Studies on antidermatophytic activity of waste leaves of *Curcuma longa* L.

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ABSTRACT

During antidermatophytic screening of some essential oils, *Curcuma longa* L. exhibited the strongest antifungal activity, completely inhibiting the mycelial growth of ringworm, caused by the fungi- *Microsporum gypseum* and *Trichophyton mentagrophytes*. The essential oil from leaves of *Curcuma longa* was fungicidal at 2.5 µl/ml at which it tolerated heavy doses of inoculum. The fungicidal activity of the oil was thermostable up to 80 °C and self life up to 24 months in storage. The oil also showed a broad fungitoxic spectrum, inhibiting the mycelial growth of other fungi, viz., *Epidermophyton floccosum*, *M. nanum*, *T. rubrum*, *T. violaceum*. Moreover, up to 5 % concentration it did not exhibit any adverse effect on mammalian skins. The oil has been formulated in the form of an ointment, 1 % w/v and subjected to topical testing on patients of the Out Patient Department (OPD) at Moti Lal Nehru Medical College, Allahabad. Patients were selected on the basis of KOH-positive results and diagnosed *tenia corporis*. After the second week of treatment, all patients were KOH- negative. At the end of medication, 75 % of patients recovered completely while 15 % showed significant improvement from the disease. The ointment thus, can be exploited commercially after ongoing successful clinical trials. Relationship of the dermatophytes to the toxicity of the oil vis-a-vis phylogeny using molecular data of the pathogens have also been discussed. [Physiol. Mol. Biol. Plants 2010; 16(2) : 177-185] E-mail : anupambplau@rediffmail.com

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INTRODUCTION

The unhealthy and poor hygienic conditions give rise to the common fungal diseases on the skin. Environmental factors (high temperature and humidity) also play a significant role to infections under tropical and sub-tropical conditions like India. Among these, ringworm infection, a superficial fungal infection, is most prevalent. Commonly, these infections are named after the affected body parts. *Tenia corporis* is a dermatophytic infection of the glabrous skin, most commonly caused by species of the genera *Trichophyton* (Mochizuki *et al.*, 2002) and *Microsporum*. The infection is generally restricted to the stratum corneum of the epidermis. *Tenia corporis* may be transmitted

through direct contact with other infected individuals or by infected animals. Transmission of infection by clothing, furniture or any other inanimate form is controversial. The usual typical lesion of *tenia corporis* is annular or polycyclic; the border being erythematous and vesicular or scaly. Synthetic antidermatophytics are mostly used to treat these infections which are largely non-renewable, non-biodegradable petro-products that also cause adverse effects and residual toxicity (Roxburgh and Borrie, 1973). Thus, screening for new treatments with higher efficacy and cheaper substitutes are desirable. Plant resources are the natural choices.

Naturally occurring antidermatophytes are mostly biodegradable (Beye, 1978) and devoid of side effects. Essential oils of plant origin besides being effective source of chemotherapeutic agents without undesirable side effects are also strong fungicides (Pandey *et al.*, 1996; Shahi *et al.*, 1996; Shahi *et al.*, 1999; Shahi *et al.*, 2000; Inouye *et al.*, 2006).

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The *Curcuma longa* L., commonly known as Haldi, Turmeric or Indian saffron belongs to family Zingiberaceae, cultivated mostly in Sri Lanka, Indonesia and in many parts of India, especially in Bengal, Tamil Nadu and Andhra Pradesh. India accounts for more than 90 % of the total output of turmeric in the world. Recent researches and market report reveal that the maximum potential of *Curcuma longa* is utilized as a spice, or in external applications and for the treatment of some common skin diseases. It is a perennial herb with simple large leaves and tubers. Rhizomes and essential oil are of great importance (Jain *et al.*, 2007).

In the present investigation the essential oil from the leaves of *Curcuma longa* L. was found effective against human pathogenic fungi, *Epidermophyton floccosum* (Hartz) Langeron and Milochevitch, *Microsporum gypseum* (Bodin) Guiart and Grigorakis, *Microsporum nanum* Fuentes, *Trichophyton mentagrophyte* (Robin) Blanchard, *Trichophyton rubrum* (Castellani) Sabouraud and *Trichophyton violaceum* Bodin causing *tenia corporis*. In Indian system of medicine, turmeric is used to some extent as a stomachic, tonic and blood purifier. The juice of fresh rhizome treats skin infections, indolent ulcers, inflamed joints and in purulent ophthalmia (Anon, 2001). The essential oil of *C. longa* is also reported to contain anti-inflammatory and anti arthritic activity (Chandra and Gupta, 1972).

MATERIALS AND METHODS

Extraction of essential oil

The essential oil was extracted from the leaves of *Curcuma longa* L. by hydrodistillation using a Clevenger apparatus (Clevenger, 1928). A clear light-yellow oily layer obtained on the top of the aqueous distillate was separated and dried over anhydrous sodium sulphate. The physiochemical properties of the oil were determined by the technique described by Langenau (1948).

GC-MS analysis

Gas Chromatography analysis of the oil was performed on a Perkin- Elmer GC 8500, using a fused capillary column (25 m × 0.55 mm i.d., film thickness 0.25µm), coated with dimethyl siloxane (BP-1). The oven temperature was programmed at 60 °C to 220 °C at 5 °C/min then held isothermal at 220 °C for 15 min injector temperature, 250 °C, detector temperature, 300 °C, carrier gas, nitrogen at a linear velocity of 10

psi: split, 1:80–GC-MS data were obtained on a Shimadzu QP-2000 mass spectrometer at 70 ev and 250 °C. GC column: Ulbon HR-1 equivalent to OV-1, fused silica capillary column 0.25 mm × 50 m, film thickness 0.25 µm. The initial temperature was 100 °C for 7 min and heated at 5 °C/min to 250 °C. Carrier gas was helium at a flow rate of 2 ml/min. The percentage composition leaf oil of *Curcuma longa* is given in Table 2.

In vitro studies

The minimum inhibitory concentrations (MICs) of the oil against test pathogens were determined *in vitro* using poisoned food technique (Grover and Moore, 1962) with slight modification (Shahi *et al.*, 1999). The requisite quantity of the oil samples were mixed in acetone (2 % of the required quantity of the medium) and then added in pre-sterilized sabouraud dextrose agar (SDA) medium, pH 5.6. In control sets, sterilized water (in place of the oil) and acetone were used in the medium in appropriate amounts. Mycelial discs of 5 mm dia, cut out from the periphery of 7-day-old cultures were aseptically inoculated upside-down on the agar surface of the medium. Inoculated petri plates were incubated at 27 ± 1 °C and the observations were recorded on the seventh day. Percentage of mycelial growth inhibition (MGI %) was calculated according to the formula:

$$\text{MGI \%} = \frac{(\text{dc} - \text{dt}) \times 100}{\text{dc}}$$

where dc = fungal colony diameter in control sets, dt = fungal colony diameter in treatment sets.

The minimum fungistatic/fungicidal concentrations of the oil at minimum inhibitory concentrations (MICs) were ascertained by the method of Garber and Houston (1959). This was done by re-inoculating the inhibited fungal discs at MICs on SDA medium. Fungal growth on the seventh day indicated a fungistatic nature, while the absence of fungal growth denoted fungicidal action of the oil. The effect of inoculum density (increased progressively up to 30 discs in multiples of five, each of 5 mm dia) of the test pathogens on MICs of the oil was determined following the procedure of Dikshit and Dixit (1982). The effect of physical factors *viz.*, temperature and expiry of toxicity during storage of the oil was evaluated according to Shahi *et al.* (1999). Five lots of 5 ml oil were kept in small vials and exposed to 40, 60 and 80 °C in an incubator for 1 h. Antidermatophytic activity was then tested at MICs by the poisoned food technique. Expiry of toxicity of the oil was determined by storing the oil at room

temperature and testing antidermatophytic activity at MICs at regular intervals of 60 days up to 48 months.

The minimum killing time (MKT) of the oil was determined by the mycelial disc killing technique [(MDKT) (Shahi *et al.*, 1999)]. Two treatment sets were maintained, one with pure oil and the other with the minimum fungicidal concentrations (MCCs) of the oil. The treatment set using MCCs of the oil was prepared by mixing the required quantity of the oil samples in acetone (5 % of the total quantity of the treatment solution) and then adding this to the appropriate quantity of distilled water. Simultaneously, controls were maintained using sterilized water (in place of the oil) and acetone, adding into the distilled water in appropriate quantities.

Mycelial discs of 5 mm diameter, cut out from the periphery of 7-day-old cultures of the test pathogens, were aseptically placed in the culture tubes of different treatment and control sets. These mycelial discs were taken out of the tubes at different time intervals and washed immediately in the washing solution (containing acetone: sterilized distilled water, 1:2) to remove the treatment solution. These washed mycelial discs were aseptically transferred upside-down to the sabouraud dextrose agar (SDA) medium (pH 5.6) in the petri plates. The same procedure was followed with the control sets. The inoculated petri plates were incubated at 27 ± 1 °C and the observations recorded as an average value of five replicates on the seventh day. The percentage of fungal growth inhibition (FGI %) was calculated according to the formula:

$$\text{FGI (\%)} = \frac{\text{dc} - \text{dt} \times 100}{\text{dc}}$$

The experiments were repeated twice each containing five replicates, and the data presented are based on their mean values.

***In vivo* studies**

Patch test method (Roxburgh and Borrie, 1973) was followed to determine the maximum tolerable concentrations (MTCs) and long-term toxicity for irritant activity of the oil.

People of both sexes aged 10-30 years were selected randomly and a group of 30 individuals of each sex was constituted. Circular areas of 5 cm² on upper hairy and lower glabrous surface of the palms and 3 cm² of neck region of each individual were first washed with distilled water followed by 70 % ethyl alcohol and then

allowed to dry for 5 min. Five drops of the graded concentrations of testing solution were applied to each individual separately for 3 weeks. The volunteers were not allowed to wash the applied area. Qualitative observations were recorded at intervals of 24 h for 3 weeks.

Further, we used the oil in the form of an ointment 1 % w/v and subjected to topical testing on patients attending the outpatient department at Moti Lal Nehru Medical College, Allahabad. Medication was administered twice a day for 3 weeks. The patients were not allowed any other systemic or topical therapy during the course of study.

The diagnosis for *tenia corporis* was confirmed by microscopic examination of the scrapping from the infected area treated with 10 % KOH. Presence of mycelia and conidia were designated as KOH-positive and their absence being recorded as KOH- negative. Only KOH-positive cases were enrolled in the study. Patients in the age group of 8-40 years were selected randomly. They were examined just before the therapy was initiated at the end of each week, or the 3 week treatment period. Where the fungal infection occurred in more than one area all the affected areas were treated. However, only one was selected and designated as the reference lesion. At each visit the same reference lesion was scrapped for fungal culture to identify the organism and for demonstration of hyphae by microscopic examination of scrapings, covered with 10 % KOH preparations. Such signs and symptoms of inflammation as erythema, scaling, itching, maceration and postulation were recorded as absent, mild, moderate or severe. At each visit, overall clinical improvement was rated as worse, none, partial, significant or completely clear by comparing the conditions with those existing at the initial visit. Satisfactory responses with KOH negative cases after third week were re-examined after 2 months to find out the relapse rate if any. The data of clinical response of the formulation was statistically analysed using two way ANOVA.

Phylogenetic study of dermatophytes

To find out the reason why the formulation is more effective against certain pathogenic fungi, phylogenetic relationship of the dermatophytes were studied including the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* and identified the species using the base pair sequences of ITS1 (Makimura *et al.*, 1999; Fig. 1). The ITS1 sequences of the standard strains used in this study and of members of the *T. mentagrophytes*

complex (*Trichophyton mentagrophytes* isolates from humans, accession no. AB011463; *T. rubrum*, accession no. AB011453; *T. violaceum*, accession no. AB017174; *Microsporum gypseum*, accession no. AB017177 and *Epidermatophyton floccosum*, accession no. AB017181), as reported by Makimura *et al.* (1998) were aligned by using the Clustal W computer program (Higgins, 1996) and GENETYX-MAC 10.1 software (Software Development Co., Ltd., Tokyo, Japan). Phylogenetic trees were then constructed by the DNA maximum-likelihood (ML) method in the PHYLIP program (Phylogeny Inference Package), version 3.5c (Felsenstein, 1995), and the neighbor-joining (NJ) (Saito and Nei 1987) method in the NJPLOT program (Gouy, 1995). Bootstrap (Felsenstein, 1985) analysis with the Clustal W program was performed by taking 1,000 random samples from the multiple alignments.

RESULTS

The leaves of *Curcuma longa* upon hydrodistillation yielded 2.2 % essential oil. The oil was characterized by various physicochemical properties (Table 1) as well as by GC-MS (Table 2). The major compounds in the oil are Terpinolene 26.4 %, α -phellendren 8 %, Terpinen-4-ol 7.4 % and Sabinyl acetate 3.2 %. The minimum static and minimum inhibitory concentration was 2.0 μ l/ml and 2.5 μ l/ml against *Microsporum gypseum* and *Trichophyton mentagrophytes*, respectively (Table 3). The oil could withstand heavy doses of inocula up to 30 discs at their respective minimum inhibitory concentration (Table 3). The activity of the oil persisted even up to 24 months of storage and was thermostable up to 80 °C.

The pure oil killed the test pathogens *Microsporum gypseum* and *Trichophyton mentagrophytes* in just 5

Table 1. Physicochemical properties of essential oil from the leaves of *Curcuma longa* L.

Properties studied	Observations
Plant height	60-90 cm
Oil yield	2.2 %
Specific gravity	0.913
Refractive index	1.5054
Optical rotation	-15.7
Ester value	3.2
Solubility	Acetone

Table 2. Chemical composition of essential oil from the leaves of *Curcuma longa* L.

S.No.	Compound	R.I.	Composition (%)
1.	n-Octane	800	0.6
2.	α -Pinene	935	1.0
3.	Sabinene	966	0.1
4.	β -Pinene	974	1.1
5.	Myrcene	984	1.7
6.	α -Phellandren	999	8.0
7.	p-Cymene	1014	2.1
8.	1,8-Cineole	1023	9.5
9.	Terpinolene	1084	26.4
10.	Linalool	1088	1.2
11.	Camphor	1130	1.3
12.	Borneol	1157	1.1
13.	Terpinen-4-ol	1166	7.4
14.	Carvone	1217	2.2
15.	Perillaketone	1230	2.1
16.	Geraniol	1242	2.4
17.	Safrole	1262	2.8
18.	Thymol	1281	0.7
19.	Sabinyl acetate	1289	3.2
20.	Linalyl propionate	1314	1.1
21.	Cis-Carvyl acetate	1321	1.1
22.	Methyl eugenol	1366	1.6
23.	β -Patchouline	1378	0.4
24.	β -Elemene	1385	1.2
25.	β -Caryophyllene	1418	0.6
26.	γ -Elemene	1423	0.8
27.	α -Cadinene	1436	1.1
28.	α -Patchouline	1456	1.3
29.	ar-Curcumene	1471	0.2
30.	Zingiberene	1487	0.3
31.	(E)-Nirolidol	1550	0.6
32.	ar-Turmerone	1640	0.5
33.	α -Turmerone	1650	0.6
34.	β -Bisabolol	1659	0.3

R.I. = Retention Indices

Table 3. *In vitro* antidermatophytic properties of essential oil from the leaves of *Curcuma longa* L.

Properties		Mycelial growth inhibition (MGI-%)			
A.	Conc. of oil (µl/ml)	<i>Microsporum gypseum</i>		<i>Trichophyton mentagrophytes</i>	
	2.5	100 ^c		100 ^c	
	2.0	100 ^s		100 ^c	
	1.5	94.1		96.4	
	1.0	73.3		85.4	
B.	Effect of Inoculum density (No. of fungal discs)	<i>Microsporum gypseum</i>		<i>Trichophyton mentagrophytes</i>	
	5	100		100	
	10	100		100	
	15	100		100	
	20	100		100	
	25	100		100	
	30	100		100	
C.	Minimum killing time (Sec.)	<i>Microsporum gypseum</i>		<i>Trichophyton mentagrophytes</i>	
		PO	MIC	PO	MIC
	75	100	100	100	100
	60	100	92	100	96
	45	100	83	100	87

S=static, C=cidal, PO= pure oil, MIC= minimum inhibitory concentration

sec. while at its minimum inhibitory concentration it required 75 sec. (Table 3). The oil also exhibited a broad range of antifungal activity inhibiting some other human pathogens viz., *Epidermophyton floccosum*, *M. nanum*, *T. rubrum* and *T. violaceum* in the range of 2.0–2.5 µl/ml concentration (Table 4; Fig. 3).

The oil at 0.5 µl/ml showed almost the same effect of toxicity i.e. 75.2, 75.0 and 74.8 per cent inhibition against closely related *Trichophyton rubrum*, *T. violaceum* and *Epidermophyton floccosum*, respectively. On the other hand *Trichophyton mentagrophytes* which is phylogenitically and distantly, related with others showed 69.4 % inhibition. Almost 71–73 % inhibition was observed with *Microsporum gypseum* and *M. nanum*, respectively (Fig. 2).

The oil did not show any irritation or adverse effect at 5 % concentration up to 3 weeks. The response of the formulation on patients after the first week of topical application showed moderate improvement in 29 % of cases, while in 30 % of the cases improvement was partial (Table 5). However, at the end of medication i.e. after the third week of application, a complete cure was seen in 72 % cases. No cases of relapse were observed when patients were re-examined after two months.

Statistical analysis:

Since the calculated value of F (15.76) was higher than the table value (3.83) at 5 % probability, it showed the significant clinical response of the formulation.

Phylogenetic relationship of dermatophytes

The phylogenetic relationship of dermatophytic genera *Trichophyton*, *Microsporum*, and *Epidermophyton* were

Table 4. Antidermatophytic spectrum of essential oil from the leaves of *Curcuma longa* L.

Human pathogenic fungi	Mycelial growth inhibition at various concentration (µl/ml)				
	0.5	1.0	1.5	2.0	2.5
<i>Epidermophyton floccosum</i>	74.8	80.1	93.0	100 ^s	100 ^s
<i>Microsporum gypseum</i>	70.9	73.3	94.1	100 ^s	100 ^c
<i>M. nanum</i>	72.1	83.2	94.1	100 ^s	100 ^c
<i>Trichophyton mentagrophyte</i>	69.4	85.4	96.4	100 ^s	100 ^c
<i>T. rubrum</i>	75.2	83.4	97.4	100 ^s	100 ^c
<i>T. violaceum</i>	75.0	80.1	97.3	100 ^s	100 ^c

C= fungicidal, S= fungistatic

Fig. 1. Alignment of ITS1 sequences of previously used dermatophytes

Fig. 2. Result of Cladogram (Neighbour Joining Tree plot) of dermatophytes on the basis of their ITS1 sequences. The NJ tree was constructed with data for standard strains of dermatophytes (see reference: Makimura *et al.*, 1999)

Fig. 3. Graphical representation of antidermatophytic spectrum of *Curcuma longa* L. leaf essential oil.

Table 5. Patients showing topical clinical response of formulation prepared from *C. longa* L. leaf essential oil

Response	% of patients showing clinical response in three weeks		
	1	2	3
Worse	0.0	0.0	0.0
None	0.0	0.0	0.0
Partial improvement	30	48	52
Moderate improvement	29	44	53
Complete cure	21	56	72

demonstrated by using internal transcribed spacer 1 (ITS1) region ribosomal DNA sequences. *Trichophyton* spp. and *Microsporum* spp. form a cluster in the phylogenetic tree with *Epidermophyton floccosum* as an outgroup, and within this cluster, all *Trichophyton* spp. except *Trichophyton terrestre* form a nested cluster (100 % bootstrap support). Dermatophytes in the cluster of *Trichophyton* spp. were classified into three groups with ITS1 homologies, each being a monophyletic cluster (100 % bootstrap support). The ITS1 sequences of 6 clinical isolates (Fig. 1) were also determined to identify the species. All strains were successfully identified by comparison of their base sequences with those in the ITS1 DNA sequence database (Makimura *et al.*, 1999). NJ tree of dermatophytes is shown in Fig. 2.

The relationship of the toxicity of the essential oil *vis-à-vis* phylogeny was analysed using molecular data. The effectiveness of the oil was equal in dermatophytes that are close in phylogenetic tree (Fig. 2).

DISCUSSION

Majority of antifungal drugs which are available in the market are less potent to control the superficial fungal infections thereby causing cosmetic embarrassment. Stock (1981) emphasized the need to discover powerful and specific antifungal agents on an increasing scale to combat fungal infections. Essential oils exhibiting wide range of antifungal activity, as suggested in the present investigation, may prove useful in the development of cheaper and potent antifungal substances against dermatophytes. The main constituents in the leaf essential oil of *Curcuma longa*, identified are Terpinolene (more than 26 %), 1,8-Cineole, α -

Phellandren, Terpinen-4-ol and Sabinyl acetate are present only as trace constituents.

Sokovic *et al.* (2009) reported that the potential antifungal effects of *Thymus vulgaris* L., *Thymus tosevii* L., *Mentha spicata* L., and *Mentha piperita* L. (Lamiaceae) essential oils were obtained by hydrodistillation of dried plant material. Their composition was determined by GC-MS. Identification of individual constituents was made by comparison with analytical standards. Many workers in India have studied the antifungal activity of candidate substances employing qualitative assay techniques (Kaul *et al.*, 1976; Lahariya and Rao, 1979) but have not described the nature of the toxic action (fungistatic/fungicidal). To the best of our knowledge, this the first report where the waste leaves of *Curcuma longa* L. which has no commercial value has been used for the extraction of essential oil and their antidermatophytic use, thereby making it economical.

The minimum static and minimum inhibitory concentration (MIC) of the oil was found to be 2.0 μ l/ml and 2.5 μ l/ml against test pathogens *Microsporum gypseum* and *Trichophyton mentagrophytes* respectively, while *Ligusticum chuanxiong* essential oil exhibited strong antidermatophytic action against *Trichophyton species* such as, *T. erinacei*, *T. mentagrophytes*, *T. rubrum*, *T. schoenleinii*, *T. tonsurans* and *T. soudanense* (Sim and Shin, 2008). Inouye *et al.* (2007) also using a wide variety of plant (*Cinnamomum zeylanicum*, *Eugenia aromatica*, *Geranium maculatum*, *Lavandula angustifolia*, *Cymbopogon citratus*, *Origanum vulgare*, *Cymbopogon martinii*, *Mentha piperita*, *Melaleuca alternifolia* and *Thymus vulgaris*) essential oils against *T. mentagrophytes* and *T. rubrum*. *Allium sativum*, *A. cepa* and *A. fistulosum* demonstrated strong antidermatophytic activity against three species of *Trichophyton*. This was confirmed by Pyun and Shin (2006).

In the present study, *Curcuma longa* L. leaf essential oil exhibited a broad spectrum antifungal activity against *Epidermophyton floccosum*, *M. nanum*, *T. rubrum*, *T. violaceum* at concentration of 2.0-2.5 μ l/ml. Antimicrobial activity of *Leptospermum petersonii*, *Syzygium aromaticum* essential oils against a wide range of dermatophytes such as *Trichophyton mentagrophytes*, *T. rubrum*, *Epidermophyton floccosum* and *Microsporum gypseum* were also noted (Park *et al.*, 2007). Likewise, different fractions, such as hexane, chloroform and essential oil of *Ocimum gratissimum* proved effective against *Microsporum canis*, *M. gypseum*, *Trichophyton*

rubrum and *T. mentagrophytes*, *Trichophyton rubrum* (Silva *et al.*, 2005).

Unlike the essential oil of *Adenocalymma allicea* where fungitoxicity expired after 21 days (Chaturvedi *et al.*, 1987), fungitoxicity of *C. longa* leaf oil persisted for 24 months. Hence, the oil can easily be used for developing a formulation without any fear of expiry.

The effectiveness of the oil was equal to those dermatophytes which are close in phylogenetic tree. To understand the relationship of the DNA sequences of the tested fungal strains and their variable response to the different concentrations of active fractions (extracted in the form of essential oil from the leaf of *Curcuma longa* L.) have been critically analyzed. Further, evaluation of the phylogenetic analysis and identification system, both of which are based on ITS1 rDNA sequences, are continuing in our laboratory with other species and strains.

CONCLUSION

The present study clearly demonstrates that the oil of *C. longa* leaves have good promise as an antifungal agent that could be used as a therapeutic remedy against human pathogenic fungi on account of its various *in vitro* and *in vivo* antifungal properties, *viz.*, strong fungicidal action, long shelf-life, its tolerability of heavy inoculum density, thermo stability, broad range of antidermatophytic activity and absence of any adverse effects. The response of the formulation on patients after 3 weeks of topical application showed that about 72 % of the cases were completely cured, thereby facing no cosmetic embarrassment. As such, the oil can be used as a potential source of effective and cheap herbal formulation after undergoing successful multicentral topical testing.

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