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Full Length Research Paper

Anti-*Malassezia furfur* activity of essential oils against causal agent of *Pityriasis versicolor* disease

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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 using two different methods: Disc diffusion and microdilution methods. In screening of lemon and orange oil by disc diffusion method, the diameter of inhibition zone was found to be 50 and 20 mm which were greater than inhibition zone of reference antibiotics, that is, gentamycin and streptomycin of 16.5 and 17 mm, respectively. Minimum inhibitory concentration (MIC) of lemon and orange oil against *M. furfur* was found to be 0.8 and 2.2 µl/ml. These findings support the use of *C. lemon* (lemon) and *C. sinensis* (orange) oil as a traditional herbal medicine for the control of *P. versicolor* infection of skin.

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

INTRODUCTION

Pityriasis versicolor (PV) is a superficial mycosis, affecting the superficial layer of stratum corneum (Marcon and Powell, 1992). The causative organism is *Malassezia furfur*, a yeast-like lipophilic fungus. Previously the mycelial form was called either *Pityrosporum ovale* or *Pityrosporum orbiculare* (Hay and Moore, 1998). 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 (Silva-Lizama, 1995). The disease is most prevalent in early adulthood and small childrens are rarely affected (Di Silverio et al., 1995; Boussida et al., 1998). PV is common in the post-pubertal age where sebaceous glands are active and in individuals who sweat more (Schmidt, 1997). There is often a positive family history of the disease. An increase in humidity, temperature and hyperhidrosis are important predisposing factors (Silva-Lizama, 1995; Boussida et al., 1998). The prevalence in colder climates is less than 1% (Rippon, 1982). *M. furfur* is a component of normal skin flora in more than 90% of adults living in tropical areas (Faergemann and Freidriksson, 1981). PV, consequently, is more common in the tropics than in temperate zones (Hay and Moore, 1998).

Infectious diseases accounts for high proportion of health problems in the developing countries. Microorganisms have developed resistance to many antibiotics and as a result, immense clinical problem in the treatment of infectious diseases has been created (Davies, 1994). The resistance of the organisms increased due to indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious disease. This situation forced the researchers to search for new antimicrobial substance from various sources including medicinal plants (Bauer et al., 1996). There are alarming reports of opportunistic fungal infections (Singh, 2001). A survey of literature reveals that there are many essential oils which possess antifungal activity. 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 (Alex et al., 1998). Essential oils have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases (Sofowora, 1993). Essential oils have

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been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Bektas et al., 2004; Burt, 2004). 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 essential oil and its components were reported (Paranagama et al., 2003). Orange oil was composed principally of d-limonene 94 and 3% myrcene. The essential oils of lemon and orange exhibited antifungal activity against common moulds *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum and Penicillium verrucosum* (Viuda-Martos et al., 2008).

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 antifungal agent for *P. versicolor* disease.

MATERIALS AND METHODS

Extraction and preparation of oil

In summer season, extraction of oil from the peels of lemon (*Citrus lemon*) and orange (*Citrus sinensis*) were 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 to 6 h, oil was collected from the apparatus, anhydrous with sodium sulphate for removal of water traces, then this 100% pure essential oil were dispensed into dark bottles and stored at 4℃ until used. Essential oil was ready to use for disc diffusion test and determination of MIC.

Screening of essential oil using disc diffusion method

Oil was screened for their antifungal activity against *M. furfur* by disc diffusion method (Rios et al., 1988). Standard size Whatman No. 1 filter paper discs, 6.0 mm in diameter, sterilized by dry heat at 140℃ in an oven for one hour were used to determine antifungal activity. Sabouraud Dextrose Agar medium for 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 was 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. Sterilized filter paper discs were soaked in neat, undiluted (100%) concentration of oils. 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 (30 mcg), streptomycin, clotrimazole and ketoconazole (10 mcg/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 oils. The plates were incubated at 37℃ for 24 h. 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 (Activity index) = Inhibition zone of sample / inhibition zone of standard.

Determination of minimum inhibitory concentration using microdilution method

The modified micro-dilution method Provine and Hadley (2000) was followed to determine MIC. 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 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, Flexiloop) of the inoculum suspension was inserted deep into each tube of medium containing a different concentration of oils, 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℃ for 48 h 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 oil-free medium as a control was detected (48 h for yeasts). Afterwards, the growth in all tubes at different concentrations of essential oils (*C. sinensis* and *C. lemon*) was compared with that of the oil-free control in order to determine inhibition.

RESULTS

In our present work, *M. furfur* was found as a major etiological agent of *P. versicolor* disease. The conventional treatment of fungal disease is limited, and part of the reason is due to the limited spectrum of the currently antifungal drugs, and the expensive treatment, particularly due to the need of 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*. The results of the present work on the antifungal activity of lemon and orange oil against *M. furfur* studied by two methods, that is, disc diffusion and microdilution method are presented in Tables 1 to 3. In our study, lemon and orange oils presented higher diameter of inhibition zones than Gentamycin, Streptomycin, Clotrimazole and Ketoconazole. The diameter of the inhibition zone obtained against lemon and orange oil at 100% concentration of pure oil was 50 and 20 mm respectively by disc diffusion method. *M. furfur* was found to be resistant against clotrimazole and ketoconazole. Other reference antibiotics, that is, gentamycin and streptomycin showed inhibition zone of 16.5 and 17 mm, 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 *M. furfur.* 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 0.8 and 2.2 µl/ml concentrations

Table 1. Antifungal activity of lemon and orange oil against *M. furfur*.

Oil	Test strain	Concentration of lemon and orange oil (%)	IZ of sample (mm)	IZ of standard, Gentamycin (mm)	AI	IZ of standard, Streptomycin (mm)	AI
Lemon	*M. furfur*	100	50	16.5	3.03	17	2.94
Orange		100	20	16.5	1.212	17	1.17

IZ = Inhibition zone (in mm) including the diameter of disc (6 mm); AI = activity index.

Table 2. MIC of lemon oil against *M. furfur*.

Test strain	Different concentrations of lemon oil used in µl/ml	Growth visually inspected in different concentrations of oil
M. furfur	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	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. µl represents the concentration of oil per ml of Brain heart infusion agar media.

Table 3. MIC of orange oil against *M. furfur.*

Test strain	Different concentrations of orange oil used in µl/ml	Growth visually inspected in different concentrations of oil
M. furfur	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 grow.

against *M. furfur.* Both oils can be used for the development of natural antifungal agent against *Pityriasis versicolor* infections.

DISCUSSION

The present results suggest that lemon and orange oils exhibits excellent antifungal activity. Essential oils derived from many plants are known to possess antifungal activities (Valero and Frances, 2006). 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 (Harris, 2002). 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 (Jayaprakasha et al., 2005).

The results showed the efficacy of lemon oil on the animal skin infections caused by *Trichophyton rubrum and Microsporum gypseum*. In our study, lemon oil exhibited the strong antimycotic activity against *M. 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 0.8 µl/ml. Our work is in agreement with the observations of Silvia et al. (2008) 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 Ezzat (2001) which revealed that the essential oil of *C. lemon* at 200 µl concentration showed diameter of inhibition zone 37 mm against *Candida albicans* 10261. According to Rusenova and Parvano (2009) *C. lemon* does not show antimicrobial activity by disc-diffusion method against yeasts, *Candida albicans* and *Malassezia pachydermatis*. 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. 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 (Ahmad and Beg, 2001).

Shahi et al. (2003) found that the MIC of oil of *C. sinensis* were in the range of 0.7 to 1 µl/ml against *Candida albicans, A. flavus, Aspergillus fumigatus, A. niger, Aspergillus ustus, Epidermophyton floccosum, Microporum audouinii, Microporum canis, M. gypseum, Microporum nanum, Rhizopus nigricans, Trichophyton tonsurans, T. rubrum, Trichophyton mentagrophytes* and *Trichophyton violaceum*. The present investigation suggests that the essential oils exhibit 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 mould strains, that is, *Fusarium* spp., *Penicillium* spp., *A. niger*, *A. flavus* and *Rhizopus* spp. In our findings, MIC of lemon oil against *M. furfur* was 0.8 µl/ml, that is, very much close to that concentration. The small differences may be due to local environmental and climatic conditions and variation in choice of microorganism tested.

Our results of orange (*C. sinensis*) and lemon (*C. lemon*) are also similar to the work of Viuda-Martos et al. (2008) which suggests that both the oils were effective in inhibiting the growth of *A. niger, A. flavus, P. chrysogenum* and *P. verrucosum*. The hydrodistilled oil of lemon and orange was also found to be more sensitive against *Penicillium digitatum* (Caccioni et al., 1998). Antimicrobial activity of Rutaceae family species have been observed on dermatophytes (Lima, 1996) and on some moulds (Megalla et al., 1980). Essential oils of some plants have recently been proven to be successful eco-friendly, bio-control agent (Chutia et al., 2006). Many authors have reported antimicrobial, antifungal, antioxidant and radical-scavenging properties of essential oils (Sawamura et al., 2005; Sokovic and Griensven, 2006).

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, incluing strong fungicidal action, long-shelf life, and withstand heavy inoculum density. 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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