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Selected essential oil components as promising topical agents against *Candida* species: *in vitro* antifungal, antioxidant, and anti-inflammatory activities

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ABSTRACT

Candida species have emerged as a global threat due to their rapid worldwide spread and multidrug-resistant properties. Therefore, novel and more effective antifungal agents, including natural-based preparations, are being actively sought. Essential oil (EO) components, including α-bisabolol, carvacrol, eugenol, linalool, menthol, and thymol, were evaluated *in vitro* for their antifungal, antioxidant, and anti-inflammatory activities. The aim of this study was to select EO compounds demonstrating the highest anticandidal activity together with promising antioxidant and anti-inflammatory properties for potential use as active ingredients in topical preparations. The activity of the tested EO compounds against five reference yeast strains belonging to *Candida* spp. was assessed *in vitro* using the broth microdilution method according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI). All EO compounds demonstrated potential anticandidal activity, with minimum inhibitory concentrations (MICs) ranging from 0.25 to 4 mg/mL, and exhibited fungicidal properties (MFC/MIC = 1–4). The highest activity was observed for α-bisabolol (MIC = 0.25–1 mg/mL), followed by carvacrol (MIC = 0.25–2 mg/mL), thymol (MIC = 0.5–2 mg/mL), and eugenol (MIC = 0.5–2 mg/mL). Eugenol exhibited the strongest antioxidant properties, even at a concentration of 0.1 mg/mL, as demonstrated by the DPPH assay. Moreover, this compound showed the highest anti-inflammatory activity, reflected by cyclooxygenase-2 (COX-2) inhibition of approximately 45% at a concentration of 0.5 mg/mL. Based on the obtained results, eugenol appears to be the most promising candidate for the development of topical preparations intended for the treatment of superficial infections caused by *Candida* spp.

INTRODUCTION

Superficial candidiasis is an opportunistic infection that commonly affects the mucous membranes of the oral cavity and vagina, as well as the skin [1,2]. Oral and oropharyngeal candidiasis, in particular, currently represent a significant challenge in clinical practice due to the frequent colonization of the oral cavity by *Candida* spp. The primary yeast species associated with oral and oropharyngeal candidiasis is *Candida albicans*, which accounts for approximately 70% of cases in healthy individuals as well as in populations presenting various predisposing factors, such as impaired salivary gland function, use of inhaled corticosteroids or broad-spectrum antibiotics, denture wearing, a high-carbohydrate diet, smoking, chronic diseases (e.g., diabetes mellitus), and immunosuppressive conditions [3].

These factors may lead to microbiota imbalance, excessive yeast proliferation, and morphological transition from the blastospore form to pseudohyphal or hyphal forms, ultimately resulting in the development of candidiasis [1,4]. Moreover, infections caused by *Candida* spp., including *C. albicans*, may promote the expression of proinflammatory cytokines [5].

Candida albicans remains the most common etiological agent of superficial candidiasis. However, a significant increase in infections caused by non-*albicans Candida* species has recently been observed, particularly *Candida*

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glabrata (*Nakaseomyces glabratus*), *Candida krusei* (*Pichia kudriavzevii*), and *C. tropicalis* [4]. Treatment of superficial candidiasis involves topical and/or systemic administration of antifungal agents, mainly belonging to the azole group (e.g., clotrimazole, fluconazole, itraconazole, ketoconazole) and polyenes (e.g., nystatin) [6]. Excessive use of antifungal drugs may contribute to the increasing resistance of *Candida* spp. Furthermore, *Candida auris*, a recently identified yeast species, is currently regarded as an emerging multidrug-resistant pathogen worldwide [7]. Therefore, novel and more effective antifungal agents, including natural-based preparations, are being actively explored.

Among the most extensively studied natural remedies are essential oils (EOs) and their individual components, owing to their multidirectional biological and pharmacological activities [6,8,9]. Previous studies have demonstrated the antifungal potential of various essential oils, including those derived from thyme, oregano, peppermint, lavender, rosemary, and eucalyptus, particularly against *Candida*, *Fusarium*, *Aspergillus*, and *Penicillium* spp. [10].

In the present study, the antifungal activity of six essential oil constituents, carvacrol, thymol, linalool, menthol, α-bisabolol, and eugenol was evaluated against *Candida* species. Thymol and carvacrol are monoterpene phenols derived from *Thymus vulgaris* and *Origanum vulgare*, respectively, and are known for their strong antifungal activity [11,12]. Linalool and menthol are monoterpene alcohols present in plants such as basil, rosemary, and peppermint, with documented antimicrobial and anti-inflammatory properties [13,14]. α-Bisabolol is a sesquiterpene alcohol found in plants such as *Arnica longifolia*, valued for its anti-inflammatory and antimicrobial activities [15]. Eugenol, a phenylpropanoid compound present in clove and cinnamon oils, exhibits broad biological activity, including antifungal, analgesic, and anti-inflammatory effects [16].

AIM

The aim of this study was to identify essential oil (EO) compounds with the highest activity against *Candida* spp., as well as promising antioxidant and anti-inflammatory properties, for potential use as active ingredients in topical pharmaceutical or cosmeceutical preparations. The investigated compounds included monoterpenes (carvacrol, thymol, linalool, and menthol), a sesquiterpene (α-bisabolol), and a phenylpropanoid (eugenol).

MATERIALS AND METHODS

Materials

Reference yeast strains obtained from the American Type Culture Collection (ATCC) were used: *Candida albicans* ATCC 10231, *Candida albicans* ATCC 2091, *Candida parapsilosis* ATCC 22019, *Candida glabrata* ATCC 90030, and *Candida krusei* ATCC 14243. Stock cultures were maintained in liquid Sabouraud medium supplemented with glycerol at −70°C.

Prior to experimentation, fungal strains were revived by culturing on Sabouraud agar (BioMaxima S.A., Lublin, Poland) at 35°C for 24 h. Fresh overnight yeast cultures were subsequently prepared at 35°C before each experiment.

The investigated EO compounds, carvacrol, thymol, linalool, menthol, α-bisabolol, and eugenol were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

Methods

In vitro antifungal activity assay

The antifungal activity of the tested EO compounds was evaluated *in vitro* using the broth microdilution method. The experiments were performed in accordance with the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [17] and the Clinical and Laboratory Standards Institute (CLSI) [11].

Yeast strains were initially subcultured on Sabouraud agar (BioMaxima S.A., Lublin, Poland) at 35°C for 18-24 h. Yeast suspensions prepared in sterile 0.85% NaCl solution were adjusted to a turbidity equivalent to the 0.5 McFarland standard (5×10^6 CFU/mL; CFU – colony-forming units per milliliter).

Stock solutions of EO compounds were prepared in dimethyl sulfoxide (DMSO) to obtain a final concentration of 200 mg/mL.

Minimum inhibitory concentration (MIC) values were determined using 96-well polystyrene microplates. Two-fold serial dilutions of the tested compounds were prepared in Mueller-Hinton (MH) broth supplemented with 2% glucose (BioMaxima S.A., Lublin, Poland). Final concentrations of EO compounds ranged from 16 to 0.008 mg/mL.

The final yeast inoculum in each well was adjusted to 5×10^4 CFU/mL. After incubation at 35°C for 18-24 h, MIC values were determined using a BioTek spectrophotometer (Biokom, Janki, Poland) at a wavelength of 570 nm. The MIC was defined as the lowest concentration of the tested compound that completely inhibited visible yeast growth.

Growth inhibition was evaluated relative to control cultures grown in medium without the tested compounds. The antifungal agent nystatin (Sigma-Aldrich Chemicals, St. Louis, MO, USA) was used as a reference compound. In addition, DMSO control and sterility control samples were included in the assay.

To determine minimum fungicidal concentration (MFC) values, aliquots from wells used for MIC determination were plated onto Sabouraud agar. Following incubation, the lowest compound concentration completely inhibiting yeast growth was recorded as the MFC. All experiments were performed in triplicate [17,18].

The MFC/MIC ratio was calculated to distinguish between fungicidal (MFC/MIC ≤ 4) and fungistatic (MFC/MIC > 4) effects of the tested EO compounds [17].

Antioxidant activity

The antioxidant potential of EO compounds was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay as previously described [19]. Standards of EO compounds were mixed with 180 µL of DPPH solution (Sigma-Aldrich Chemicals, St. Louis, MO, USA) prepared in methanol (78 µg/mL).

The 96-well microplates were incubated in the dark at room temperature for 30 min. Subsequently, absorbance was measured at a wavelength of 515 nm using a BioTek ELx808 microplate reader (BioTek, USA). The positive control consisted of DPPH solution supplemented with methanol instead of the tested compounds.

Free radical scavenging activity was expressed as percentage inhibition according to the following equation:

$$\text{Inhibition (\%)} = (A_{control} - A_{sample})/A_{control} \times 100$$

All experiments were performed in triplicate.

Cyclooxygenase-2 (COX-2) inhibition assay

Cyclooxygenase-2 (COX-2) inhibitory activity of the tested compounds was evaluated using a COX Colorimetric Inhibitor Screening Assay Kit (Cayman Chemical, MI, USA). In this assay, arachidonic acid serves as a substrate for the peroxidase component of cyclooxygenase, and the formation of oxidized *N,N,N',N'-tetramethyl-p-phenylenediamine* (TMPD) is measured colorimetrically.

Dimethyl sulfoxide (DMSO) was used as the solvent, as it does not affect COX-2 activity. Three concentrations of each compound were tested: 0.5 mg/mL, 1 mg/mL, and 2 mg/mL. Each concentration, as well as negative (background) and positive (100% initial activity) controls, was analyzed in triplicate.

Absorbance of the colorimetric reaction was measured at a wavelength of 590 nm, and the percentage inhibition of COX-2 activity was subsequently calculated.

RESULTS

The *in vitro* antifungal activity of EO components, namely α-bisabolol, carvacrol, eugenol, linalool, menthol, and thymol, was evaluated using the quantitative broth microdilution method recommended by EUCAST [17], allowing determination of minimum inhibitory concentration (MIC) values.

As shown in Table 1, all tested EO compounds demonstrated variable antifungal activity against *Candida* spp., ranked from the highest to the lowest activity as follows: α-bisabolol (MIC = 0.25-1 mg/mL) > carvacrol (MIC = 0.25-2 mg/mL) > thymol (MIC = 0.5-2 mg/mL) = eugenol (MIC = 0.5-2 mg/mL) > menthol (MIC = 2-4 mg/mL) = linalool (MIC = 2-4 mg/mL).

Species-dependent antifungal susceptibility to EO components was observed as follows: *C. parapsilosis* ATCC 22019 (MIC = 0.25-2 mg/mL), *C. albicans* ATCC 2091 (MIC = 0.25-4 mg/mL), *C. glabrata* ATCC 90030 (MIC = 0.5-4 mg/mL), *C. krusei* ATCC 14243 (MIC = 0.5-4 mg/mL), and *C. albicans* ATCC 10231 (MIC = 1-4 mg/mL).

Minimum fungicidal concentration (MFC) values were also determined for all tested EO compounds. Both MIC and MFC parameters were used to calculate the MFC/MIC ratio in order to distinguish between fungicidal and fungistatic activity (Table 2). All EO components exhibited fungicidal activity, as evidenced by MFC/MIC ratios ≤ 4 (range: 1-4).

Table 1. Antifungal activity of studied essential oils (EOs) components against reference strains of Candida spp. based on estimation of minimum inhibitory concentration (MIC)

Candida spp. strains	MIC (mg/mL)						MIC (μg/mL)
	α-bisabolol	carvacrol	eugenol	linalool	menthol	thymol	nystatin
C. albicans ATCC 10231	1	1	2	4	4	1	0.48
C. albicans ATCC 2091	1	0.25	1	4	4	0.5	0.24
C. parapsilosis ATCC 22019	0.25	1	1	2	2	0.5	0.24
C. glabrata ATCC 90030	0.5	1	2	4	4	1	0.24
C. krusei ATCC 14243	0.5	2	0.5	4	4	2	0.24

Table 2. Fungicidal activity of studied essential oils (EOs) components against reference strains of *Candida* spp. based on estimation of minimum fungicidal concentration (MFC) and of minimum fungicidal concentration/minimum inhibitory concentration (MFC/MIC) ratio

Candida spp. strains	MFC (mg/mL)/(MFC/MIC)					
	α-bisabolol	carvacrol	eugenol	linalool	menthol	thymol
C. albicans ATCC 10231	4/4	2/2	4/2	8/2	8/2	2/2
C. albicans ATCC 2091	4/4	1/2	2/2	4/1	4/1	2/4
C. parapsilosis ATCC 22019	1/4	2/2	2/2	4/2	2/1	2/4
C. glabrata ATCC 90030	2/4	2/2	2/1	8/2	8/2	2/2
C. krusei ATCC 14243	2/4	4/2	2/4	8/2	8/2	2/1

Subsequently, antioxidant activity was evaluated for EO components demonstrating the highest antifungal activity against reference *Candida* spp. strains, namely α-bisabolol, carvacrol, thymol, and eugenol. As presented in Figure 1, eugenol exhibited the strongest antioxidant activity at all tested concentrations.

Thymol and carvacrol showed lower antioxidant activity at concentrations of 0.1 mg/mL and 1 mg/mL compared with eugenol at corresponding concentrations. Their antioxidant activity was comparable to that of eugenol only at the highest tested concentration (10 mg/mL). The lowest antioxidant activity was observed for α-bisabolol at all tested concentrations; therefore, this compound was excluded from further analyses.

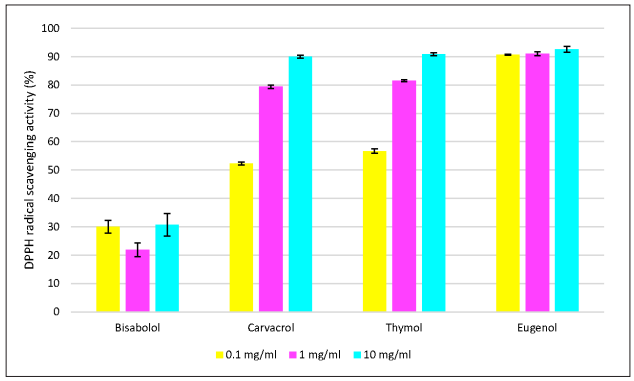

Figure 1. Antioxidant activity of tested essential oil (EO) components determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.

The next stage of the study involved *in vitro* evaluation of the anti-inflammatory activity of three EO components, thymol, carvacrol, and eugenol, using the COX-2 inhibition assay. As shown in Figure 2, eugenol exhibited the highest anti-inflammatory activity, as demonstrated by COX-2 inhibition ranging from 41% to 46.5% across all tested concentrations.

The remaining EO compounds demonstrated markedly lower anti-inflammatory activity, with COX-2 inhibition ranging from 25.6% to 29.5% for carvacrol and from 21.5% to 22.2% for thymol. No significant differences in anti-inflammatory activity were observed among the tested concentrations of EO compounds.

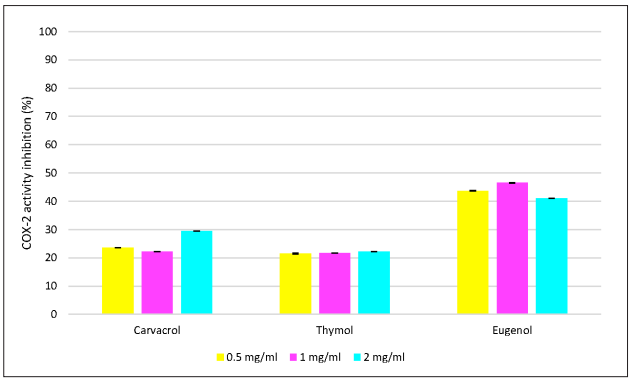

Figure 2. Anti-inflammatory activity of tested essential oil (EO) components determined using the cyclooxygenase-2 (COX-2) inhibition assay

DISCUSSION

Natural bioactive products and their applications in the pharmaceutical and cosmetic industries have attracted considerable interest in recent years. Numerous plants, their secondary metabolites, and essential oils are increasingly recognized as valuable sources of compounds exhibiting multidirectional biological and pharmacological activities that may contribute to the development of novel medicines or cosmeceutical products [20-22].

According to available literature, several essential oils and their individual components have demonstrated potential effectiveness against superficial infections caused by *Candida* spp., as evidenced by both *in vitro* and *in vivo* studies [23-25]. Recently, El-Shiekh et al. investigated plumieride, an iridoid isolated from *Plumeria obtusa* L. leaves, in a murine model of *C. albicans*-induced dermatitis. Under *in vivo* conditions, plumieride exhibited antifungal activity superior to that of fluconazole. Moreover, this compound was shown to interfere with the expression of *C. albicans* genes encoding virulence factors and to modulate the skin inflammatory response [26].

Although all EO compounds investigated in the present study (α-bisabolol, carvacrol, eugenol, linalool, menthol, and thymol) have previously been reported to possess antifungal activity [9,27-30], α-bisabolol, carvacrol, eugenol, and thymol demonstrated the highest activity against the tested reference *Candida* spp. strains. Clove oil and its main constituent, eugenol, have been reported to exhibit strong antioxidant properties [30], which is consistent with the findings of the present study.

Several EO components, including carvacrol, thymol, and eugenol, have also been shown to possess immunomodulatory properties [31-33]. Among the compounds evaluated in this study, eugenol demonstrated the most pronounced anti-inflammatory activity, as evidenced by COX-2 inhibition. Previous studies have shown that eugenol inhibits not only cyclooxygenase-2 (COX-2) but also 5-lipoxygenase (5-LOX), another enzyme involved in inflammatory processes [34,35]. Molecular modeling studies have further demonstrated interactions between the eugenol molecule and amino acid residues located within the active sites of both enzymes [34].

Considering the combined antifungal, antioxidant, and anti-inflammatory activities observed in the present study, eugenol may be regarded as a promising multifunctional antifungal agent. These findings are consistent with previously published data [30]. Importantly, eugenol has also been reported to act synergistically with conventional antifungal agents [36,37], which may be beneficial in the treatment of infections caused by drug-resistant *Candida* strains.

Furthermore, the World Health Organization (WHO) has classified eugenol as generally recognized as safe (GRAS), with a dose of 2.5 mg/kg body weight considered safe [30]. Eugenol is commonly present in essential oils derived from plants belonging to the Lamiaceae, Lauraceae, Myrtaceae, and Myristicaceae families. However, its primary natural source is clove essential oil obtained from *Syzygium aromaticum* (L.) Merr. & L.M. Perry (Myrtaceae). According to the European Union herbal monograph, this essential oil is recommended by the Committee for Herbal Medicinal Products for the relief of inflammation within the oral cavity and throat, as well as toothache, based on traditional medicinal use for at least 30 years, including a minimum of 15 years within the European Union [38].

Currently, eugenol is widely used in the food and cosmetic industries as a flavoring and fragrance ingredient. It has also been applied as a topical antiseptic and as a component of zinc oxide-based dental formulations used for root canal sealing and pain management [39]. Nevertheless, further studies are required to develop novel topical formulations containing eugenol and to evaluate their efficacy and safety in the treatment of localized infections such as superficial candidiasis [40,41].

Several limitations of the present study should be acknowledged. One limitation is the relatively small number of yeast isolates tested, which may limit the generalizability of the findings. Additionally, the study was conducted exclusively using reference strains obtained from ATCC which, although standardized and widely used in research, may not fully reflect the genetic and phenotypic diversity of clinical isolates encountered in clinical practice.

Therefore, future studies assessing the medical applicability of EO compounds should include clinical yeast strains isolated from patients with confirmed candidiasis. Such an approach would enable a more comprehensive evaluation of antifungal efficacy and facilitate identification of potential strain-specific differences in susceptibility to natural agents.

CONCLUSION

The data presented in this study suggest that, among the EO compounds evaluated, eugenol represents the most promising candidate for the development of topical anti-*Candida* preparations, owing to its potent antifungal, antioxidant, and anti-inflammatory properties. These findings, together with evidence from the literature, provide a basis for further *in vitro* investigations, as well as for the development of optimized formulations of eugenol, either alone or in combination with other antifungal agents, for topical use, including validation of its efficacy under *in vivo* conditions.

ABBREVIATIONS

ATCC – American Type Culture Collection
CFU – Colony Forming Units/mL
CLSI – Clinical and Laboratory Standards Institute
COX-2 – cyclooxygenase-2
DMSO – dimethyl sulfoxide
DPPH – 2,2-diphenyl-1-picrylhydrazyl radical
EOs – essential oils
EUCAST – European Committee on Antimicrobial Susceptibility Testing
GRAS – generally recognized as safe
5-LOX – 5-lipoxygenase
MFC – minimal fungicidal concentration
MH – Mueller-Hinton
MIC – minimal inhibitory concentration
TMPD – N,N,N',N'-tetramethyl-p-phenylenediamine

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