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Screening of some essential oils against *Trichosporon* species

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Abstract

White Piedra is a superficial mycoses characterized by nodules on the hair shaft, caused by the basidiomycetous yeast *Trichosporon* species. In this study 25 essential oils were extracted and screened against two *Trichosporon* species i.e. *Trichosporon asahii* and *Trichosporon cutaneum*. Both these fungi procured from MTCC Chandigarh were maintained on yeast malt agar plates and tubes at 25°C. Two screening methods viz., agar well diffusion assay and minimum inhibitory concentration were adopted for the study. The results showed that the maximum anti-yeast activity against *T. asahii* and *T. cutaneum* was demonstrated by oil of *Mentha piperita* showing full inhibition of both the fungi, *Melaleuca alternifolia* with an inhibition zone of 45 and 40 mm, *Cymbopogon winterians* with inhibition zone of 45 and 45 mm and *Cymbopogon flexuosus* with 35 and 30 mm inhibition zones. The oil of *Trachyspermum ammi* exhibited 10 and 20 mm, *Abelmoschus moschatus* exhibited 30 and 20 mm, *Salvia sclarea* showed 20 and 18 mm and *Jasminum officinale* exhibited 25 and 15 mm inhibition zones showing moderate activity. The oil of *Cyperus scariosus*, *Pogostemon patchouli* and *Rosa damascene* showed no inhibition zone against both the fungi while *Vetiveria zizanoides* exhibited no inhibition in case of *T. asahii* and inhibition zone of 10 mm in case of *T. cutaneum* demonstrating comparatively low activity against both the fungi. These results support that the essential oils can be used to cure superficial mycoses and these oils may have significant role as pharmaceuticals and preservatives.

Key words

Trichosporon, White Piedra, Essential Oils, Antifungal activity, Medicinal plants,

Introduction

Piedra, meaning stone in Spanish, is an asymptomatic fungal infection of the hair shaft, resulting in the formation of nodules of different hardness on the infected hair. The infection, also known as *Trichomycosis nodularis*, is a superficial fungal infection arising from the pathogen being restricted to the stratum corneum. The conditions caused are considered superficial mycoses because they neither invade living tissue nor provoke an immune response by the host (De Hoog and De Hoog, 1995, 1998). Two varieties of Piedra i.e. Black Piedra and White Piedra are often observed.

White Piedra is found on scalp, beard, moustache hair, eyebrows, eyelashes, groin, genital and perigenital hairs (Kwon-Chung *et al*., 1992; De Hoog *et al.,* 1995; De Hoog *et al.,* 1998) and is characterized by white to light brown soft and smaller nodules. Nodules consist of fungal mass with encapsulated arthroconidia or blastoconidia (Kwon-Chung *et al.,* 1992; De Hoog *et al.,* 1995; De Hoog *et al.,* 1998). The nodules produce a gritty sensation when palpated (Gupta *et al.,* 2003) and may be detached easily resulting into the split or broken hair (Ghorpade, 2004). Genital white Piedra can indicate a co-infection with *Corynebacterium* (Therizol-Ferley *et al*., 1994). White Piedra has a wide geographic distribution and has been described in tropical as well as

temperate countries including India (Therizol-Ferley *et al*., 1994; Schwinn *et al*., 1996; Palungwachira *et al*., 1991; Gupta *et al*., 2003; Ghorpade *et al*., 2004).

White Piedra is caused by *Trichosporon* genus of Class Basidiomycetes which is sub divided into six distinct species viz. *Trichosporon asahii*, *T. ovoides*, *T. inkin*, *T. mucoides*, *T. cutaneum* and *T. asteroides* (Gueho *et al*., 1992, 1994; Chagas-Neto *et al*., 2008). The natural habitats of all these species are soil, lake water, plants and occasionally seen as normal flora of the human skin and mouth (Sugita *et al*., 2000). *T. asahii* involved in systemic mycosis, *T. asteroids* and *T. cutaneum* both are associated with skin infections, the latter occasionally producing axillary White Piedra. *T. inkin* exclusively isolated from human crural areas, *T. mucoides* involved in systemic mycoses, onychomycoses and crural white Piedra and *T. ovoides* in capital white Piedra and occasionally with superficial mycosis (De Hoog *et al*., 1998; Gueho *et al*., 1994; Howard *et al*., 1995). *Trichosporon*, in particular has also been considered urophilic due to its ability to occupy strongly acidic localisations when colonising pubic hair and its capacity to utilize urea and uric acid (De Hoog and De Hoog, 1995, 1998).

The use of medicinal herbs in the treatment of skin diseases including mycotic infections is an age old practise in many parts of the world (Irobi *et al.,* 1993).There is very scanty information regarding the treatment of Piedras by plant derived medicines which are safer than the drugs of chemical origin. Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population (Canter *et al*., 2005). Bioactive compounds currently extracted from plants are used as medicines; many of the plant species that proved medicinal herbs have been scientifically evaluated for their possible medical applications (Patwardhan *et al*., 2004).

In the present study, the antifungal activity of 25 essential oils was assessed against two species of *Trichosporon*: *Trichosporon asahii* and *Trichosporon cutaneum* using agar well diffusion and minimum inhibitory concentration assay.

Materials and Methods

Maintenance of cultures: The cultures of two test fungi viz. *Trichosporon asahii* (MTCC No.6179) and *Trichosporon cutaneum* (MTCC No.255) were procured from Microbial Type Culture Collection, Chandigarh for the study and maintained on yeast malt agar media at optimum temperature of 25ºC.

Extraction of essential oils: The seeds, roots and leaves of 25 aromatic plants collected from different regions of Dehradun district were dried and ground to semi powdered state. The plant specimens were submitted to the Division of Botany, Forest Research Institute, Dehradun for identification. Steam distillation of the air dried parts was done in a Clevenger apparatus for 2 hrs in accordance with the British Pharmacopoeia (1993). The aqueous phase was extracted with dichloromethane. The organic phase was dried with sodium sulphate, filtered and the solvent was evaporated until dryness by air drying. The oils were stored in a refrigerator at 4^0C until required. These oils were screened for their anti-mycotic activity.

Antifungal assays :

Agar well diffusion assay: Preliminary analysis of antifungal activity was conducted using agar well diffusion assay as described by Garcia *et al.* (2002). Fungal inoculum was prepared in Tween 80 saline solution and incubated for 1 hr. 1 ml of this solution was homogeneously inoculated into petriplates containing Sabouraud dextrose agar (SDA) medium and kept for solidification. After solidification, wells of 6mm diameter were punctured in the culture medium using sterile cork borer. A fixed volume(100 µl) of respective essential oil was loaded in the well using sterilized micropipettes. Plates were incubated for 2-3 days at 25^0C and zone of inhibition of different oils was determined after 48 hrs in mm. Sterile 5% aqueous dimethyl sulphoxide (DMSO) was used as negative control while ketoconazole (50µg $disc^{-1}$) and nystatin (100µg $disc^{-1}$) were used as the positive control. All experiments were carried out in triplicates.

Minimum inhibitory concentration:

Broth dilution assay: MIC of the oils against the test fungi was determined using the broth dilution method (Sahm and Washington, 1990).1 ml of the essential oil (100µl ml^{-1}) was added to 1 ml of Sabouraud dextrose broth and subsequent concentrations were prepared by using serial dilution technique. 1 ml fungal culture prepared in saline water was inoculated into each test tube and mixed thoroughly on a vortex mixer. The test tubes were then incubated at 25°C for 2 days. DMSO was used as a negative control. The tube with the lowest dilution with no detectable growth was considered as the MIC.

Statistical analysis: The inhibitory zones of essential oils were expressed as the mean $\pm$ S.D. and compared using Student Waller Ducan test at $P \leq 0.05$.

Results and Discussion

All the oils tested in the present study exhibited different degrees of antifungal activity against *T. asahii* and *T. cutaneum* (Table 1). The maximum anti-mycotic activity was shown by oil of *Mentha piperita* which gave

the most promising antifungal effects showing full inhibition of growth in the petriplates against both the fungal species followed by *Cymbopogon winterians* which showed inhibition zones of 45 and 45 mm and *Melaleuca alternifolia* showing inhibition zones of 45 and 40 mm against *T. asahii* and *T. cutaneum*. *Cymbopogon flexuosus* showed inhibition zone of 35 and 30 mm, *Ocimum basilicum* and *Citrus aurantifolia* showed similar inhibition zones of 31 and 28 mm, *Citrus aurantius* showed inhibition zone of 28 and 30 mm, *Eucalyptus globulus* showed inhibition zone of 24 and 27 mm, *Palargonium graveolens* showed inhibition zone of 30 and 20 mm and *Abelmoschus moschatus* showed inhibition zone of 30 and 20 mm. (Fig.1). The oil of *Zingiber officinalis* showed moderate activity by exhibiting inhibition zone of 26 and 22 mm against *T. asahii* and *T. cutaneum* respectively followed by *Cinnamomum zeylanicum* (25 and 21 mm), *Citrus bergamia* (23 and 21mm), *Citrus limon* (22 and 21mm), *Juniperus communis* (22 and 18 mm), *Jasminum officinale* (25 and 15 mm), *Salvia sclarea* (20 and 18 mm), *Boswellia carterii* (16 and 19 mm), *Trachyspermum ammi* (10 and 20 mm), *Commiphora myrrha* (12 and 15 mm) and *Cedrus atlantica* (10 and 12 mm). Some of the oils like *Cyperus scariosus, Pogostemon patchouli* and *Rosa damascene* were not effective at all showing no inhibition zones whereas *Vetiveria zizanoides* showed very small inhibition zone of 10 mm against *T. cutaneum* and was not effective against *T. asahii*.

Cinnamomum zeylanicum (cinnamon) and *Palargonium graveolens* (geranium) had lowest MIC's of 1.55µl ml^{-1} against *T. asahii*. *Mentha piperita* (Peppermint), *Eucalyptus globulus* (eucalyptus) and *Salvia sclarea* (clarysage) inhibited the visible growth of *T. asahii* at the concentration of 3.1µl ml^{-1} and oils of *Jasminum officinale* (jasminum) and *Zingiber officinalis* (ginger) showed inhibition of *T. asahii* at 6.2 µl ml^{-1}. *Cymbopogon flexuosus* (lemongrass), *Melaleuca alternifolia* (tea tree), *Ocimum basilicum* (basil), *Citrus bergamia* (bergamot), *Citrus limon* (lemon) and *Citrus aurantifolia* (lime) inhibited the visible growth of *T. asahii* at 12.5 µl ml^{-1}. *Citrus aurantius* (orange), *Juniperus communis* (juniper), *Cymbopogon winterians* and *Abelmoschus moschatus* (muskdana) showed inhibition at 25 µl ml^{-1}. *Trachyspermum ammi* (ajowain), *Pogostemon patchouli* (patchouli), *Commiphora myrrha* (myrrh) and *Boswellia carterii* (frankincense) showed MIC of 50 µl ml^{-1}. *Cedrus atlantica* (cedarwood) and *Vetivaria zizanoides* (khus) showed inhibition at 100µl ml^{-1} and *Cyperus scariosus* (Nagarmotha) and *Rosa damascene* (rose) showed inhibition at 200 µl ml^{-1}, respectively (Table 1).

In case of *T. cutaneum*, lowest MIC was shown by the oils of *Eucalyptus globulus, Palargonium graveolens, Cinnamomum zeylanicum* and *Salvia sclarea* at 1.55 µl ml^{-1}. Oils of *Juniperus communis* and *Citrus aurantifolia* showed inhibition at 3.1µl ml^{-1}. *Mentha piperita, Melaleuca altemifolia*, *Ocimum basilicum, Citrus aurantius, Citrus*

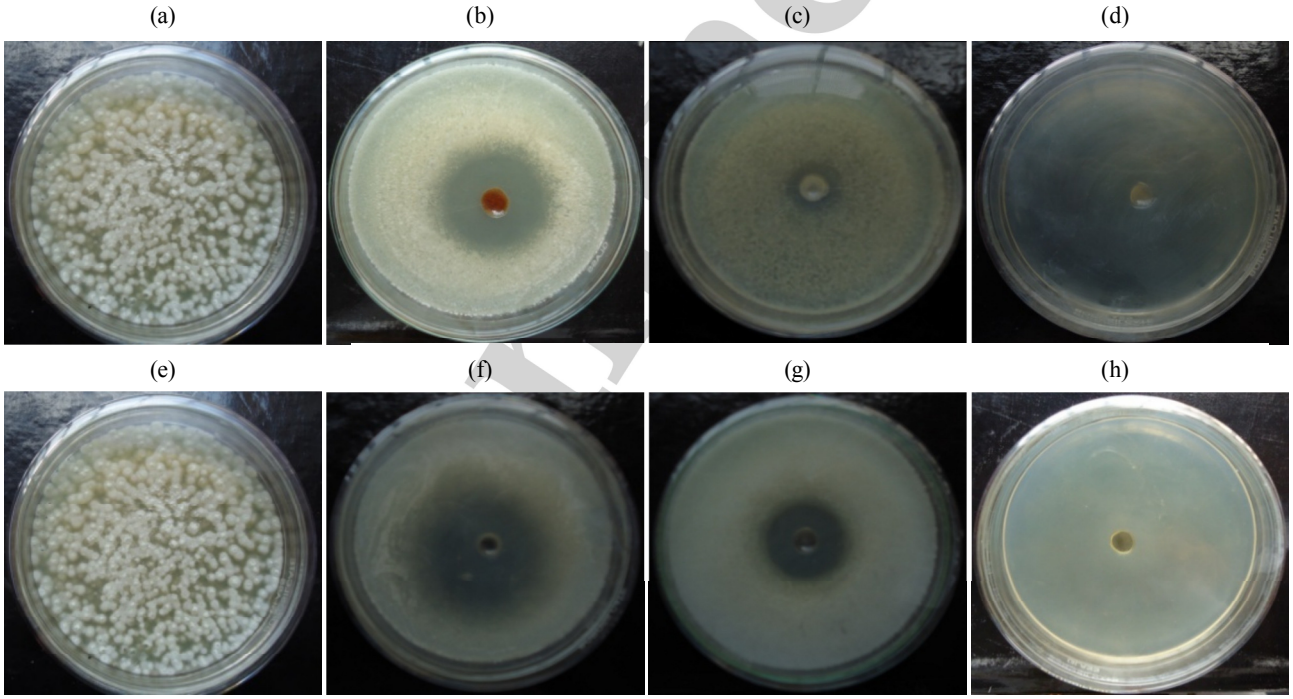

Fig.1 : Results showing Agar Well Diffusion Assay. (a) *T. asahii* pure culture (b) Eucalyptus oil against *T. asahii* (c) Nagarmotha oil against *T. asahii* (d) Peppermint oil against *T. asahii* (e) *T. cutaneum* pure culture (f) Lemongrass oil against *T.cutaneum* (g) Myrrh oil against *T. cutaneum* (h) Peppermint oil against *T. cutaneum*

Table 1. Mean zone of inhibition and minimum inhibitory concentration of essential oils against *T. asahii* and *T. cutaneum*

Essential oil	Botanical names	Family	Parts used	Average zone of inhibition (mm)		Minimum inhibitory concentration (µl ml^{-1})	
				T. asahii	*T. cutaneum*	*T. asahii*	*T. cutaneum*
Juniper	*Juniperus communis*	Cupressaceae	Berries and Twig	22±1.5	18±1.0	25	3.1
Myrrh	*Commiphora myrrha*	Burseraceae	Gum, Resin, sap	12±1.1	15±1.5	50	25
Ginger	*Zingiber officinalis*	Zingiberaceae	Root and Rhizome	26±1.5	22±1.0	6.2	6.2
Frankincense	*Boswellia carterii*	Burseraceae	Resin	16±1.0	19±1.1	50	25
Eucalyptus	*Eucalyptus globulus*	Myrtaceae	Leaves, Fruit, sap	24±1.0	27±1.1	3.1	1.55
Lime	*Citrus aurantifolia*	Rutaceae	Leaves, Fruit and peel	31±1.0	28±1.0	12.5	3.1
Orange	*Citrus aurantius*	Rutaceae	Fruit and Peel	28±1.5	30±1.0	25	6.2
Cinnamon	*Cinnamomum zeylanicum*	Lauraceae	Bark, leaves	25±1.0	21±1.1	1.55	1.55
Cedar wood	*Cedrus atlantica*	Pinaceae	Twig andLeaf	10±1.0	12±1.0	100	200
Lemon	*Citrus limon*	Rutaceae	Fruit, Peel, Seeds	22±1.1	21±1.5	12.5	6.2
Tea tree	*Melaleuca alternifolia*	Myrtaceae	Leaves	45±1.0	40±1.0	12.5	6.2
Nagarmotha	*Cyperus scariosus*	Cyperaceae	Roots	NI	NI	200	100
Jasmine	*Jasminum officinale*	Oleaceae	Flower	25±1.5	15±1.0	6.2	12.5
Patchouli	*Pogostemon patchouli*	Labiatae	Leaves	NI	NI	50	50
Bergamot	*Citrus bergamia*	Rutaceae	Flower and Fruit	23±1.0	21±1.1	12.5	6.2
Basil	*Ocimum basilicum*	Lamiaceae	Leaves and stem	31±1.5	28±1.1	12.5	6.2
Geranium	*Palargonium graveolens*	Geraniaceae	Leaves and flowers	30±1.0	20±1.1	1.55	1.55
Clarysage	*Salvia sclarea*	Labiatae	leaves	20±1.0	18±1.1	3.1	1.55
Peppermint	*Mentha piperita*	Labiatae	leaves	NG	NG	3.1	6.2
Musk	*Abelmoschus moschatus*	Malvaceae	seeds	30±1.5	20±1.0	25	12.5
Citronella	*Cymbopogon winterians*	Poaceae	Leaves, Grass	45±1.0	45±1.1	25	12.5
Ajowain	*Trachyspermum ammi*	Apiaceae	seeds	10±1.0	20±1.0	50	25
Lemongrass	*Cymbopogon flexuosus*	Poaceae	Leaves and stem	35±1.1	30±1.5	12.5	25
Khus	*Vetiveria zizanoides*	Poaceae	root	NI	10±1.0	100	100
Rose	*Rosa damascene*	Rosaceae	Flower and leaves	NI	NI	200	100
Ketoconazole (50µg disc^{-1})	-	-	-	21±0.0	20±0.0	-	-
Nystatin (100µg disc^{-1})	-	-	-	12±0.0	12±0.0	-	-

NI = No inhibition; NG= No growth; Values are mean of three replicates ± S.D.; The mean values for zone of inhibition measured in two directions after 48-72 hrs incubation at 25ºC; Ketoconazole and Nystatin are used as the positive controls in well diffusion assay

bergamia, Citrus limon and *Zingiber officinale* inhibited the visible growth at the concentration of 6.2µl ml^{-1}. *Cymbopogon winterians, Abelmoschus moschatus and Jasminum officinale* showed MIC of 12.5µl ml^{-1}. *Cymbopogon flexuosus, Trachyspermum ammi*, *Commiphora myrrha and Boswellia carterii* showed inhibition at 25 µl ml^{-1}. *Pogostemon patcholi* inhibited the growth at the concentration of 50 µl ml^{-1}. *Cyperus scariosus, Rosa damascene* and *Vetivaria zizonoides* were less effective and inhibited the growth at 100 µl ml^{-1} while *Cedrus atlantica* inhibited the growth at concentration of 200µl ml^{-1} (Table 1).

The traditional use of plants as medicines provide the basis for indicating which essential oils and plant oils may be useful for specific medical conditions. Historically, many plant oils and extracts, such as tea tree, myrrh and clove, have been used as topical antiseptics, or have been reported to have antimicrobial properties (Lawless,1995). In recent years, research on aromatic plants and particularly their essential oils, has attracted many investigators. Essential oils have traditionally been used for centuries for their antifungal properties (Rios and Recio, 2005). More recently, several studies have confirmed the huge potential of these natural products as antifungal agents (Bakkali *et al.*, 2008; Cavaleiro *et al.*, 2006; Pina-Vaz *et al.*, 2004; Pinto *et al.*, 2006; Zuzarte *et al.*, 2009). Therefore, it is not surprising that essential oils are one of the most promising groups of natural products for the development of broad spectrum, safer and cheaper antifungal agents. Nowadays, the increasing impact of the infections, the limitations encountered in their treatment (e.g. resistance, side effects and high toxicity) and the rising over prescription and overuse of conventional antifungal (Perez-Parra *et al.*, 2009; Ferris *et al.*, 2002) all stimulate a search for alternative natural drugs. The mechanism of action of essential oils remains somewhat controversial. While some studies suggest that the compounds may penetrate the micro-organism and react with active sites of enzymes and/or interfere with cellular

metabolism, most evidences support direct disruption of cellular membranes and concentration-dependent pro-oxidant cytotoxic effects. This leads to changes in permeability leading to leakage and ultimately resulting in cell death (Bakkali *et al*., 2008). According to our investigations, all the oils tested exhibited different degrees of antifungal activity against *T. asahii* and *T. cutaneum*. Our results confirm that *Mentha piperita* (peppermint), *Melaleuca alternifolia* (tea tree), *Cinnamomum zeylanicum* (cinnamon), *Cymbopogon winterians* (citronella), *Citrus limon* (lemon) and *Citrus aurantifolia* (lime) confirmed the antifungal activity against both *T. asahii* and *T. cutaneum* showing large inhibitions. Some oils like *Cyperus scariosus* (nagarmotha), *Cedrus atlantica* (cedarwood), *Rosa damascene* (Rose), *Pogostemon patchouli* (patchouli), and *Commiphora myrrha* (myrrh) showed no effectiveness against the two *Trichosporon* species. Kishore *et al*. (1993) also reported that the essential oil of mint showed high anti-mycotic activity against dermatophytes.

The study confirms that majority of essential oils tested are an important source of antifungal compounds that may provide renewable sources of useful antifungal drugs against superficial infections in humans. Since some of these plants *viz Mentha piperita*, *Melaleuca alternifolia*, *Cinnamomum zeylanicum*, *Cymbopogon winterians*, *Citrus limon* and *Citrus aurantifolia* showed broad spectrum anti-mycotic activity are cheap and could be useful in antiseptic and disinfectant formulations. The study suggests that therapeutic measures involving cutting the hair and application of ointments and shampoos synthesized from drugs of chemical origin can be replaced by drugs of plant origin. However, further studies are needed including *in vivo* investigations. Purification and identification of the active components from some of the effective plants are in progress.

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