

ORIGINAL ARTICLE

Essential Oils and their Components in Combating Fungal Pathogens of Human Skin-lipophilic Yeast *Malassezia furfur*

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Abstract: The most common problem of modern medicine is the development of increasing antifungal drug resistance nowadays including fungal infections of the skin and its appendages caused by dermatomycetes, yeast-like fungi, and molds so, there will be a need for alternative antifungal treatment. Natural substances with high antifungal properties like essential oils and their components show potential in this regard. Citrus lemon and Citrus sinensis essential oils were screened against *Malassezia furfur* by disc diffusion method and Minimum Inhibitory Concentration was determined by Modified Microdilution method. Citrus lemon (lemon) and Citrus sinensis (orange) show excellent inhibitory effects on the lipophilic, yeast-like fungus *Malassezia furfur* which causes Pityriasis versicolor, a chronic superficial fungal disease of the skin. The diameter of the inhibition zone was found to be 70 mm and 60 mm respectively. *Malassezia furfur* was found to be resistant against Clotrimazole & Ketoconazole. The minimum inhibitory concentration (MIC) of lemon and orange oil against *Malassezia furfur* was found to be 0.6 µl/ml and 0.8 µl/ml. These findings concluded that the use of Citrus lemon (lemon) and Citrus sinensis (orange) oil showed remarkable and excellent antifungal activity, and can be used as a traditional herbal medicine for the control of Pityriasis versicolor infection of the skin after undergoing clinical trials.

Keywords: Pityriasis versicolor, skin, *Malassezia furfur*, Citrus lemon and Citrus sinensis oils

INTRODUCTION

Pityriasis versicolor (PV) is a superficial mycosis, that affects the superficial layer of stratum corneum [1]. The causative organism is *Malassezia furfur*, a yeast-like lipophilic fungus. Previously the mycelial form was called either *Pityrosporum ovale* or *Pityrosporum orbiculare* [2]. In 1951, Gordon isolated the organism *M.furfur* and renamed it *P.orbiculare*. It was recognized that *M.furfur* is the correct name and that *P.orbiculare* and *P.ovale* are synonyms [3]. The disease is most prevalent in early adulthood and small children are rarely affected [4-5]. PV is common in the post-pubertal age where sebaceous glands are active and in individuals who sweat more [6]. The prevalence in colder climates is less than 1% [7]. *M. furfur* is a component of normal skin flora in more than 90% of adults living in tropical areas [8]. Infectious diseases account for a high proportion of health problems in developing countries including India. Microorganisms have developed

resistance to many antibiotics and as a result, immense clinical problem in the treatment of infectious diseases has been created [9]. The resistance of the organisms increased due to the indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases. This situation forced the researchers to search for new antimicrobial substances from various sources including medicinal plants [10-11]. A survey of the literature reveals that many essential oils possess antifungal activity. Therefore, we need to search for plant-derived antifungal drugs which are safe and without side effects. Herbal medicines may be in the form of powders, liquids, or mixtures, which may be raw or boiled, ointments, liniments, and incisions [12]. Essential oils have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases [13]. Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal, and antioxidant properties

[14-15]. Numerous essential oils have been tested for both in vitro as well as in vivo antifungal activity and some pose much potential as antifungal agents. Studies on the antimicrobial, especially antibacterial and antifungal activity of lemon grass oil and its components were reported [16-18]. Orange oil was composed principally of d-limonene, at 94%, and myrcene at about 3%. The essential oils of lemon and orange exhibited antifungal activity against common molds *Aspergillus niger*, *A.flavus*, *Penicillium chrysogenum*, and *P.verrucosum*. In the present study, the antifungal activities of lemon and orange oils were investigated to discover the medicinal potential of these oils for future application as an antifungal agent for Pityriasis versicolor disease.

MATERIALS AND METHODS

Extraction and Preparation of Oil

In the summer season, extraction of oil from the peels of lemon (*Citrus lemon*) and orange (*Citrus sinensis*) was carried out by standard hydrodistillation method, Clevenger's apparatus, and all operations were carried out at room temperature. The fresh peels of lemon and orange were washed to remove soil and sliced. Sliced pieces of fresh peels of lemon and orange (250 g) were placed in a separate flask, together with distilled water (1 L). After 5-6 hours, oil was collected from the apparatus, anhydrous with sodium sulfate for removal of water traces, then this 100% pure essential oil was dispensed into dark bottles and stored at 4°C until used. Essential oil was ready to use for disc diffusion and determination of MIC.

Screening of essential oil using Disc Diffusion method

Oil was screened for its antifungal activity against *M.furfur* by disc diffusion method [19]. Standard size Whatman No.1 filter paper discs, 6.0 mm in diameter, sterilized by dry heat at 140°C in an oven for one hour were used to determine antifungal activity. SDA medium for the disc diffusion test was prepared. After sterilization, it was poured into sterilized Petri plates and allowed to solidify. Then one day old, fresh culture of yeast will be used for inoculum preparation. A suspension that was just turbid (~0.5 McFarland standard) by visual inspection was prepared by suspending yeast in 0.9% NaCl solution and the homogeneous suspension was used for inoculation. Using a sterile cotton swab, yeast cultures were swabbed on the surface of sterile Sabourauds dextrose agar plates. Sterilized filter paper discs were soaked in neat, undiluted (100%) concentration of oils. Using ethanol-dipped and flamed forceps, oil-saturated discs of 100% concentration were aseptically placed over Sabourauds dextrose agar plates seeded with the respective test microorganism. The antibiotic discs of Clotrimazole and Ketoconazole (10 mcg/disc) were

also aseptically placed over the seeded Sabouraud's dextrose agar plates as a standard drug for comparison of antifungal activity of lemon and orange oils. The plates were incubated at 37°C for 24 hours. The diameter of the inhibition zones was measured in mm. Three replicates were kept in each case and average values were calculated. The activity of oils was measured by the following formula:

$$AI \text{ (Activity index)} = \frac{\text{Inhibition zone of sample}}{\text{Inhibition zone of standard}}$$

Determination of Minimum inhibitory concentration using Microdilution method

The modified microdilution method by Provine and Hadley (2000) was followed to determine MIC [20]. Media used for MIC was semisolid agar media (Brain Heart Infusion Agar) aliquots of semisolid agar media (Bacto Agar; Difco Laboratories) at a pH of approximately 7.4 were poured into a 16-by 125-mm glass tube and autoclaved. A suspension that was just turbid (~0.5 McFarland standard) by visual inspection was prepared by suspending the selected fungi in 0.9% NaCl solution, vortexing, and homogeneous suspension was used for inoculation. Different concentrations of lemon and orange oils were added in media containing test tubes, afterwards a standard platinum loopful (~0.001 ml, Himedia, Flexiloon) of the inoculum suspension was inserted deep into each tube of medium containing a different concentration of oils, as well as an oil-free control, by a centered down-up motion to form a two-dimensional inoculum. The tubes were then incubated at 30°C for 48 hours to determine the MIC. MIC was read to be the lowest concentration at which there was no visible growth of the organism. Then, by visual inspection, good growth of the respective fungi in an oil-free medium as a control was detected (48 hrs for yeasts). Afterward, the growth in all tubes at different concentrations of lemon and orange oil was compared with that of the oil-free control to determine inhibition.

RESULTS

In our present work, *M.furfur* was found main etiological agent of Pityriasis versicolor disease hence antimycotic studies were carried out on *M.furfur* (Figure 1). The conventional treatment of fungal disease is limited, and part of the reason is due to the limited spectrum of the current antifungal drugs, and the expensive treatment, particularly due to the need for prolonged therapy. Thus, new drugs and alternative therapies are necessary, including natural products. We report here the antimycotic study of lemon and orange oil against *M.furfur* in vitro (Figure 2). The results of the present work on the antifungal activity of lemon and orange oil against *M. furfur* studied by two methods i.e Disc diffusion and Microdilution

method are presented in Tables 1, 2, and 3.

Table 1: Antifungal activity of lemon and orange oil against *Malassezia furfur*

Essential Oil	Test Strain	IZ of Essential Oil
<i>C. limon</i>	<i>Molassezia furfur</i>	70 mm
<i>C. sinensis</i>	<i>Molassezia furfur</i>	60 mm

Table 2: MIC of *C. limon* oil against *Malassezia furfur*

Test Strain	Different concentrations of lemon Oil used in $\mu\text{l/ml}$	Growth visually inspected in different concentrations of oil
<i>Malassezia furfur</i>	0.1	+4
	0.2	+3
	0.3	+3
	0.4	+2
	0.5	+1
	0.6	0
	0.7	0
	0.8	0
	0.9	0
	1	0
	Control without oil	100% growth

Growth was scored in the following manner: 4+, growth comparable to that of the oil free control; 3+, growth approximately 75% that of the control; 2+, growth approximately 50% that of the control; 1+, growth 25% or less that of the control; and 0, no visible growth

Table 3: MIC of *C. sinensis* oil against *Malassezia furfur*

Test Strain	Different concentrations of lemon Oil used in $\mu\text{l/ml}$	Growth visually inspected in different concentrations of oil
<i>Malassezia furfur</i>	0.1	+4
	0.2	+3
	0.3	+2
	0.4	+2
	0.5	+1
	0.6	+1
	0.7	+1
	0.8	0
	0.9	0
	1	0
	Control without oil	100% growth

Growth was scored in the following manner: 4+, growth comparable to that of the oil free control; 3+, growth approximately 75% that of the control; 2+, growth approximately 50% that of the control; 1+, growth 25% or less that of the control; and 0, no visible growth

In our study, lemon and orange oils presented higher diameters of inhibition zones. The diameter of the inhibition zone obtained against lemon and orange oil at 100% concentration of pure oil was 70 mm and 60 mm respectively by disc diffusion method (Figure 3). According to our study by comparing with the reference drugs, lemon and orange oil was found to be more effective in inhibiting the growth of *M. furfur*. Clotrimazole and ketoconazole were found to be resistant to *Malassezia furfur* (Figure 4). Results of MIC of

lemon and orange oils against *M. furfur*. are summarized in Tables 2 and 3. The results show that the lemon and orange oil exhibited inhibitory action at 0.6 $\mu\text{l/ml}$ and 0.8 $\mu\text{l/ml}$ concentrations against *M. furfur*. Both oils can be used for the development of natural antifungal agents against Pityriasis versicolor infections.



Figure 1: *M. furfur* on SDA media with olive oil & Bottle shape yeast cells under microscopic examination

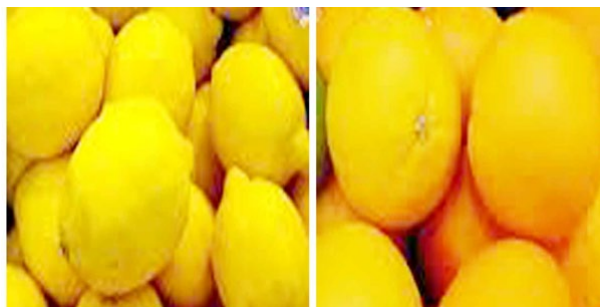


Figure 2: Fruit of Citrus lemon (lemon) & Citrus sinensis (sweet orange)

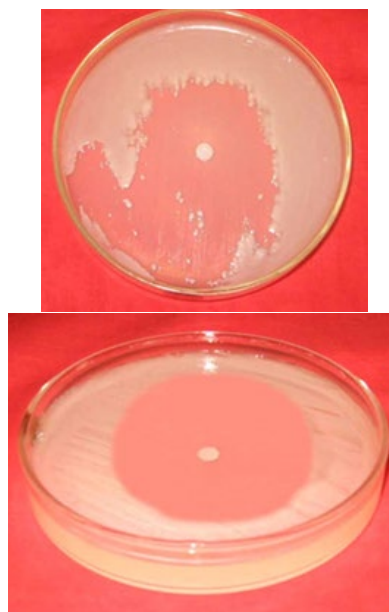


Figure 3: Antifungal activity of *C. limon* & *C. sinensis* against *Malassezia furfur*

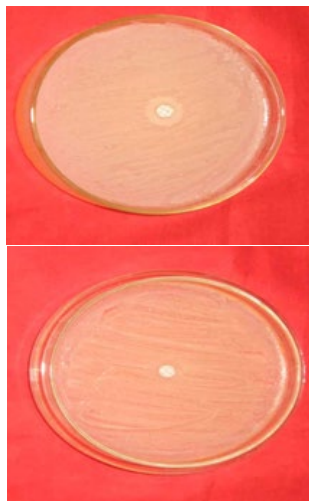


Figure 4: Resistance of Clotrimazole & Ketoconazole against *Malassezia furfur*

Their mechanism of action appears to be predominantly on the fungal cell membrane, disrupting its structure causing leakage and cell death; blocking the membrane synthesis; and inhibition the spore germination, fungal proliferation, and cellular respiration [22]. Because of the high volatility and lipophilicity of the essential oils, they are readily attached to penetrate the cell membrane to exert their biological effect [23]. The results showed the efficacy of lemon oil on animal skin infections caused by *Trichophyton rubrum* and *Microsporum gypseum*. In our study, lemon oil exhibited strong antimycotic activity against *M. furfur*. In the screening of lemon oil, the diameter of the inhibition zone by the disc-diffusion method was found to be 50 mm. MIC of oil obtained by microdilution method was 0.8 µl/ml. Our work is in agreement with the observations of Silvia et al in which screening of lemon grass oil against *Candida* species was studied and it was found as an effective antifungal agent [24]. Our work also coincides with the previous findings of Ezzat et al which revealed that the essential oil of *Citrus lemon* at 200 µl concentration showed a diameter of inhibition zone 37 mm against *Candida albicans* 10261 [25]. According to Rusenova and Parvano *Citrus lemon* does not show antimicrobial activity by disc-diffusion method against both yeasts, *Candida albicans* and *Malassezia pachydermatis* [26]. These results are not in agreement with our studies which showed that antifungal activity by disc-diffusion and minimum inhibitory concentration by microdilution method, lemon oil was found to be effective in inhibiting the growth of *M. furfur* at a low concentration of oil. Lemon oil is also preferentially more active against Gram-positive bacteria as compared to Gram-negative bacteria. The antimicrobial activity of lemon oil is due to the presence of terpenoids- α -pinene, camphene, β -pinene, sabinene, myrcene, terpinene, β -bisabolene, limonene, trans-

α -bergamotene, nerol and neral [27]. Essential oils from citrus offer the potential for all-natural antimicrobials for use in improving the safety of organic or all-natural foods [28]. Shahi et al in 2003 found that the MIC of oil of *Citrus sinensis* were in the range of 0.7-1 µl/ml against *Candida albicans*, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. ustus*, *Epidermophyton floccosum*, *Microporum audouinii*, *M. canis*, *M. gypseum*, *M. nanum*, *Rhizopus nigricans*, *Trichophyton tonsurans*, *T. rubrum*, *T. mentagrophytes* and *T. violaceum* [29]. The present investigation suggests that essential oils exhibit a narrow or wide range of antifungal activity, which may prove useful in the development of effective antifungal substances. Souza et al (2005) reported MIC of lemon oil at 0.5% concentration against mold strains i.e. *Fusarium* spp, *Penicillium* spp, *Aspergillus niger*, *A. flavus*, and *Rhizopus* spp [30]. In our findings, MIC of lemon oil against *M. furfur* was 0.8 µl/ml i.e. very close to that concentration. The small differences may be due to local environmental and climatic conditions and variations in the choice of microorganism tested.

Our results of orange (*Citrus sinensis*) and lemon (*Citrus lemon*) are also similar to the work of Viuda-Martos et al which suggests that both oils were effective in inhibiting the growth of *Aspergillus niger*, *A. flavus*, *Penicillium chrysogenum* and *P. verrucosum* [18]. The hydrodistilled oil of lemon and orange was also found to be more sensitive against *Penicillium digitatum* [31]. Antimicrobial activity of Rutaceae family species has been observed on dermatophytes [32] and on some molds [33]. Essential oils of some plants have recently been proven to be successful eco-friendly, bio-control agents [34]. Many authors have reported antimicrobial, antifungal, antioxidant, and radical-scavenging properties of essential oils [35-36].

DISCUSSION

The present results suggest that lemon and orange oils exhibit strong antifungal activity. This is in agreement with the findings of Valero and Frances who reported narrow and wide ranges of antifungal activities. Essential oils derived from many plants are known to possess antifungal activities [21].

CONCLUSIONS

The present study suggests that the oils extracted from the waste product (peels) of *C. sinensis* and *C. lemon* hold good promise as an antifungal agent, which could be used in therapeutic remedy against human pathogenic fungi on account of its various antifungal properties, viz. strong fungicidal action, long-shelf life, withstand heavy inoculum density, thermostable, broad range of antifungal activity and absence of any adverse effects. The waste product of *C.*

sinensis and C. lemon can be used for the development of a potential source of effective and economically viable herbal antifungal against Pityriasis versicolor (fungal skin infection) after undergoing successful clinical trials.

Conflicts of interest and sources of funding

The authors declare no conflict of interest. This research received no external funding.

Authors' contribution

RS contributed to design of experiments, review, discussion, and editing of the

manuscript. GS contributed to the review and writing of the manuscript. RS contributed to all editing of the manuscript. ER contributed to the review of the manuscript. MS contributed to the editing and review of the manuscript.

Ethics approval and consent to participate

This article does not contain any studies with human and animal participants performed by any of the authors,

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