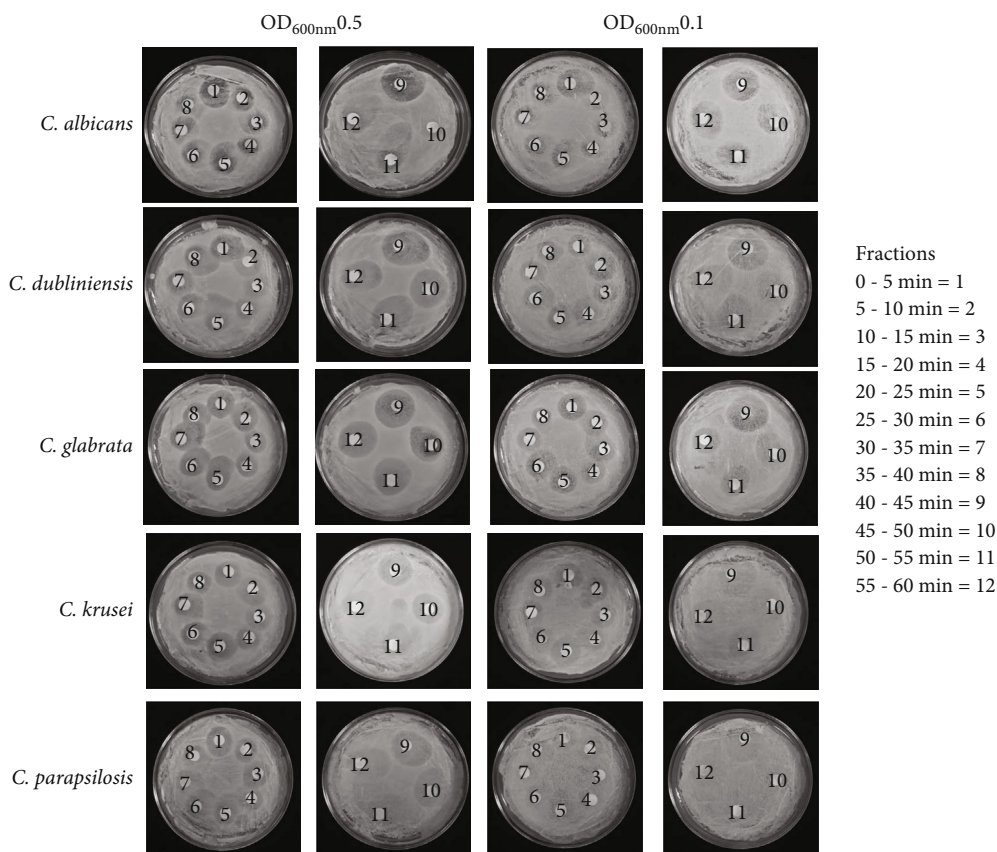
Figure 5: Susceptibility assays of the 12 pure fractions obtained from HPLC.

extracts were fractionated at 12-elution times through HPLC, collecting 12 fractions in 60 min. Each fraction was used to perform susceptibility assays against *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*. Some of these fractions exhibited inhibitory activity against these fungal species, indicating that the fraction must contain some compound with antifungal activity. Figure 5 and Table 2 summarize the results of these assays.

The susceptibility assays with the different fractions obtained through HPLC revealed specific activity against some of the *Candida* species (Figure 5). To analyze these results, a table was constructed considering all experiments performed in triplicate and independently (Table 2).

For the *P. tithymaloides* stem extract, fractions 8, 9, and 12 showed antifungal activity against the five *Candida* species. The *T. vulgaris* leaf extract presented greater antifungal activity in fractions 1, 3, 5, 6, 9, 10, and 12, whereas only fraction 12 of the stem extracts presented antifungal activity. The *O. basilicum* leaf extract presented the largest number of antifungal fractions against *Candida*, these fractions were 1, 5, 6, 7, 8, 9, 10, 11, and 12 (Table 2) during the susceptibility assays. For the stem, fractions 10, 11, and 12 (Table 2) presented susceptibility to the five *Candida* species. To assess whether the four metabolites identified in the three analyzed plants could be good candidates against *Candida*, but apparently innocuous to the human cells, the targets of these metabolites were mapped using the SwissTargetPrediction software. The bioinformatics analyses revealed that the

probability that any of the proteins of human cells could be an actual target was practically inexistent for the four secondary metabolites (Figure 6).

4. Discussion

In the last years, it has been reported that *Candida* species represent one of the most important causes of systemic nosocomial infections. *C. albicans* is the most important opportunistic pathogen; however, the prevalence of other species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and, recently, *C. auris* is increasing. The developments in medicine, associated with an increase in invasive procedures, increasingly aggressive immunosuppressing treatments, and the generalized use of wide-spectrum antibiotics, have favored the increase in the selective pressure and the development of antifungal resistance, contributing to the incidence of candidemia and IC. Although a variety of antifungal agents is available and because patients do not respond adequately to treatment, it is necessary to identify new antifungal agents that are safe for the patients, but highly selective against *Candida* species. The main inconvenience when designing new antifungal compounds is that the only difference between mammalian and fungal cells is the cell wall of *Candida* and, thus, the target site of excellence, leading to a lesser possibility for the effect of antifungal agents. For this reason, the search and use of new active principles, derived from natural products for alternative therapies, are utterly important. Among these natural

TABLE 2: Susceptibility assays with the HPLC obtained fractions.

Hexane plant extract	Number of fraction	Collecting time	Inhibitory activity on each species				
			C. albicans	C. dubliniensis	C. glabrata	C. krusei	C. parapsilosis
Stem of P. tithymaloides	1	0–5 min	−	−	−	−	−
	2	5–10 min	−	−	−	−	−
	3	10–15 min	−	−	−	−	−
	4	15–20 min	−	−	−	−	−
	5	20–25 min	−	−	−	−	−
	6	25–30 min	−	−	−	−	−
	7	30–35 min	−	−	−	−	−
	8	35–40 min	+	+	+	+	+
	9	40–45 min	+	+	+	+	+
	10	45–50 min	−	−	−	−	−
	11	50–55 min	−	−	−	−	−
	12	55–60 min	+	+	+	+	+
Leaf of T. vulgaris	1	0–5 min	−	+	+	+	+
	2	5–10 min	−	−	−	−	−
	3	10–15 min	+	−	−	−	+
	4	15–20 min	−	−	−	−	−
	5	20–25 min	+	+	+	+	−
	6	25–30 min	−	+	+	+	−
	7	30–35 min	−	−	−	−	−
	8	35–40 min	−	−	−	−	−
	9	40–45 min	−	+	+	+	+
	10	45–50 min	−	−	+	−	−
	11	50–55 min	−	−	−	−	−
	12	55–60 min	+	+	+	+	+
Stem of T. vulgaris	1	0–5 min	−	−	−	−	−
	2	5–10 min	−	−	−	−	−
	3	10–15 min	−	−	−	−	−
	4	15–20 min	−	−	−	−	−
	5	20–25 min	−	−	−	−	−
	6	25–30 min	−	−	−	−	−
	7	30–35 min	−	−	−	−	−
	8	35–40 min	−	−	−	−	−
	9	40–45 min	−	−	−	−	−
	10	45–50 min	−	−	−	−	−
	11	50–55 min	−	−	−	−	−
	12	55–60 min	−	−	+	+	+
Leaf of O. basilicum	1	0–5 min	+	+	+	−	+
	2	5–10 min	−	−	+	−	−
	3	10–15 min	−	−	−	−	−
	4	15–20 min	−	−	−	−	−
	5	20–25 min	+	−	+	+	−
	6	25–30 min	−	+	+	−	−
	7	30–35 min	+	−	+	+	−
	8	35–40 min	+	+	+	−	−
	9	40–45 min	+	+	+	+	+
	10	45–50 min	−	+	+	+	+
	11	50–55 min	+	+	+	+	+
	12	55–60 min	+	+	+	+	+

TABLE 2: Continued.

| Hexane plant extract	Number of fraction	Collecting time	Inhibitory activity on each species				
			C. albicans	C. dubliniensis	C. glabrata	C. krusei	C. parapsilosis
	1	0–5 min	–	–	–	–	–
	2	5–10 min	–	–	–	–	–
	3	10–15 min	–	–	–	–	–
	4	15–20 min	–	–	–	–	–
	5	20–25 min	–	–	–	–	–
Stem of O. basilicum	6	25–30 min	–	–		–	–
	7	30–35 min	–	–	–	–	–
	8	35–40 min	–	–	+	+	+
	9	40–45 min	+	+	–	+	+
	10	45–50 min	+	+	+	+	+
	11	50–55 min	+	+	+	+	+
	12	55–60 min	+	+	+	+	+

– resistant; + susceptible.

products are plants which contain a large variety of biologically active molecules, which can have different target sites and action mechanisms from those of traditional antimicrobials [35, 36]; in fact, the antifungal activity of different crude plant extracts against different microorganisms has been reported.

To identify the metabolites with antifungal activity against *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*, we chose *P. tithymaloides*, *T. vulgaris*, and *O. basilicum*, which are plants used in traditional medicine to treat different diseases. Crude extracts were prepared with two different solvents: ethanol that is a solvent of polar character and hexane that is a polar. The variety of the solvents allowed obtaining different metabolites from the plants. The ethanol extracts presented higher antifungal activity (Figure 2(a)) against the five studied *Candida* species, whereas for the hexane extracts, the *T. vulgaris* leaf extract was the only one with antifungal activity against the *Candida* species (Figure 2(b)). The anatomical parts, i.e., leaves and stems, were assessed independently to analyze whether there is some difference in the composition and concentration of metabolites. This was done because some of the secondary metabolites in plant exert defense functions against predators and can also inhibit the development of insects [37], fungi [38], and bacteria [39], which determine their preferential location in one or another anatomical site. Results allowed identifying a total of 30 metabolites in *P. tithymaloides*, *T. vulgaris*, and *O. basilicum* in leaves and/or stems. Among these metabolites, monoterpene phenols like thymol, carvacrol, and eugenol were identified in *T. vulgaris*. However, of the identified metabolites, we focused in those identified in at least two plants. In this way, linalool was identified in three plants, and eugenol, limonene, and borneol were found in *T. vulgaris* and *O. basilicum* (Table 1).

Linalool (3,7-dimethylocta-1,6-dien-3-ol) has been reported to have properties as acaricide, bactericide, and fungicide. Its antifungal activity has been tested against diverse microorganisms, such as *Fusarium moniliforme* at a dose of 1000 ppm, *Candida* species, and *Acinetobacter baumannii*

[40–48]. Our results agree with other reports that have evaluated linalool as an antifungal against *Candida* species; in strains isolated from individuals with oral candidiasis, the antifungal activity was good against *C. tropicalis* and moderate against *C. albicans* [42]. In another study, the fungicide activity of linalool against 39 *C. albicans* isolates and 9 isolates of *Candida* non-*C. albicans* was evaluated and found to be effective against both *Candida* species [46, 47]. Linalool has also been found in the essential oil of *Lavandula angustifolia*; in this work, the antifungal activity against *C. albicans* was shown, as well as its participation in avoiding the morphological transition from yeast to mycelium, because linalool can inhibit the formation of the germ tube and reduce the elongation of hyphae. This finding indicates that linalool, by avoiding dimorphism, can be considered as an effective compound against *C. albicans* [41]. Besides, by preventing the morphology change in *Candida*, linalool also avoids the formation of biofilms by *C. albicans* [43] and *C. tropicalis* [44]. Biofilms are considered as virulence factors in *Candida* species, because they induce recurring candidiasis symptoms, leading to fatal outcomes. As a whole, these data show that linalool is a compound with efficient antifungal activity against the studied *Candida* species, and it is present in different plant species, as shown in this and other studies; hence, it is a good candidate to be used against these fungi. The mechanism by which terpenoids, like linalool, act as antifungal agents has not been elucidated yet; but a model has been described proposing that terpenoids induce membrane fluidization, modulating the functions of the proteins bound to the membrane and involved in signaling and transport [47]. Besides, it has been reported that terpenoids arrest the cell cycle in *Candida* [47] and other organisms, like *Staphylococcus aureus* and *Escherichia coli* [49]. Linalool exhibited an estimated low affinity for distinct families of proteins from *Homo sapiens* (Figure 6(a)).

Another identified metabolite in *T. vulgaris* and *O. basilicum* was eugenol (4-allyl-2-methoxyphenol). This compound is found in a variety of plants, like *Syzygium aromaticum* (L.) and *Myristica fragrans* (Houtt); besides, finding eugenol in *O. basilicum* agrees with other works [50, 51]. Its common
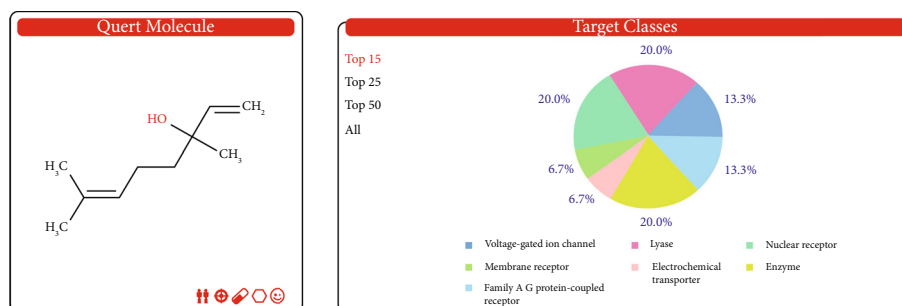
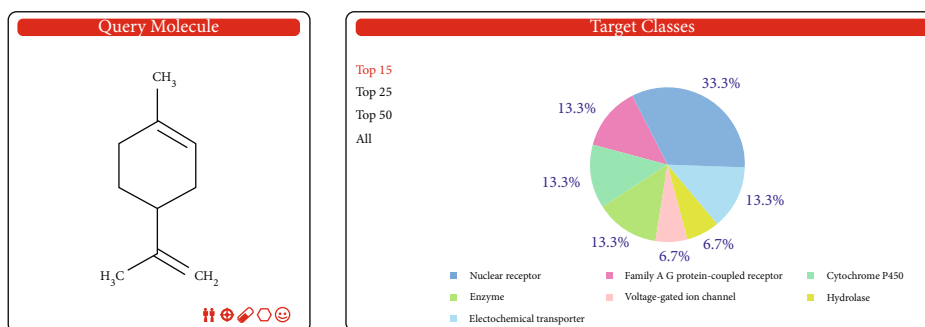| Target	Common name	Uniprot ID	chEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Transient receptor potential cation channel subfamily V member 3	TRPV3	Q8NET8	CHEMBL5522	Voltage-gated ion channel	0.0630257148888	1 / 0
Carbonic anhydrase II	CA2	P00918	CHEMBL205	Lyase	0.0630257148888	33 / 0
Carbonic anhydrase I	CA1	P00915	CHEMBL261	Lyase	0.0630257148888	27/ 0
Carbonic anhydrase IV	CA4	P22748	CHEMBL3729	Lyase	0.0630257148888	11 / 0
Transient receptor potential cation channel subfamily M member 8	TRPM8	Q7Z2W7	CHEMBL1075319	Voltage-gated ion channel	0.0630257148888	1 / 0
Mineralocorticoid receptor	NR3C2	P08235	CHEMBL1994	Nuclear receptor	0.053517944289	34 / 0
Glucocorticoid receptor	NR3C1	P04150	CHEMBL2034	Nuclear receptor	0.053517944289	52 / 0
Progesterone receptor	PGR	P06401	CHEMBL208	Nuclear receptor	0.053517944289	74 / 0
Sigma opioid receptor	SIGMAR1	Q99720	CHEMBL287	Membrane receptor	0.053517944289	27 / 0
Dopamine transporter (by homology)	SLC6A3	Q01959	CHEMBL238	Electrochemical transporter	0.053517944289	101 / 0
Squalene monooxygenase	SQLE	Q14534	CHEMBL3592	Enzyme	0.053517944289	0 / 4
Indoleamine 2,3-dioxygenase	IDO1	P14902	CHEMBL4685	Enzyme	0.053517944289	9 / 0
Estradiol 17-beta-dehydrogenase 2	HSD17B2	P37059	CHEMBL2789	Enzyme	0.053517944289	57 / 0
Dopamine D2 receptor (by homology	DRD2	P14416	CHEMBL217	Family A G protien coupled receptor	0.053517944289	13 / 0
Muscarinic acetylcholine receptor M4	CHRM4	P08173	CHEMBL1821	Family A G protien coupled receptor	0.053517944289	11 / 0
Mu opioid receptor	OPRM1	P35372	CHEMBL233	Family A G protien coupled receptor	0.053517944289	8 / 0
Delta opioid receptor	OPRD1	P41143	CHEMBL236	Family A G protien coupled receptor	0.053517944289	5 / 0
Kappa opioid receptor	OPRK1	P41145	CHEMBL237	Family A G protien coupled receptor	0.053517944289	29 / 0
Adrenergic receptor alpha-2	ADRA2C	P18825	CHEMBL1916	Family A G protien coupled receptor	0.0439186325197	13 / 0
Heme oxygenase 1 (by homology)	HMOX1	P09601	CHEMBL2823	Enzyme	0.0439186325197	36 /0
Tyrosine-protien kinase JAK 1	JAK1	P23458	CHEMBL2835	Kinase	0.0439186325197	60 /0
Tyrosine-protien kinase JAK 2	JAK2	O60674	CHEMBL2971	Kinase	0.0439186325197	86 /0

(a)

FIGURE 6: Continued.
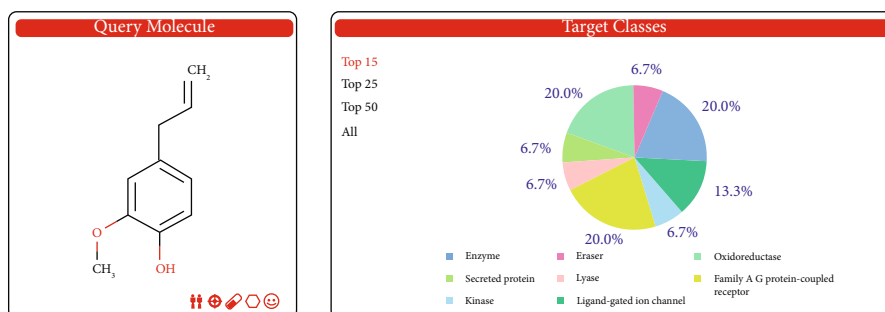
Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Fatty acid desaturase 1	FADS1	O60427	CHEMBL5840	Enzyme	0.133391037839	8 / 0
Histone deacetylase 6	HDAC6	Q9UBN7	CHEMBL1865	Eraser	0.133391037839	10 / 0
Egl nine homolog 1	EGLN1	Q9GZT9	CHEMBL5697	Oxidoreductase	0.125075959828	4 / 0
Vascular endothelial growth factor A	VEGFA	P15692	CHEMBL1783	Secreted protein	0.125075959828	1 / 0
Carbonic anhydrase II	CA2	P00918	CHEMBL205	Lyase	0.125075959828	199 / 15
G-protein coupled receptor 84	GPR84	Q9NQS5	CHEMBL3714079	Family A G protein-coupled receptor	0.125075959828	18 / 0
Cyclooxygenase-1	PTGS1	P23219	CHEMBL221	Oxidoreductase	0.125075959828	35 / 13
D-amino-acid oxidase	DAO	P14920	CHEMBL5485	Enzyme	0.125075959828	27 / 0
Poly [ADP-ribose] polymerase-1	PARP1	P09874	CHEMBL3105	Enzyme	0.125075959828	32 / 0
Tyrosine-protein kinase SRC	SRC	P12931	CHEMBL267	Kinase	0.125075959828	10 / 0
Adenosine A1 receptor	ADORA1	P30542	CHEMBL226	Family A G protein-coupled receptor	0.125075959828	28 / 0
Adenosine A2a receptor	ADORA2A	P29274	CHEMBL251	Family A G protein-coupled receptor	0.125075959828	17 / 0
Steroid 5-alpha-reductase 1	SRD5A1	P18405	CHEMBL1787	Oxidoreductase	0.125075959828	3 / 0
Neuronal acetylcholine receptor subunit alpha-3	CHRNA3	P32297	CHEMBL3068	Ligand-gated ion channel	0.125075959828	1 / 0
Neuronal acetylcholine receptor protein alpha-4 subunit (by homology)	CHRNA4	P43681	CHEMBL1882	Ligand-gated ion channel	0.125075959828	3 / 0
Interleukin-8 receptor B	CXCR2	P25025	CHEMBL2434	Family A G protein-coupled receptor	0.125075959828	20 / 0
dCTP pyrophosphatase 1	DCTPP1	Q9H773	CHEMBL3769292	Enzyme	0.125075959828	5 / 0
Alkaline phosphatase, tissue-nonspecific isozyme	ALPL	P05186	CHEMBL5979	Enzyme	0.125075959828	15 / 0
Methionine aminopeptidase 2	METAP2	P50579	CHEMBL3922	Protease	0.116739032206	5 / 0
Carbonyl reductase [NADPH] 1	CBR1	P16152	CHEMBL5586	Enzyme	0.116739032206	1 / 0
Calcium-activated potassium channel subunit alpha-1	KCNMA1	Q12791	CHEMBL4304	Voltage-gated ion channel	0.116739032206	11 / 0
Arachidonate 15-lipoxygenase	ALOX15	P16050	CHEMBL2903	Enzyme	0.116739032206	33 / 11
Neuronal acetylcholine receptor; alpha3/ beta4	CHRNA3 CHRNB4	P32297 P30926	CHEMBL1907594	Ligand-gated ion channel	0.116739032206	1 / 0
Vascular endothelial	KDR	P35968	CHEMBL279	Kinase	0.116739032206	32 / 0

(b)

Figure 6: Continued.

Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Peroxisome proliferator-activated receptor alpha	PPARA	Q07869	CHEMBL239	Nuclear receptor	0.146856850103	1 / 7
Cannabinoid receptor 2	CNR2	P34972	CHEMBL253	Family A G protein-coupled receptor	0.146856850103	1 / 1
LXR-alpha	NR1H3	Q13133	CHEMBL2808	Nuclear receptor	0.0043083186041	0 / 14
Cytochrome P450 19A1	CYP19A1	P11511	CHEMBL1978	Cytochrome P450	0.023827432783	0 / 127
Anandamide amidohydrolase	FAAH	000519	CHEMBL2243	Enzyme	0.0	0 / 10
Vanilloid receptor	TRPV1	Q8NER1	CHEMBL4794	Voltage-gated ion channel	0.0	0 / 2
Androgen Receptor (by homology)	AR	P10275	CHEMBL1871	Nuclear receptor	0.0	0 / 47
Estrogen receptor alpha	ESR1	P03372	CHEMBL206	Nuclear receptor	0.0	0 / 27
Muscarinic acetylcholine receptor M2	CHRM2	P08172	CHEMBL211	Family A G protein-coupled receptor	0.0	0 / 1
Acetylcholinesterase	ACHE	P22303	CHEMBL220	Hydrolase	0.0	4 / 1
Norepinephrine transporter	SLC6A2	P23975	CHEMBL222	Electochemical transporter	0.0	0 / 1
Serotonin transporter	SLC6A4	P31645	CHEMBL228	Electochemical transporter	0.0	0 / 1
Cytochrome P450 2C19	CYP2C19	P33261	CHEMBL3622	Cytochrome P450	0.0	0 / 1
Squalene monooxygenase	SQLE	Q14534	CHEMBL3592	Enzyme	0.0	0 / 5
Estrogen receptor beta	ESR2	Q92731	CHEMBL242	Nuclear receptor	0.0	0 / 32
Butyrylcholinesterase	BCHE	P06276	CHEMBL1914	Hydrolase	0.0	0 / 1
Protein-tyrosine phosphatase 1B	PTPN1	P18031	CHEMBL335	Phosphatase	0.0	0 / 25
Nuclear receptor subfamily 1 gropu 1 member 3 (by homology)	NR1I3	Q14994	CHEMBL5503	Nuclear receptor	0.0	0 / 2
Cyclooxygenase-1	PTGS1	P23219	CHEMBL221	Oxidoreductase	0.0	0 / 2
DNA topoisomerase I	TOP1	P11387	CHEMBL1781	Isomerase	0.0	0 / 2
Adenosine A1 receptor	ADORA1	P30542	CHEMBL226	Family A G protein-coupled receptor	0.0	0 / 1
Adenosine A2a receptor	ADORA2A	P29274	CHEMBL251	Family A G protein-coupled receptor	0.0	0 / 1
Adenosine A3 receptor	ADORA3	P0DMS8	CHEMBL256	Family A G protein-coupled receptor	0.0	0 / 2
Arachidonate 5-lipoxygenase	ALOX5	P09917	CHEMBL215	Oxidoreductase	0.0	0 / 4
Nuclear receptor ROR-gamma	RORC	P51449	CHEMBL1741186	Nuclear receptor	0.0	0 / 9
Sterol regulatory element-binding protein 2	SREBF2	Q12772	CHEMBL1795166	Unclassified protein	0.0	0 / 1
Niemann-Pick C1-like	NPC1L1	Q9UHC9	CHEMBL2027	Other membrane	0.0	0 / 5

(c)

FIGURE 6: Continued.

Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Carbonic anhydrase II	CA2	P00918	CHEMBL205	Lyase	0.340608982509	3 / 2
Carbonic anhydrase I	CA1	P00915	CHEMBL261	Lyase	0.340608982509	3 / 2
Carbonic anhydrase IV	CA4	P22748	CHEMBL3729	Lyase	0.340608982509	2 / 2
Transient receptor potential cation channel subfamily M member 8	TRPM8	Q7Z2W7	CHEMBL1075319	Voltage-gated ion channel	0.302383432167	1 / 1
Dual specificity phosphatase Cdc25A	CDC25A	P30304	CHEMBL3775	Phosphatase	0.197404892104	0 / 7
Dual specificity phosphatase Cdc25B	CDC25B	P30305	CHEMBL4804	Phosphatase	0.197404892104	0 / 5
Nuclear receptor subfamily 1 group I member 3	NR1I3	Q14994	CHEMBL5503	Nuclear receptor	0.197404892104	0 / 2
Androgen Receptor	AR	P10275	CHEMBL1871	Nuclear receptor	0.177829917574	34 / 17
Bile acid receptor FXR	NR1H4	Q96RI1	CHEMBL2047	Nuclear receptor	0.139879779447	0 / 12
G-protein coupled bile acid receptor 1	GPBAR1	Q8TDU6	CHEMBL5409	Family A G protein-coupled receptor	0.139879779447	0 / 16
Sonic hedgehog protein (by homology)	SHH	Q15465	CHEMBL5602	Unclassified protein	0.139879779447	0 / 10
Estrogen receptor beta	ESR2	Q92731	CHEMBL242	Nuclear receptor	0.101228839251	5 / 5
Estrogen receptor alpha	ESR1	P03372	CHEMBL206	Nuclear receptor	0.0918391658524	1 / 6
UDP-glucuronosyltransferase 2B7	UGT2B7	P16662	CHEMBL4370	Enzyme	0.0822880706974	8 / 24
Testis-specific androgen-binding protein	SHBG	P04278	CHEMBL3305	Secreted protein	0.0822880706974	2 / 22
DNA polymerase alpha subunit	POLA1	P09884	CHEMBL1828	Transferase	0.0727163610114	0 / 1
Niemann-Pick C1-like protein 1	NPC1L1	Q9UHC9	CHEMBL2027	Other membrane protein	0.0727163610114	0 / 7
11-beta-hydroxysteroid dehydrogenase 1	HSD11B1	P28845	CHEMBL4235	Enzyme	0.053517944289	9 / 11
LXR-alpha	NR1H3	Q13133	CHEMBL2808	Nuclear receptor	0.053517944289	0 / 10
Glucose-6-phosphate 1-dehydrogenase	G6PD	P11413	CHEMBL5347	Enzyme	0.0439186325197	0 / 7
GABA-B receptor (by homology)	GABBR1	Q9UBS5	CHEMBL2064	Family A G protein-coupled receptor	0.0439186325197	0 / 4
Glucocorticoid receptor	NR3C1	P04150	CHEMBL2034	Nuclear receptor	0.0439186325197	20 / 0
Dopamine D2 receptor (by homology)	DRD2	P14416	CHEMBL217	Family A G protein-coupled	0.0439186325197	5 / 0

(d)

FIGURE 6: Results of the target sites prediction in human cells: (a) Linalool (3,7-dimethylocta-1,6-dien-3-ol; SMILE: CC(O)(C=C)CCC=C(C)C. B) Eugenol (4-Allyl-2-methoxyphenol; SMILE: COC1=CC(CC=C)=CC=C1O. (c) Limonene (1-methyl-4-(1-methyletenyl)-cyclohexene; SMILE: CC1=CCC(CC1)C(=C)C. (d) Borneol ([(2R)-1,7,7-trimethyl-2-bicyclo[2.2.1]heptanyl] formate; SMILE: CC1(C2CCC1(C(C2)O)C)C. Green bars indicate the estimated probability that a protein could be an actual target of the secondary metabolite, at less probability the lesser it is that an actual target is implicated.

name comes from the plant *Eugenia caryophyllata* (syn. *Syzygium aromaticum*) [50] where it was found for the first time. This compound, like linalool, has been shown to have antifungal activity against *Candida* species [47]. The antifungal activity of this compound has been investigated for almost four decades [52]. These authors analyzed the antifungal activity of eugenol in 31 strains of *C. albicans* and found that it had indeed antifungal activity against this fungus [52]. Several years later, the activity of eugenol against several opportunistic fungi, including *C. albicans*, was investigated, and authors found that the analyzed strains were inhibited by this metabolite [53]. Aiming at administering eugenol as a treatment against fungi, toxicity studies were performed in mice, but the maximal tolerated dose was of 62.5 mg/kg; therefore, treatment of mycoses with eugenol is not allowed [52]. To be able to use eugenol as an antifungal drug, derivatives of this metabolite have been synthetized, finding that a derivative known as peracetyl glucoside, which is more potent and less cytotoxic than eugenol, inhibited the growth of *C. albicans*, *C. glabrata*, and *C. tropicalis* [54]. In a later work, it was demonstrated that the use of a new derivative of eugenol against these fungi inhibited 90% the growth of *C. glabrata* [44, 45]. These data together with our findings indicated that the eugenol in the fractions of two of the analyzed plants is possibly responsible for the observed antifungal activity (Table 1). The action mechanism of eugenol in fungi has already been studied, and it is suggested that because eugenol is lipophilic, it can enter the fatty acid chains of the lipid bilayer of the membrane, upsetting its fluidity and permeability [55, 56]. It has also been reported that eugenol inhibits the ATPase activity and that it is capable of producing oxidative stress [57]. The analysis by SwissTargetPrediction revealed a low affinity of eugenol for enzymes from *H. sapiens* (Figure 6(b)) which would be beneficial for the treatment of candidiasis.

Limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) is another of the compounds identified in *T. vulgaris* and *O. basilicum*; this cyclic monoterpene has been identified in a large variety of citrus plants and other plants, i.e., *Thapsia villosa*, *Dyssodia decipiens*, *Helichrysum italicum* [58, 59]. Other reports agree with our results by reporting limonene as a compound of *Thymus vulgaris* [60–62]. Limonene is widely used in the pharmaceutical industry as insecticide and antimicrobial [63–67]. Few studies have analyzed the antimicrobial effects of limonene; however, there are some works that evaluated the biological activity of limonene against species of genera like *Aspergillus*, *Trichophyton*, and *Candida*, reporting the antifungal efficiency of this compound [68, 69]. Our results agree with studies that have evaluated limonene as an antifungal against species of *Candida*, in which it was efficient against clinical isolates of *C. albicans* strains, with a MIC of 12.5–188.4 μg mL^{-1} [59]. Another study demonstrated the antifungal activity of limonene on the planktonic growth of 35 clinical isolates and two standard strains of *C. albicans*, in which the fungicide activity was efficient in all cases at a 20 mM concentration [68]. No specific mechanisms on how limonene induces cellular disturbances have been proposed; however, it is known that limonene exerts an inhibitory effect on the formation of the germ tube in *C. albicans*, affecting the morphological change

from yeast to mycelium [46, 47, 70, 71], which is an important virulence factor for the pathogenicity of this species [72]. The hydrophobicity of limonene apparently facilitates the dissolution of lipids located in the microbial plasmatic membrane, inducing a loss of the membrane's integrity and, thereby, affecting functions like permeability, signaling, and transport [73, 74]. Besides, it affects respiration and the energetic metabolism by interfering with the ATP synthesis through the inhibition of the respiratory complex and ATPase activities [75, 76]. These data support the capacity of limonene to act as an efficient antifungal against *Candida*, because it is a component of a large variety of plants, and as shown in this and other studies, it is capable of acting on multiple targets. The aforementioned makes this compound a good candidate to be used against fungi. This compound was identified in this work as one of the metabolites responsible for the antifungal activity of *T. vulgaris* and *O. basilicum*. In addition, limonene exhibits a low activity on family A of G protein-coupled receptor, nuclear receptor, and cytochrome P450 (Figure 6(c)).

The fourth metabolite identified in *T. vulgaris* and *O. basilicum* was borneol ([(2*R*)-1,7,7-trimethyl-2-bicyclo[2.2.1]heptanyl] formate). It is an organic compound belonging to the bicyclic monoterpenoid class. Its presence has been reported in more than 260 plants, mainly in those belonging to the *Lamiaceae* family, including *T. vulgaris* and *O. basilicum* species [60–62]. These reports agree with our present results (Table 1). Borneol is widely used in the pharmaceutical industry and as an antimicrobial agent [77–82]. The fungicide activity reported in other studies on borneol against *C. albicans* is effective at a MIC of 0.320 mg/mL [83], which places this compound as a potential candidate for the treatment of candidiasis. Like other terpenoid compounds, as linalool and limonene, borneol interrupts the integrity of the microbial plasmatic membrane [46, 47, 73, 74] and affects the yeast to mycelium morphological change in *C. albicans* [71, 84]. In addition, borneol is found in a large variety of plants, and the analysis *in silico* indicated a low affinity for proteins such as lyase, phosphatase, nuclear receptor, and family A of G protein-coupled receptor (Figure 6(d)). These observations and results described in this work allowed us to propose thus terpenoid as one of the compounds mainly responsible for the antifungal activity of *T. vulgaris* and *O. basilicum*. However, besides borneol, linalool, eugenol, and limonene were identified as additional candidate drugs with anti-*Candida* activity. These results together with our *in silico* studies suggest a low percentage of binding of these drugs to human targets, but future studies will be required to fully elucidate their mechanism of action.

5. Conclusions

Linalool, eugenol, limonene, and borneol found in *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* plants, traditionally of everyday use, are effective compounds against *Candida* species, thus allowing them to be considered as viable alternatives to traditional antifungal agents once their toxicity has been discarded in animal models.

Data Availability

The data used to support the findings of this study are included within this article.

Conflicts of Interest

The authors declare that there were no conflicts of interest with any organization or entity with a financial interest or financial conflict with the material discussed in this work.

Acknowledgments

Supplementary Materials

Figure S1: representative chromatograms obtained from each extract that were analyzed with the AMDIS software, in which some of the secondary metabolites present in the studied plants could be identified. Table S1: diameters of the inhibition halos of the *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* extracts against the five *Candida* species at an OD_{600nm} 0.5. Superscript letters indicate significant differences between treatments (Tukey's test $p \leq 0.05$), $n = 3$. Lowercase letters indicate significant differences on inhibition halos between stem extracts for each *Candida* species. Uppercase letters indicate significant differences on inhibition halos between leave extracts for each *Candida* species. EtOH: ethanol; Hex: hexane; NGI: no growth inhibition; SD: standard deviation. Table S2: diameters of the inhibition halos of the *P. tithymaloides*, *O. basilicum*, and *T. vulgaris* extracts against the five *Candida* species at an OD_{600nm} 0.1. Superscript letters indicate significant differences between treatments (Tukey's test $p \leq 0.05$), $n = 3$. Lowercase letters indicate significant differences on inhibition halos between stem extracts for each *Candida* species. Uppercase letters indicate significant differences on inhibition halos between leave extracts for each *Candida* species. EtOH: ethanol; Hex: hexane; NGI: no growth inhibition; SD: standard deviation. (*Supplementary Materials*)

References

[1] G. Brown, D. Denning, and S. Levitz, "Tackling human fungal infections," *Science*, vol. 336, no. 6082, p. 647, 2012.

[2] N. Papon, V. Courdavault, M. Clastre, and R. Bennett, "Emerging and emerged pathogenic *Candida* species: beyond the *Candida albicans* paradigm," *PLoS Pathogens*, vol. 9, no. 9, article e1003550, 2013.

[3] R. López, "Candidosis, a new challenge," *Clinics in Dermatology*, vol. 28, no. 2, pp. 178–184, 2010.

[4] J. Naglik, J. Richardson, and D. Moyes, "*Candida albicans* pathogenicity and epithelial immunity," *PLoS Pathogens*, vol. 10, no. 8, article e1004257, 2014.

[5] C. Spampinato and D. Leonardi, "Candida Infections, Causes, Targets, and Resistance Mechanisms: Traditional and Alternative Antifungal Agents," *BioMed Research International*, vol. 2013, Article ID 204237, 13 pages, 2013.

[6] C. Sriphannam, N. Nuanmuang, K. Saengsawang, D. Amornthipayawong, and A. Kummasook, "Anti-fungal susceptibility and virulence factors of *Candida spp.* isolated from blood cultures," *Journal de Mycologie Médicale*, vol. 29, no. 4, pp. 325–330, 2019.

[7] M. Cheng, Y. Yang, T. Yao et al., "Risk factors for fatal candidemia caused by *Candida albicans* and non-*albicans Candida* species," *BMC Infectious Diseases*, vol. 5, no. 1, 2005.

[8] M. Morrell, V. J. Fraser, and M. H. Kollef, "Delaying the empiric treatment of *Candida* bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality," *Antimicrobial Agents and Chemotherapy*, vol. 49, no. 9, pp. 3640–3645, 2005.

[9] M. Cuéllar-Cruz, J. C. Villagómez-Castro, E. Ruiz-Baca, and E. López-Romero, "Geographic distribution of *Candida* species and its correlation whit blood type and biofilm formation," *Current Trends in Microbiology*, vol. 7, pp. 61–86, 2011.

[10] M. Pfaller, M. Castanheira, S. A. Messer, G. J. Moet, and R. N. Jones, "Variation in *Candida spp.* distribution and antifungal resistance rates among bloodstream infection isolates by patient age: report from the SENTRY Antimicrobial Surveillance Program (2008–2009)," *Diagnostic Microbiology and Infectious Disease*, vol. 68, no. 3, pp. 278–283, 2010.

[11] E. S. Spivak and K. E. Hanson, "*Candida auris*: an emerging fungal pathogen," *Journal of Clinical Microbiology*, vol. 56, no. 2, article e01588-17, 2018.

[12] G. Moran, C. Stokes, S. Thewes, B. Hube, D. Coleman, and D. Sullivan, "Comparative genomics using *Candida albicans* DNA microarrays reveals absence and divergence of virulence-associated genes in *Candida dubliniensis*," *Microbiology*, vol. 150, no. 10, pp. 3363–3382, 2004.

[13] G. R. Thompson, P. K. Patel, W. Kirkpatrick et al., "Oropharyngeal candidiasis in the era of antiretroviral therapy," *Oral surgery, oral medicine, oral pathology, oral radiology and endodontics*, vol. 109, no. 4, pp. 488–495, 2010.

[14] K. Zomorodian, N. Haghighi, N. Rajaee et al., "Assessment of *Candida* species colonization and denture-related stomatitis in complete denturewearers," *Medical Mycology*, vol. 49, no. 2, pp. 208–211, 2011.

[15] A. L. Colombo, T. Guimarães, L. R. B. F. Silva et al., "Prospective observational study of candidemia in Sao Paulo: Brazil incidence rate, epidemiology, and predictors of mortality," *Infection Control and Hospital Epidemiology*, vol. 28, no. 5, pp. 570–576, 2007.

[16] O. Gudlaugsson, S. Gillespie, K. Lee et al., "Attributable mortality of nosocomial candidemia, revisited," *Clinical Infectious Diseases*, vol. 37, no. 9, pp. 1172–1177, 2003.

[17] P. Pappas, C. Kauffman, D. Andes et al., "Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America," *Clinical Infectious Diseases*, vol. 62, no. 4, pp. e1–e50, 2016.

[18] C. Sibylle, M. Von Bergwelt, H. Schößer, and O. Cornely, "A novel approach to candidemia? The potential role of checkpoint inhibition," *Medical Mycology*, vol. 57, no. 2, pp. 151–154, 2019.

[19] S. Chapman, J. Cleary, and P. Rogers, "Amphotericin B," in *Essentials of Clinical Mycol*, pp. 41–55, Springer, N.Y., 2011.

[20] P. Manzano, L. Méndez, F. Hernández, and R. López, "La resistencia a los antifúngicos: un problema emergente en México," *Gaceta Médica de México*, vol. 144, no. 1, pp. 23–26, 2008.

[21] M. Cuenca, "Antifungal drug resistance mechanisms in pathogenic fungi: from bench to bedside," *Clinical Microbiology and Infection*, vol. 20, pp. 54–59, 2014.

[22] M. Madigan, J. Martinko, P. Dunlap, and D. Clark, *Brock. Biología de los microorganismos*, Pearson, 2009.

[23] J. C. Orlandi-Sardi, A. M. Fusco-Almeida, and M. J. Soares-Mendes, "New antimicrobial therapies used against fungi present in subgingival sites – a brief review," *Archives of Oral Biology*, vol. 56, no. 10, pp. 951–959, 2011.

[24] F. B. Holetz, G. L. Pessini, N. RogerioSanches, D. A. García-Cortez, C. V. Nakamura, and B. P. Dias-Filho, "Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases," *Memorias do Instituto Oswaldo Cruz*, vol. 97, no. 7, pp. 1027–1031, 2002.

[25] G. Baulies and R. Torres, "Actualización en fitoterapia y plantas medicinales," *FMC: Formación Médica Continuada en Atención Primaria*, vol. 19, no. 3, pp. 149–160, 2012.

[26] O. J. M. Hamza, C. J. P. van den Bout-van, M. I. N. Matee et al., "Antifungal activity of some Tanzanian plants used traditionally for the treatment of fungal infections," *Journal of Ethnopharmacology*, vol. 108, no. 1, pp. 124–132, 2006.

[27] B. P. Mathew and M. Nath, "Recent approaches to antifungal therapy for invasive mycoses," *ChemMedChem: Chemistry Enabling Drug Discovery*, vol. 4, no. 3, pp. 310–323, 2009.

[28] E. Martínez-Meyer, J. E. Sosa-Escalante, and F. Álvarez, "El estudio de la biodiversidad en México: ¿una ruta con dirección?," *Revista mexicana de biodiversidad*, vol. 85, pp. 1–S9, 2014.

[29] R. Verpoorte and A. Alfermann, *Metabolic engineering of plant secondary metabolism*, Springer Science & Business Media., 2000.

[30] F. M. Ausubel, R. Brent, R. E. Kingston et al., *Current Protocols in Molecular Biology*, John Wiley & Sons Inc, NY USA, 2003.

[31] I. Serrano-Fujarte, E. López-Romero, G. E. Reyna-López, M. A. Martínez-Gámez, A. Vega-González, and M. Cuéllar-Cruz, "Influence of culture media on biofilm formation by Candida species and response of sessile cells to antifungals and oxidative stress," *BioMed Research International*, vol. 2015, Article ID 783639, 15 pages, 2015.

[32] O. Fiehn, "Metabolomics by gas chromatography-mass spectrometry: combined targeted and untargeted profiling," *Current protocols in molecular biology*, vol. 114, pp. 30.4.1–30.4.32, 2016.

[33] A. A. Gonzalez-Ibarra, K. Wrobel, E. Yanez-Barrientos et al., "Changes of metabolomics profile in *Helianthus annuus* under exposure to chromium(VI) studied by capHPLC-ESI-QTOF-MS and MS/MS," *Journal of Analytical Methods in Chemistry*, vol. 2017, Article ID 3568621, 18 pages, 2017.

[34] D. Gfeller, A. Grosdidier, M. Wirth, A. Daina, O. Michielin, and V. Zoete, "SwissTargetPrediction: a web server for target prediction of bioactive small molecules," *Nucleic Acids Research*, vol. 42, no. W1, pp. W32–W38, 2014.

[35] I. Ahmad and A. Z. Beg, "Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens," *Journal of Ethnopharmacology*, vol. 74, no. 2, pp. 113–123, 2001.

[36] A. Upadhyay, I. Upadhyaya, A. Kollanoor, and K. Venkitanarayanan, "Combating pathogenic microorganisms using plant-derived antimicrobials: a minireview of the mechanistic basis," *Biomed Research International*, vol. 2014, Article ID 761741, 18 pages, 2014.

[37] G. Zhang, W. Zhang, B. Lian, and L. Gu, "Insecticidal effects of extracts from two rice varieties to brown plan hopper *Nilaparvatalugens*," *Journal of Chemical Ecology*, vol. 25, pp. 1843–1853, 1999.

[38] A. Oliva, E. Lahoz, R. Contillo, and G. Aliota, "Fungistatic activity of rutagraveolens extract and its allelochemicals," *Journal of Chemical Ecology*, vol. 25, no. 3, pp. 519–526, 1999.

[39] R. Grayer and J. Harborne, "A survey of antifungal compound from higher plants, 1982-1983," *Phytochemistry*, vol. 37, no. 1, pp. 19–42, 1994.

[40] L. Bravo, K. Bermúdez, and R. Montes, "Inhibition of *Fusarium moniliforme* by plant powders and some of their chemical components," *Journal of Integrated Pest Management*, vol. 57, pp. 29–34, 2000.

[41] F. D'Auria, M. Tecca, V. Strippoli, G. Salvatore, L. Battinelli, and G. Mazzanti, "Antifungal activity of *Lavandulaangustifolia* essential oil against *Candida albicans* yeast and mycelial form," *Medical Mycology*, vol. 43, pp. 391–396, 2005.

[42] I. J. Dias, E. R. I. S. Trajano, R. D. Castro, G. L. S. Ferreira, H. Medeiros, and D. Gomes, "Antifungal activity of linalool in cases of *Candida spp.* isolated from individuals with oral candidiasis," *Brazilian Journal of Biology*, vol. 78, no. 2, pp. 368–374, 2018.

[43] R. K. Manoharan, J. H. Lee, Y. G. Kim, S. I. Kim, and J. Lee, "Inhibitory effects of the essential oils α-longipinene and linalool on biofilm formation andhyphal growth of *Candida albicans*," *Biofouling*, vol. 33, no. 2, pp. 143–155, 2017.

[44] C. M. Souza, S. A. Pereira Junior, T. D. Moraes et al., "Antifungal activity of plantderived essential oils on *Candida tropicalis*planktonic and biofilms cells," *Medical Mycology*, vol. 54, no. 5, pp. 515–523, 2016.

[45] T. B. de Souza, K. M. de Oliveira Brito, N. C. Silva et al., "New eugenol glucoside-based derivative shows fungistatic and fungicidal activity against opportunistic *Candida glabrata*," *Chemical Biology and Drug Design*, vol. 87, no. 1, pp. 83–90, 2016.

[46] G. Zore, A. Thakre, S. Jadhav, and S. Karuppayil, "Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle," *Phytomedicine*, vol. 18, no. 13, pp. 1181–1190, 2011.

[47] G. Zore, A. Thakre, S. Jadhav, and S. Karuppayil, "Evaluation of anti-*Candida* potential of geranium oil constituents against clinical isolates of *Candida albicans*differentially sensitive to fluconazole: inhibition of growth, dimorphism and sensitization," *Mycoses*, vol. 54, no. 4, pp. e99–e109, 2011.

[48] S. Alves, A. Duarte, S. Sousa, and F. Domingues, "Study of the major essential oil compounds of *Coriandrumsativum*against *Acinetobacter baumannii*and the effect of linalool on adhesion, biofilms and quorum sensing," *Biofouling*, vol. 32, no. 2, pp. 155–165, 2016.

[49] B. F. Brehm and E. A. Johnson, "Sensitization of *Staphylococcus aureus* and *Escherichia coli* to antibiotics by the sequiterpenoids, nerolidol, farnesol, bisabolol and apritone," *Antimicrobial Agents and Chemotherapy*, vol. 47, no. 10, pp. 3357–3360, 2003.

[50] A. Marchese, R. Barbieri, E. Coppo et al., "Antimicrobial activity of eugenol and essential oils containing eugenol: a

mechanistic viewpoint," *Critical Reviews in Microbiology*, vol. 43, no. 6, pp. 668–689, 2017.

[51] N. Slougui, M. Mahammed, Z. Rahmani, and A. Baaliouamer, "Composition of essential oils of six varieties of *Ocimumbasilicum L.* grown in Algeria Mustaghanem: West of Algeria," *Asian Journal of Chemistry*, vol. 27, article 3895, 2015.

[52] C. Boonchird and T. Flegel, "*In vitro* antifungal activity of eugenol and vanillin against *Candida albicans* and *Cryptococcus neoformans*," *Canadian Journal of Microbiology*, vol. 28, no. 11, pp. 1235–1241, 1982.

[53] H. Carrasco, M. Raimondi, L. Svetaz et al., "Antifungal activity of eugenol analogues. Influence of different substituents and studies on mechanism of action," *Molecules*, vol. 17, no. 1, pp. 1002–1024, 2012.

[54] T. B. de Souza, M. Orlandi, L. F. L. Coelho et al., "Synthesis and in vitro evaluation of antifungal and cytotoxic activities of eugenol glycosides," *Medicinal Chemistry Research*, vol. 23, no. 1, pp. 496–502, 2014.

[55] S. Bennis, F. Chami, N. Chami, T. Bouchikhi, and A. Remmal, "Surface alteration of *Saccharomyces cerevisiae* induced by thymol and eugenol," *Letter in Applied in Microbiology*, vol. 38, no. 6, pp. 454–458, 2004.

[56] P. Braga, M. Dal Sasso, M. Culici, and M. Alfieri, "Eugenol and thymol, alone or in combination, induce morphological alterations in the envelope of *Candida albicans*," *Fitoterapia*, vol. 78, no. 6, pp. 396–400, 2007.

[57] A. Khan, A. Ahmad, F. Akhtar et al., "Induction of oxidative stress as a possible mechanism of the antifungal action of three phenylpropanoids," *FEMS Yeast Research*, vol. 11, no. 1, pp. 114–122, 2011.

[58] B. Djihane, N. Wafa, S. Elkhamssa, H. Pedro, A. Maria, and Z. Mohamed, "Chemical constituents of *Helichrysumitalicum*(Roth) G. Don essential oil and their antimicrobial activity against Gram-positive and Gram-negative bacteria, filamentous fungi and *Candida albicans*," *Saudi Pharmaceutical Journal*, vol. 25, no. 5, pp. 780–787, 2017.

[59] Y. Pacheco-Hernández, G. R. Sánchez-Hernández, E. Reyes-Cervantes, O. Romero-Arenas, I. Pérez-Xochipa, and N. Villa-Ruano, "Chemical variation and pharmacological properties of *Dyssodiadecipiens* essential oil," *Chemistry and Biodiversity*, vol. 17, no. 10, article e2000487, 2020.

[60] K. Rajkowska, P. Nowicka-Krawczyk, and A. Kunicka-Stycznska, "Effect of clove and thyme essential oils on *Candida* biofilm formation and the oil distribution in yeast cells," *Molecules*, vol. 24, no. 10, p. 1954, 2019.

[61] P. Satyal, B. Murray, R. McFeeters, and W. Setzer, "Essential oil characterization of *Thymus vulgaris* from various geographical locations," *Foods*, vol. 5, no. 4, p. 70, 2016.

[62] A. Gedikoğlu, M. Sökmen, and A. Çivit, "Evaluation of *Thymus vulgaris* and *Thymbraspicata* essential oils and plant extracts for chemical composition, antioxidant, and antimicrobial properties," *Food Science and Nutrition*, vol. 7, no. 5, pp. 1704–1714, 2019.

[63] J. Sun, "D-Limonene: safety and clinical applications," *Alternative Medicine Review*, vol. 12, no. 3, pp. 259–264, 2007.

[64] V. Sfara, E. N. Zerba, and R. A. Alzogaray, "Fumigant insecticidal activity and repellent effect of five essential oils and seven monoterpenes on first-instar nymphs of *Rhodniusprolixus*," *Journal of Medical Entomology*, vol. 46, no. 3, pp. 511–515, 2009.

[65] T. Feitosa-Machado, N. A. P. Nogueira, R. C. Alves-Pereira, C. Texeira-de Sousa, and V. Chaves-Vasconcelos-Batista,

"The antimicrobial efficacy of *Lippia alba* essential oil and its interaction with food ingredients," *Brazilian Journal of Microbiology*, vol. 45, no. 2, pp. 699–705, 2014.

[66] L. Juiz, P. José, A. M. Lucchese et al., "Essential oils and isolated compounds from *Lippia alba* leaves and flowers: antimicrobial activity and osteoclast apoptosis," *International Journal of Molecular Medicine*, vol. 35, no. 1, pp. 211–217, 2015.

[67] J. Benites, C. Moiteiro, G. Miguel et al., "Composition and biological activity of the essential oil of *Peruvian Lantana*camara," *Journal of the Chilean Chemical Society*, vol. 54, no. 4, pp. 379–384, 2009.

[68] A. Thakre, G. Zore, S. Kodgire et al., "Limonene inhibits *Candida albicans* growth by inducing apoptosis," *Medical Mycology*, vol. 56, no. 5, pp. 565–578, 2018.

[69] H. Chee, H. Mm, and M. Lee, "In *vitro* Antifungal Activity of Limonene against *Trichophyton rubrum*," *Mycobiology*, vol. 37, no. 3, pp. 243–246, 2009.

[70] A. Devkatte, G. Zore, and S. Karuppayil, "Potential of plant oils as inhibitors of growth," *FEMS Yeast Research*, vol. 5, no. 9, pp. 867–873, 2005.

[71] J. Liu, Y. Zhu, G. Du, J. Zhou, and J. Chen, "Response of *Saccharomyces cerevisiae* to D-limonene-induced oxidative stress," *Applied Microbial and Biotechnology*, vol. 97, no. 14, pp. 6467–6475, 2013.

[72] S. P. Saville, A. L. Lazzell, A. P. Bryant et al., "Inhibition of filamentation can be used to treat disseminated candidiasis," *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 10, pp. 3312–3316, 2006.

[73] R. Di Pasqua, N. Hoskins, G. Betts, and G. MaurielloChanges in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 7, pp. 2745–2749, 2006.

[74] L. Espina, T. Gelaw, S. Lamo, R. Pagán, and D. García, "Mechanism of bacterial inactivation by (+)-limonene and its potential use in food preservation combined processes," *PLoS one*, vol. 8, article e56769, 2013.

[75] B. Chueca, R. Pagán, and D. García, "Differential mechanism of *Escherichia coli* inactivation by (+)-limonene as a function of cell physiological state and drug's concentration," *PloS one*, vol. 9, no. 4, article e94072, 2014.

[76] Y. Han, Z. Sun, and W. Chen, "Antimicrobial susceptibility and antibacterial mechanism of limonene against *Listeria monocytogenes*," *Molecules*, vol. 25, no. 1, 2020.

[77] R. M. Belmont, V. C. Cruz, G. M. Martínez et al., "Propiedades antifúngicas en plantas superiores. Análisis retrospectivo de investigaciones," *Revista Mexicana de Fitopatología*, vol. 18, no. 2, pp. 125–131, 2000.

[78] T. Nurhayat, K. Neşe, D. Betül, D. Fatih, and K. Hüsnü, "Composition and antimicrobial activity of the essential oils of *Micromeriacristata subsp. phrygia* and the enantiomeric distribution of borneol," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 9, pp. 4300–4303, 2001.

[79] N. Pazyar, R. Yaghoobi, E. Rafiee, A. Mehrabian, and A. Feily, "Skin wound healing and phytomedicine: a review," *Skin Pharmacology and Physiology*, vol. 27, no. 6, pp. 303–310, 2014.

[80] Q. Zheng, Z. Chen, M. Xu et al., "Borneol, a messenger agent, improves central nervous system drug delivery through

enhancing blood-brain barrier permeability: a preclinical systematic review and meta-analysis," *Drug Delivery*, vol. 25, no. 1, pp. 1617–1633, 2018.

[81] W. Zhong, Y. Cui, Q. Yu et al., "Modulation of LPS-stimulated pulmonary inflammation by borneol in murine acute lung injury model," *Inflammation*, vol. 37, no. 4, pp. 1148–1157, 2014.

[82] G. Li, H. Zhao, J. Hong, K. Quan, Q. Yuan, and X. Wang, "Antifungal graphene oxide-borneol composite," *Colloids and Surfaces B: Biointerfaces*, vol. 160, no. 2017, pp. 220–227, 2017.

[83] Y. Liu, Y. Zhao, D. Guo, W. Liu, and Y. Liu, "Synergistic antimicrobial activity of berberine hydrochloride, baicalein and borneol against *Candida albicans*," *Chinese Herbal Medicines*, vol. 9, no. 4, pp. 353–357, 2017.

[84] M. Alves, M. J. Gonçalves, M. Zuzarte et al., "Unveiling the antifungal potential of two Iberian thyme essential oils: effect on *C. albicans* germ tube and preformed biofilms," *Frontiers in Pharmacology*, vol. 10, article 446, 2019.