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Antifungal and Antibiofilm Activities of Some Essential Oils Against *Candida* spp

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ABSTRACT

Candida species are commonly encountered strains associated with a wide range of infections. Unlike bacterial pathogens, fungal pathogens treatment is difficult and the development of resistance has been increasing at an alarming rate. In this study, the antifungal and antibiofilm effect of thyme oil, rosemary oil, mint oil, citronella oil, was tested on *Candida albicans, Candida tropicalis, Candida kefyr, Candida glabrata, Candida parapsilosis* isolated from clinical samples. The agar disc diffusion method was employed to determine the antifungal effect of the essential oils, and the inhibition of biofilm formation was assessed using microtiter biofilm inhibition assay. The results indicated that all the essential oils inhibited *Candida* strains and their biofilm in varying degrees. The highest antifungal activity in all isolates was observed in the thyme oil (>50mm), while rosemary oil showed the highest antibiofilm effect (>77%) in all tested strains. These findings led us to assume that the active components found in essential oils might be potential antifungal agents, adding to the repertoire of therapeutic options for the treatment of candidiasis.

Keywords: Antibiofilm, Antifungal, Citronella oil, Mint oil, Thyme oil.

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Introduction

Candida species are fungi that exist as commensals in human and animal mucous membranes. *Candida* spp has long been a leading cause of hospital-acquired infections. Human fungal infections have risen in recent years [1].

Candida albicans is the most commonly isolated in human candidiasis [2]. *Candida* spp. produces dangerous opportunistic infections, particularly in immunocompromised patients (HIV infections), because the immune system normally suppresses the spread of *Candida* [3].

Antifungal drugs are used to treat infections that are caused by fungi. They include the azoles group such as: clotrimazole, fluconazole, itraconazole, ketoconazole and polyenes which include: amphotericin B and nystatin [4].

Azoles have a fungistatic effect that inhibits the growth of fungal cell [5]. However, they are often related to excessive toxicity to the host tissues and drug resistance hence fungal infection becomes a serious clinical problem. Infections caused by Candida biofilm is particularly difficult to eradicate because the biofilm is more resistant to antifungal agents than planktonic cells [6]. Biofilm formation is considered to be important virulence factor as it protects microbes against adverse environmental factors, antibiotics, reduces the effectiveness of host immune mechanisms, and facilitates the acquisition of nutrients [7]. Some species of the *Candida* genus cause infections related to biofilm formation and it associated with higher patients mortality, probably correlated with the poor permeability of the matrix to the antifungal drugs [8].

Plants and their derivatives have been used previously in traditional medicine in the treatment of many diseases. Essential oils (EO) are naturally formed by the aromatic plant as a secondary metabolite. They are volatile, composed of a complex mixture of organic compounds, and characterized by a strong fragrance [9]. Essential oils are known for their activities against bacteria, fungi, viruses, and cancers. They are employed as analgesics, anti-inflammatory medications, spasmolytics, and local anesthetics [10].

The literature provides a lot of information regarding the effect of essential oils on the growth of *C. albicans* but, a little data on their impacts on biofilm formation and on the other *Candida* species. In this study, we tested the antimicrobial and antibiofilm effect of six essential oils obtained commercially, against ten strains of *Candida* clinical isolates and a standard strain.

Materials and Methods

Essential Oils

Essential oils are used in traditional and alternative medicine methods with the relevant literature review selected from the oils used and obtained commercially. The oils included; thyme oil (*Thymus vulgaris*), rosemary oil (*Salvia rosmarinus*), mint oil (*Mentha piperita*), citronella oil (*Cymbopogon nardus*).

Microorganisms

A total of Ten fungal strains of Candida cultures were prospectively obtained from the Clinical Microbiology

Laboratory of Sivas Cumhuriyet University Practice and Research Hospital in Sivas, Turkey. clinical isolates of Candida albicans, Candida tropicalis, Candida kefyr, Candida glabrata, Candida parapsilosis, and a standard strain Candida albicans ATCC 10231 were used for quality control of the study. Yeasts were cultured from clinical samples that included urine, sputum, vaginal swabs, blood, oral swabs and abscesses. Those samples were refrigerated until further processing.

Inoculum Preparation

The yeast to be used in the study, were reproduced by incubating overnight at 37C° in yeast peptone agar. Yeast suspension 0.5 McFarland (1x10^6 CFU/ml) to be at McFarland turbidity standard has been prepared. Inoculum suspensions of all Mueller Hinton Agar (MHA) media homogeneously spread on the surface.

Sensitivity Test Of The Essential Oils

The agar disc diffusion method was used to determine the sensitivity to the essential oils. The disc (6 mm in diameter) was placed on inoculated agar. Discs with this diameter can absorb 10-15 µl of liquid [11].

As the negative control, DMSO, which is the solvent of the EO, was used. Incubation of the inoculated plates was performed at 37 ºC. The inhibition zone was measured to assess the antimicrobial activity against the Candida spp. All the tests were conducted three times. All plates were incubated at 37ºC for 18-24 hours [12].

Microtiter Biofilm Inhibition Assay

The inhibition of biofilm formation was assessed using microtiter biofilm inhibition assay. Briefly, 100 µL of *Candida* strains 0.5 McFarland (1x106 CFU/ ml) were inoculated into 96-well microtiter plate wells and 100 µL of Essential oil (EO) were added into all the wells. The final volume was 200 µL per well on the microtiter plate. Since the densities of essential oils vary, the oils were weighed on precision scales to determine the volume. Stock solutions are prepared by dissolving essential oils in DMSO at 50 % and 25 %. The final DMSO concentration in the microplate wells was set at 1%. Incubation of the microplates was conducted at 37 C° for 48 h. The positive control was the suspension without EO. Following the 48 h incubation, the suspension was discarded, left to dry, then washes three times by phosphate buffer saline (PBS). A volume of 200 µL of 0.1% crystal violet was added of the 96-well. Incubated at room temperature for 30 min. Then, crystal violet was discarded and wells were washed with PBS three times. The plate was left to dry at room temperature for 30 min. Then, 95% ethanol was used to solubilize the crystal violet and the absorbance was read through a microplate reader (BMG LABTECK, Offenburg, Germany) at 550 nm. All experiments were performed in triplicate [11]. Percent inhibition of biofilm was calculated using the equation described by Onsare and Arora [13].

% Inhibition = 100 -((OD 570 sample)/(OD 570 control) X 100)

Results

In this study, the anti-candidal activity of the four essential oils was investigated against ten clinical isolates of Candida spp.

Antifungal Activity Assays

The in vitro results of anti-candidal activity of the essential oils by the disc diffusion method are shown in Table 1. The different levels of oil activity were observed in the inhibition zone. The essential oils inhibited the growth of Candida strains, producing a zone diameter of inhibition from 8.0 mm to ≥ 50 mm based on the susceptibility of the Candida species. As the highest anti-candidal activity of the essential oils were observed in the thyme oil.

Table 1. Antifungal activity results of essential oils (Inhibition zone diameters)

Strains	Essential oils			
	Thyme Oil	***Rosemary Oil***	***Mint Oil***	***Citronella***
C.tropicalis (1)	≥ 50 mm	12 mm	17 mm	25 mm
C.tropicalis (2)	≥ 50 mm	19 mm	14 mm	34 mm
C.parapsilosis (1)	≥ 50 mm	24 mm	14 mm	32 mm
C.parapsilosis (2)	≥ 50 mm	27 mm	16 mm	20 mm
C. albicans (1)	≥ 50 mm	≥ 50 mm	≥ 50 mm	29 mm
C. albicans (2)	≥ 50 mm	≥ 50 mm	≥ 50 mm	38 mm
C. kefyr	≥ 50 mm	≥ 50 mm	≥ 50 mm	21 mm
C.glabrata (1)	≥ 50 mm	≥ 50 mm	≥ 50 mm	21 mm
C.glabrata (2)	≥ 50 mm	≥ 50 mm	≥ 50 mm	32 mm
C. albicans ATCC 10231	≥ 50 mm	≥ 50 mm	≥ 50 mm	33 mm

The potential of the oils to prevent biofilm cell formation was investigated through the biofilm inhibition assay. The most effective oil was Rosemary oil. Which was observed to reduce the biofilm of all the strains under study. The biofilm of C.parapsilosis showed the highest reduction value, while C.glabrata had the least one. Mint oil had a significant reduction as well. The reduction activity was highest in the isolates of C.tropicalis. Similarly to rosemary oil, the lowest reduction value was seen in C.glabrata isolates. Furthermore, citronella oil was mostly effective against C.parapsilosis and least effective against the strains of C.tropicalis. Thyme Oil was found to be the weakest oil in the terms of biofilm reduction. It had activity on C.tropicalis and C.parapsilosis. However, It showed a little to no effect against C.glabrata and C. albicans.

The results are shown in the following table.

Table 2. biofilm inhibition assay results of essential oils (%)

Strains	Essential oils							
	Thyme Oil		*Rosemary oil*		*Mint oil*		*Citronella*	
	Reduction in biofilm formation (%)		Reduction in biofilm formation (%)		Reduction in biofilm formation (%)		Reduction in biofilm formation (%)	
	1/2	1/4	1/2	1/4	1/2	1/4	1/2	1/4
C.tropicalis (1)	14±0.30	18±0.013	78±0.31	76±0.31	61±0.27	57±0.29	28±0.40	18±0.47
C.tropicalis (2)	52±0.13	62±0.24	85±0.16	78±0.41	70±0.10	78±0.36	64±0.39	61±0.39
C.parapsilosis (1)	47±0.22	49±0.17	86±0.21	84±0.40	54±0.32	67±0.37	54±0.42	58±0.40
C.parapsilosis (2)	6±0.27	13±0.07	83±0.11	77±0.38	56±0.12	66±0.46	32±0.45	30±0.50
C. albicans (1)	2±0.13	13±0.22	60±0.9	80±0.33	37±0.13	60±0.46	37±0.44	31±0.46
C. albicans (2)	21±0.13	27±0.08	65±0.44	88±0.35	38±0.43	63±0.43	25±0	15±0.59
C. kefyr	11±0.08	9±0.17	52±o.50	77±0.35	60±0.41	30±0.37	16±0.6	39±0.53
C.glabrata (1)	9±0.10	NE	59±0.30	77±0.28	54±.18	30±0.53	35±0.45	14±0.59
C.glabrata (2)	5±0.18	80±0.04	52±0.42	78±0.27	35±0.33	35±0.30	23±0.53	42±0.47
C. albicans ATCC 10231	2±0.17	NE	34±0.09	78±0.30	58±0.21	58±0.53	40±0.35	39±0.37

NE: No effect

Discussion

Plants as sources of antimicrobial compounds have sparked increased interest in recent years. The plant-derived antimicrobial agents have been reported to be safe and without side effects and antimicrobial properties of plant volatile oils have been recognized [14].

Previous studies have indeed demonstrated the antimicrobial activity of essential oils, mainly focusing on *C. albicans* [15]. However, in this study, we tested the antifungal activities of the EO against 5 clinical strains including *C. albicans*. The observation in this study is consistent with previous reports that EOs from thyme, rosemary, mint oil, citronella possess anti-candidal activity and exhibit anti-biofilm action as well [16] [17] [18].

Candida biofilm structure is particularly difficult to eradicate since biofilm is much more resistant to antifungal agents than planktonic cells. In this context, a more effective strategy seems to assess the prevention of biofilm formation than its eradication plus the direct effect on the planktonic cells.

Mint essential oil is one of the most well-known and extensively utilized essential oils. *Mentha piperita*, (family Lamiaceae) is a species found in many parts of the world which has a financial worth because of its flavoring, odor, and medicinal qualities in foods and cosmetic industrial products. In addition, the leaves and flowers of *M. piperita* have medicinal properties [19]. Several studies have shown that mint oil extracted from *M. piperita* exhibited strong inhibitory activity against many organisms [20]. In our study, the Mint oil exhibited antifungal and antibiofilm activities against both the standard and clinical strains of Candida species, with inhibition zone diameters ranging from 17 mm to more than 50 mm. Bona and his colleagues observed similar results [21]. The most sensitive strains to Mint oil (with inhibition zone ≥ 50 mm) were: *C. albicans, C. kefyr, and C. glabrata*. Mint oil showed a reduction in biofilm formation of the tested strains. The highest inhibitory effect was observed in *C. tropicalis* isolates (78%), while the lowest effect was observed in *C. glabrata* strains (35%). The antibiofilm effect of mint oil on Candida isolates growth reported in this study support those of previous studies [22,23]. In a study carried out by Samber and others, they investigated the synergistic anti-candidal activity and mode of action of Mentha piperita essential oil and its major components against *candida* spp and mode of action as well. According to their results, the mint EO and its lead compounds influence antifungal activity by reducing ergosterol levels, inhibiting PM-ATPase, and leading to intracellular acidification and cell death [24].

Citronella oil is mostly derived from *Cymbopogon nardus*. It has long been used in traditional practices by many ancient cultures. It's widely used in the perfume industry and soap manufacturing [25]. Citronella oil is well-known for its antibacterial properties. It has been recommended as home remedy for the treatment of oral and vaginal fungal infections by numerous books and articles and products containing these essential oils for the treatment of such infections are being used in many parts of the world [26]. The results of the present study have shown that the inhibitory effect of EO from citronella was less than that of thyme oil, rosemary oil, and Mint oils in planktonic and biofilm-forming isolates. The Inhibition zone diameters were between (20-38 mm). The least susceptible strains were *C. kefir* and *C. glabrata* (21 mm), while *C. albicans* strains were the highest susceptible to citronella. The antibiofilm inhibition of Citronella showed different values among the tested strains. The highest reduction was observed in C. tropicalis (64%) and *C. parapsilosis* (58%), while the lowest reduction was observed in *C. albicans* strains (15%) which is lower than the reduction that was achieved in a previous study [27]. The difference could be due to the use of standard instead of clinical strains. Furthermore, the geographical location from which the plants were collected can influence resulting outcome. When comparing antibiofilm inhibition of Citronella to other EOs used in this study, Citronella oil along with thyme oil, rosemary oil, and mint oil has a high reduction in biofilm formation of *C. tropicalis* and *C. parapsilosis* isolates. *C. albicans* isolates were less susceptible to Citronella than the others. Our result disagrees with that of a previously published work submitted by Trindade and his colleagues. They suggest that Citronella EO has a strong in vitro antifungal and antibiofilm activity on *C. albicans* [28].

Rosemary is a plant that originates in the Mediterranean region and belongs to the *Lamiaceae* family. It might, however, be found all over the planet. It is a perennial and aromatic plant. Rosemary can be used as a spice in cooking, as a natural preservative in the food industry, and as an ornamental and medicinal plant [29]. In our result, rosemary oil showed an inhibition zone in all tested clinical isolates and standard (12-50 mm). The highest effect of rosemary oil (≥50) was seen against *C. albicans, C. kefyr* and *C. glabrata*. Our results regarding *C. albicans* agree with that of previous work by Matsuzaki et al, which suggests that Rosemary EO has significant antifungal activity against *Candida albicans* [30].

Rosemary oil also inhibits biofilm formation in all tested clinical isolates and the standard isolate. Significant effect (>83%) is seen particularly against *C. tropicalis*, *C. parapsilosis* and *C. albicans*. The antifungal activity of rosemary essential oil in this study was similar to the known literature with a little difference, which could be for many reasons such as a different growing environment of Rosemary, and different extraction methods of essential oils [31].

Thyme essential oil is used as a flavor in a wide variety of foods, beverages, confectionery products, and perfumery to synthesize soaps and lotions. It possesses some antiseptic, bronchiolitis, antispasmodic, and antimicrobial properties [32]. It was documented that thyme EO has an inhibitory impact on Candida albicans virulence factors.

Alves et al suggested that Thyme EO is very effective in inhibiting *C. albicans* germ tube formation. Also, it was found to disrupt C. albicans biofilms [33].

Rajkowska and others demonstrated the role of the effect of thyme essential oils on candida cell distribution and biofilm formation. Their results showed a statistically significant reduction in biofilm formation [34].

In this present study, thyme oil showed a high effect (inhibition zone ≥ 50 mm) against all tested clinical isolates and the standard. However, in biofilm inhibition, thyme oil shows a significant effect (> 60%) in only *C. tropicalis* and *C. glabrata*. Antifungal activities of thyme oil found in this study are consistent with several reports where thyme oil shows a high effect on *Candida* species [35].

Taken together these results show that *thyme, rosemary, mint, and citronella* essential oils have anti-candidal and anti-biofilm potential.

Conclusion

In this study, the inhibitory effect of thyme oil, rosemary oil, mint oil, and citronella oil on *Candida* spp were tested. In conclusion, rosemary and mint oils were found to be the most effective. they can be used as effective anti-biofilm against *Candida* spp. However, if essential oils are to be used for medicinal purposes, molecular mechanisms, mode of action, stability, toxicity, and efficiency of active components derived from essential oils must be explored and assessed further. The results of this work support the research for new alternatives or complementary therapies against candidal infections.

Conflicts of interest

There are no conflicts of interest in this work.

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