

## ORIGINAL RESEARCH ARTICLE

## Essential Oils against Lipophilic Yeast like Fungus

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**ABSTRACT**

Potential inhibitory effect of *Citrus lemon* (lemon) and *Citrus sinensis* (orange) on lipophilic, yeast like fungus *Malassezia furfur* which causes Pityriasis versicolor, chronic superficial fungal disease of the skin have been studied by using two different methods: Disc diffusion and Microdilution methods. In screening of IT and orange oil by disc diffusion method, the diameter of inhibition zone was found to be 50 mm and 20 mm which were greater than inhibition zone of reference antibiotics i.e Gentamycin and Streptomycin of 16.5 mm and 17 mm respectively. Minimum inhibitory concentration (MIC) of lemon and orange oil against *Malassezia furfur* was found to be 0.8 $\mu$ l/ml and 2.2 $\mu$ l/ml. These findings support the use of *Citrus lemon* (lemon) and *Citrus sinensis* (orange) oil as a traditional herbal medicine for the control of Pityriasis versicolor infection of skin.

**Key words:** Pityriasis versicolor, skin, *Malassezia furfur*, *Citrus lemon* and *Citrus sinensis* oils.

**INTRODUCTION**

Pityriasis versicolor (Tinea versicolor) is a chronic, superficial fungal disease of the skin caused by the lipophilic, yeast like fungus *Malassezia*. This organism is a saprophytic yeast that is part of the normal skin flora. *Malassezia* (formerly known as Pityrosporum) is a genus of related fungi, classified as yeasts naturally found on the skin surfaces of many animals including human beings. Synonyms of this disease are Pityriasis versicolor, Tinea flava, Dermatomyces furfuracea and Tinea versicolor. *Malassezia furfur* is the causative agent of Pityriasis versicolor disease. Various environmental factors like high temperature, humidity and hyperhidrosis are the main factors responsible for the occurrence of this disease. Versicolor refers to the variety and changing shades of colors present in this disease. Affected areas include the back, chest, abdomen, neck, and upper limbs. The most common symptom of Pityriasis versicolor is light brown or white patches on the skin. These patches are most noticeable during summer season. Infectious diseases accounts for high proportion of health problems in the 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<sup>[10]</sup>. This situation forced the researchers to search for new antimicrobial

substance from various sources including medicinal plants<sup>[4]</sup>. There are alarming reports of opportunistic fungal infections<sup>[23]</sup>. In recent years, there has been a gradual revival of interest in the use of medicinal and aromatic plants in developed as well as in developing countries, because plant-derived drugs have been reported to be safe and without side-effects. Treatments contain high doses of antibiotic due to resistance and side effects of this antibiotic, patients take more time for cure. Therefore, we need to search plant derived antifungal drugs which are safe and without side-effects. The herbal medicines may be in form of powders, liquids, or mixtures, which may be raw, or boiled, ointments, liniments, and incisions<sup>[2]</sup>. In diseases of microbial origin, the plants function as a result of antimicrobial activity against the causative agents<sup>[3]</sup>. Down the ages essential oils and other extracts of plants have evoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases<sup>[24]</sup>. Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties<sup>[5]</sup>. Thus, new drugs and alternative therapies are necessary, including natural products. 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. Production of

essential oils by plants is believed to be predominantly a defence mechanism against microorganism and indeed, essential oils have been shown to possess antimicrobial and antifungal properties. Essential oils and their components are gaining increasing interest because of their relatively safe status and their wide acceptance by consumers. Lemon oil is characterized for monoterpenes compounds, and citral is the major component, present at levels of, approximately, 65-85%. Orange essence oil was composed principally of d-limonene, 94%, and myrcene at about 3%. Studies on the antimicrobial, especially antibacterial and antifungal, activity of lemon grass oil and its components were reported<sup>[15,17]</sup>. In the present study, antifungal activities of lemon and orange oils were investigated with the aim to discover the medicinal potential of these oils for future application as an anti-*Malassezia furfur* agent in Pityriasis versicolor disease.

### Materials and Methods

Oil of *Citrus lemon* (lemon) and *Citrus sinensis* (orange) were tested for their antifungal activity against test organism, lipophilic yeast like fungus, *Malassezia furfur*, the causal organism of Pityriasis versicolor infection. In summer season, extraction of oil from peels of lemon (*Citrus lemon*) and in winter season, from the peels of orange (*Citrus sinensis*) was carried out by standard hydrodistillation method. The antifungal activity of the lemon and orange oils against yeast was determined using the disc diffusion method<sup>[19]</sup>. 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 culture was swabbed on the surface of sterile Sabourauds Dextrose Agar plates. Filter paper discs of 6 mm diameter were prepared and sterilised. Using an 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 gentamycin (30mcg), streptomycin, clotrimazole and ketoconazole (10mcg/disc) were also aseptically placed over the seeded Sabourauds dextrose agar plates as a standard drugs for comparison of antifungal activity of lemon and orange oil. The plates were incubated at 37°C for 24 hours. The diameter of the

inhibition zones was measured in mm. The activity of oils was measured by the following formula.

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

The modified Microdilution method<sup>[18]</sup> was followed to determine MIC. In this method, 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 tubes, autoclaved ,different concentrations of lemon and orange oils were added in media containing test-tubes, afterwards a standard platinum loopful (~0.001 ml, Himedia, Flexiloop) of the inoculum suspension was inserted deep into each tube of medium containing a different concentration of oil, as well as a 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 yeast in oil-free medium as a control was detected (within 48 h for yeasts) afterwards, the growth in all tubes at different concentrations of lemon and orange oils was compared with that of the oil-free control in order to determine inhibition.

### RESULTS AND DISCUSSION

The results of the present work on the antifungal activity of lemon and orange oil against *Malassezia furfur* studied by two methods i.e disc diffusion and microdilution method are presented in (Table 1, 2 & 3). In our study lemon and orange oils presented higher diameter of inhibition zones than gentamycin, streptomycin, clotrimazole and asketoconazole. The diameter of the inhibition zone obtained against lemon and orange oil at 100% concentration of pure oil was 50 mm and 20 mm respectively by disc diffusion method. *Malassezia furfur* was found to be resistant against clotrimazole and ketoconazole. Other reference antibiotics i.e gentamycin and streptomycin showed inhibition zone of 16.5mm and 17mm respectively. 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 *Malassezia furfur*. Results of MIC of lemon and orange oils against *Malassezia furfur* are summarized in table 2 and table 3. The results show that the lemon and orange oil exhibited inhibitory action at 0.8 ul/ml and 2.2 ul/ml concentrations against *Malassezia*

*furfur*. Both the oils can be used for the development of natural antifungal agent against Pityriasis versicolor infections. The present results suggest that lemon and orange oils exhibits strong antifungal activity. This is in agreement to findings where the essential oils have been found to exhibit narrow as well as wide ranges of activity. Essential oils derived from many plants are known to possess antifungal activities<sup>[28]</sup>. Numerous essential oils have been tested for *in-vivo* and *in-vitro* antimycotic activity and some have been recognized to be potential antifungal agents. 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; inhibition of the spore germination, fungal proliferation and cellular respiration<sup>[12]</sup>. Because of high volatility and lipophilicity of the essential oils, they are readily attached to penetrate into the cell membrane to exert their biological effect<sup>[13]</sup>. The results showed the efficacy of lemon oil on the animal skin infections caused by *T. rubrum* and *M. gypseum*. In our study, lemon oil exhibited the strong antimycotic activity against the *Malassezia furfur*. In screening of lemon oil, the diameter of inhibition zone by disc-diffusion method was found to be 50 mm. MIC of oil obtained by microdilution method was 0.8µl/ml. Our work is in agreement with the observations<sup>[25]</sup>, in which screening of lemon grass oil against *Candida* species was studied and it was found as an effective antifungal agent. The diameter of inhibition zone against *Candida* species was found to be more than 40 mm. Our work also coincide with the previous findings of<sup>[11]</sup> which revealed that the essential oil of *Citrus lemon* at 200µl concentration showed diameter of inhibition zone 37 mm against *Candida albicans* 10261. *Citrus lemon* does not show antimicrobial activity by disc-diffusion method against yeasts, *Candida albicans* and *Malassezia pachydermatis*<sup>[20]</sup>. These results are not in agreement with our studies which showed that according to antifungal activity by disc-diffusion method and minimum inhibitory concentration by microdilution method, lemon oil were effective in inhibiting the growth of *Malassezia furfur* at a low concentration of oil. Our results of orange (*Citrus sinensis*) and lemon (*Citrus lemon*) are also similar to the work of<sup>[29]</sup>,

which suggests that both the oils were effective in inhibiting the growth of *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum* and *Penicillium verrucosum*. The hydrodistilled oil of lemon and orange was also found to be more sensitive against *Penicillium digitatum*<sup>[8]</sup>. Antimicrobial activity of Rutaceae family species have been observed on dermatophytes<sup>[15]</sup> and on some moulds<sup>[16]</sup>. Essential oils of some plants have recently been proven to be successful eco-friendly, bio-control agent<sup>[9]</sup>. Many authors have reported antimicrobial, antifungal, antioxidant and radical-scavenging properties of essential oils<sup>[21]</sup>. Appreciable antifungal activity of lemon grass oil has been also noted against clinical isolates of *Candida*, *Aspergillus fumigatus*, *Microsporium gypseum* and *Trichophyton mentagrophytes*. 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-a-pinene, camphene, b-pinene, sabinene, myrcene, terpinene, b-bisabolene, limonene, trans-a-bergamotene, nerol and neral<sup>[1]</sup>. Essential oils from citrus offer the potential for all natural antimicrobials for use in improving the safety of organic or all natural foods<sup>[6]</sup>. The MIC of oil of *Citrus sinensis* were in the range of 0.7-1µl/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*<sup>[22]</sup>. The present study matches with the work of<sup>[29]</sup>, which suggests that the essential oils of lemon, mandarin, grapefruit and orange all exhibited antifungal activity. The present investigation suggests that the essential oils exhibit narrow or wide range of antifungal activity, which may prove useful in the development of effective antifungal substances. MIC of lemon oil at 0.5% concentration against mould strains i.e *Fusarium spp*, *Penicillium spp*, *Aspergillus niger*, *A.flavus* and *Rhizopus spp*<sup>[27]</sup>. In our findings, MIC of lemon oil against *Malassezia furfur* was 0.8µl/ml i.e very much close to that concentration. The small differences are possibly due to local environmental and climatic conditions and variation of choice of microorganism tested.

**Table No.1 Antifungal activity of essential oils against *Malassezia furfur***

Oil	Test strain	Concentration of lemon	IZ of sample	IZ of standard (Gentamycin)	AI	IZ of standard (Streptomycin)	AI
Lemon	<i>Malassezia furfur</i>	100%	50mm	16.5 mm	3.03	17 mm	2.94
Orange	<i>Malassezia furfur</i>	100%	20mm	16.5 mm	1.212	17 mm	1.17

IZ= Inhibition zone (in mm) including the diameter of disc (6mm), AI (Activity index)

**Table 2: MIC of lemon oil against *Malassezia furfur***

Test Strain	Different concentrations of compound used in µl/ml	Growth visually inspected in different concentrations of compound
<i>Malassezia furfur</i>	0.1	+4
	0.3	+3
	0.5	+2
	0.7	+1
	0.9	0
	1.1	0
	1.3	0
	1.5	0
	1.7	0
	1.9	0
		Control without oil

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 orange oil against *Malassezia furfur***

Test Strain	Different concentrations of used in µl/ml	Growth visually inspected in different concentrations of oil
<i>Malassezia furfur</i>	0.1	+4
	0.3	+4
	0.5	+4
	0.7	+4
	0.9	+3
	1.1	+3
	1.3	+3
	1.5	+3
	1.7	+2
	1.9	+2
	2.1	+1
	2.2	0
	2.4	0
	2.6	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.

## CONCLUSION

The present study clearly 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.

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