Broad spectrum antimycotic drug for the control of fungal infection in human beings

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During antifungal evaluation of the secondary metabolites (essential oil) of some *Eucalyptus* species, *E. citriodora* was found to be most effective as an antifungal against human pathogenic fungi *Microsporum nanum*, *Trichophyton mentagrophytes* and *T. rubrum*. While the pure oil (100%) killed the *M. nanum* in 20 s and *T. mentagrophytes*, *T. rubrum* in just 15 s, at their minimum fungicidal concentrations (MFCs) it required 5 h 30 min against *M. nanum* and 4 h against *T. mentagrophytes* and *T. rubrum*. On comparing the minimum effective concentration of the oil with those of prevalent synthetic antifungal drugs, the oil was found to be more effective. Moreover, it did not exhibit any adverse side effects on mammalian skin up to 5% concentration. The oil in the form of ointment broad spectrum antimycotic drug (BSAD) was subjected to topical testing on patients attending out patient department of M.L.N. Medical College, Allahabad. All patients showed positive potassium hydroxide (KOH) results at the beginning of the trial. Patients were diagnosed as either *tinea pedis* or *tinea cruris*. After the second week of treatment, all patients were KOH-negative. At the end of the third week of treatment, every patient was KOH-negative and remained negative when re-examined after one month of treatment, thereby leaving no scope for relapse. At the end of treatment, while 55.5% of patients recoverd completely, 44.5% showed significant improvement from the disease. The ointment thus not only showed maximum effectivity, but was also found to be cost effective, had long shelf life, and showed absence of any adverse side effects.

INFECTION caused by fungi in man and animal, mycosis, are common throughout the world. Dermatophytoses poses a serious concern to sociologically backward and economically poor populations of India.

Dermatophytoses is the disease caused by a group of fungi known as dermatophytes. It is also known as ringworm or *tinea* and involves superficial infections of keratinized tissue of the skin of animals and human beings. Clinical surveys carried out in India have shown ringworm as one of the most common dermatomycoses caused by the species of *Epidermophyton*, *Microsporum* and *Trichophyton*. The disease is predominant in tropical and subtropical countries due to their prevailing moisture and temperature regimes, and poses a therapeutic problem despite several antimycotic drugs available in the market. These drugs are mostly fungistatic. Besides, these drugs have recently been found to possess various side effects[1].

In recent years there has been a gradual revival of interest in the use of medicinal plants in developed as well as in developing countries, because herbal medicines have been reported to be safe and without any adverse side effects. Thus, a search for new drugs with better and cheaper substitutes, plant resources are a natural choice. Recently, some products of higher plants origin have been shown to be effective source of chemotherapeutic agents without undesirable side effects and with strong fungicidal activity[1–4]. These findings prompted us to explore other plant products which could be exploited as effective antifungals. We report here the detailed antifungal study of the essential oil, obtained from the leaves of *E. citriodora in vitro* as well as in clinical trials against dermatophytoses in human beings.

For our studies we extracted the essential oil from the fresh leaves of *E. citriodora* Hook by hydrodistillation, using Clevenger's apparatus[5]. The oil, thus obtained was evaluated for its antifungal properties against the test pathogens, *M. nanum*, *Fuentes*, *T. mentagrophytes* (Robin) Blanchards, and *T. rubrum* (Castellani) Sabouraud.

For *in vitro* investigations, the minimum effective concentrations (MECs) of the oil were determined following the poisoned food technique[6] with slight modifications[1]. Minimum fungistatic and fungicidal concentrations of the oil were determined by the method of Garber and Houston[7]. This was done by reinoculating the inhibited discs on sabouraud dextrose agar (SDA) medium in culture tubes[1]. Inoculated culture tubes were incubated at 27 ± 1°C and the observations recorded on seventh day. While fungal growth indicated fungistatic activity, its absence denoted fungicidal action.

The minimum killing time (MKT) of the oil was determined by mycelial disc killing technique[1]. Two treatment sets were maintained, one with pure oil and the other with the minimum fungicidal concentrations (MFCs) of the oil. The treatment set using MFCs of the oil was prepared by mixing the required quantity of the oil in acetone (5% of the total quantity of the treatment solution) and then added to required quantity of distilled water. Simultaneously, controls were maintained using sterilized water (in place of the oil) and acetone was added to the distilled water in required quantities.

Mycelial discs of 5 mm diameter, cut out from the periphery of 7-day-old cultures of the test pathogens, were aseptically placed inside 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 and sterilized distilled water in 1:2 ratio) to remove the treatment solution. These washed mycelial discs were aseptically transferred upside-down to the 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 on seventh day. Percentage of fungal growth inhibition (FGI) was calculated according to the formula[1].

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

where dc = colony diameter of control disc, and dt = colony diameter of treated disc.

The effect of inoculum density (increased progressively up to 30 discs in multiples of 5 and each of 5 mm diameter) of the test pathogens on toxicity of the oil was determined according to the procedure outlined by Dikshit and Dixit[8], using the agar-free medium as adopted by Shahi *et al.*[9]. The expiry of toxicity of the oil was determined by storing them at room temperature and testing their antifungal activity at minimum effective concentrations (MECs) at regular intervals of 60 days up to 60 months following the usual poisoned food technique[6].

For determination of the effect of temperature, five lots of the oil were kept in small vials, each containing 5 ml of oil separately. These were exposed to different temperatures, 40, 60, 80 and 100°C in the incubator for one hour. The antifungal activity of the oil was then tested at their MECs by the usual poisoned food technique[6].

Antifungal spectrum of the oil was determined at various concentrations, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 µl/ml, against some other test pathogens by the usual poisoned food technique[6]. Nature of toxicity, i.e. fungistatic/fungicidal of the oil was determined by the method of Garber and Houston[7].

The comparative study of the oil with some synthetic antifungal drugs was carried out by comparing MECs. The antifungal activity was assayed following the poisoned food technique[6] against the test pathogens. The nature of toxicity (i.e., fungistatic/fungicidal, at their MECs) of the antifungal compounds was ascertained by the method of Garber and Houston[7].

For *in vivo* investigations, experiments were carried out to find the maximum tolerable concentrations (MTCs) for irritant activity, if any, of the oil by their topical application on human skin, following the patch test method of Roxburgh and Borrie[10]. People of different sex in the age group of 10 to 30 years were selected randomly and a group of 30 individuals of each sex was constituted. Circular areas of 5 cm^2 on upper hairy and lower glabrous surface of palms and 3 cm^2 of neck region of each individual were first washed with distilled water followed by 70% ethyl alcohol and then allowed to dry for five min. Five drops of the graded concentrations of testing solution was applied to each individual separately. The volunteers were not allowed to wash the applied area. The qualitative observation have been recorded after 24 h.

For the clinical response of ointment 'BSAD' prepared from the oil, 1% concentration v/v of BSAD was applied topically on human patients for the control of fungal infections (dermatophytoses)[1]. The patients were treated with the ointment. Medication was administered twice a day for 3 weeks. The patients were not allowed

Table 1. Minimum effective concentrations of the oil of *Eucalyptus citriodora* against test pathogens

Concentrations (µl/ml)	Mycelial growth inhibition (%)		
	Microsporum nanum	*Trichophyton mentagrophytes*	*Trichophyton rubrum*
1.0	100[c]	100[c]	100[c]
0.9	100[s]	100[c]	100[c]
0.8	100[s]	100[c]	100[c]
0.7	89.9	100[s]	100[s]
0.6	65.0	100[s]	100[s]
0.5	35.1	100[s]	100[s]
0.4	–	100[s]	100[s]
0.3	–	83.1	76.6

[s]Fungistatic.
[c]Fungicidal.

Table 2. Minimum killing time of the oil of *E. citriodora* against test pathogens

Minimum killing time	Fungal growth inhibition (%)					
	M. nanum		*T. mentagrophytes*		*T. rubrum*	
	PO	MFC	PO	MFC	PO	MFC
6 h	100	100	100	100	100	100
5 h 30 m	100	100	100	100	100	100
5 h	100	79.1	100	100	100	100
4 h 30 m	100	65.2	100	100	100	100
4 h	100	30.3	100	100	100	100
3 h 30 m	100	–	100	75.1	100	77.3
3 h	100	–	100	51.2	100	69.3
2 h 30 m	100	–	100	30.2	100	51.0
2 h	100	–	100	–	100	–
1 h 30 m	100	–	100	–	100	–
1 h	100	–	100	–	100	–
30 m	100	–	100	–	100	–
15 m	100	–	100	–	100	–
5 m	100	–	100	–	100	–
60 s	100	–	100	–	100	–
30 s	100	–	100	–	100	–
20 s	100	–	100	–	100	–
15 s	89.2	–	100	–	100	–
10 s	61.3	–	87.1	–	76.6	–

PO = pure oil; MFC = minimum fungicidal concentration.

Table 3. Comparison of efficacy of the oil of *E. citriodora* with commercial antifungal drugs

Oil and trade name of antifungals	Active ingredients	Minimum effective concentrations (µl/ml)		
		M. nanum	*T. mentagrophytes*	*T. rubrum*
BSAD	Essential oil	0.8	0.4	0.4
Dactrine	Miconazol nitrate	6.0	5.0	6.0
Nizral	Ketoconazole	0.5	5.0	5.0
Tenaderm	Tolnaftate	1.5	0.4	0.8

Table 4. Patients showing clinical response of ointment prepared from the oil of *E. citriodora*

Response	Percentage of patients showing clinical response for three weeks		
	1	2	3
Worse	0.0	0.0	0.0
None	0.0	0.0	0.0
Partial improvement	62.5	25.0	0.0
Significant improvement	37.5	50.0	44.5
Completely clear	0.0	25.0	55.5

to take any other systemic or topical therapy during the course of study.

Patients of either sex were required to have a diagnosis for either *tinea pedis*, *tinea corporis* or *tinea cruris* based on site and type of infection. The diagnosis was further confirmed by microscopic examination of the scraping (from the infected area) treated with 10% potassium hydroxide (KOH). While the patients designated showing mycelium and/or conidia have been designated as KOH-positive, those showing their absence have been designated as KOH-negative. Only KOH-positive cases were enrolled for the study. Patients within the age group of 8 to 40 years were selected randomly, and a group of 30 individuals was formed. Patients having *onychomycosis*, *candidiosis* or *tinea versicolour* were excluded from the study.

Patients were examined just before the therapy was initiated and at the end of each week of the 3 weeks of treatment. Although when the cutaneous fungal disease manifested itself in several body areas, all affected areas could be treated, but only one was selected and designated as the reference lesion. At each visit of the patient, the same reference lesion was scraped for fungal culture to identify the organism and for demonstration of presence of hyphae by microscopic examination of the scrapings which were covered with 10% KOH preparations. Signs and symptoms of inflammation as erythema, scaling, itching, maceration, vesiculation and pustulation were recorded as absent, mild, moderate, or severe. At each visit of the patients, overall clinical improvement was rated as worse, none, partial, significant or completely clear by comparing the conditions with those existing at the time of initial visit. Any adverse systemic or local reaction was noted at each visit and recorded as mild, moderate or severe. Satisfactory response with KOH-negative cases after third week were re-examined after one month to find out the relapse rates, if any.

The results show that while the MECs of the oil of *E. citriodora* were found to be 0.8, 0.4 and 0.4 µl/ml against the test pathogens *M. nanum*, *T. mentagrophytes* and *T. rubrum*, the MFCs were found to be 1.0, 0.8 and 0.9 µl/ml against the same pathogens respectively (Table 1). The oil tolerated the heavy doses of inocula that inhibited the mycelial growth completely at their respective MECs.

While the pure oil killed the fungi in 20, 15, 15 s against *M. nanum*, *T. mentagrophytes* and *T. rubrum* respectively, at their MFCs it required 5 h 30 min against *M. nanum* and 4 h against *T. mentagrophytes* and *T. rubrum* (Table 2). The toxicity of the oil persisted up to 100°C, and its activity did not expire even up to 60 months, the maximum time taken into consideration.

The oil showed broad range of antifungal activity at 1.0 µl/ml inhibiting all the tested pathogens completely with fungistatic activity. The oil at 1.5 µl/ml was found to be fungicidal against all the tested pathogens, *Epidermophyton floccosum*, *M. audouinii*, *M. canis*, *M. gypseum*, *T. tonsurans* and *T. violaceum*. On comparing the MECs of the oil with those of the synthetic antifungal drugs, the MECs of the oil were found to be more active than MECs of dactrine, nizral and tenaderm (Table 3).

The oil when tested for its irritant activity on human skin, did not show any irritation or adverse effect up to 5% concentration. The response of the formulation 'BSAD' on patients after first week of topical application showed that while in 37.5% cases improvement was significant, in 62.5% cases improvement was partial. However, at the end of treatment, i.e. after third week of application, while 55.5% patients were completely cured, 44.5% patients showed significant improvement, i.e. all those patients who showed positive KOH results at the beginning of the trial. No KOH-negative cases of relapse were observed when patients were re-examined after a month following the end of treatment.

During cost-benefit analysis of the botanical product compared with synthetic antifungal drugs, the ointment was found to be most cost effective, with long shelf life, and showed absence of any adverse side effects (Table 5).

Thus, for assessing the total antifungal activity, candidate samples (drugs) have to be tested *in vitro* first and effective ones subjected to animal trials. But the experimental experiences of earlier investigators[2,11,12] showed that the result of topical testing of antifungal

Table 5. Comparison of cost-benefit analysis of botanical drug (BSAD) with commercial synthetic antimycotic drugs

Antimycotic drugs	Drugs percentage	Cost (Rs)		Adverse effects	Expiry duration mth	Environmental impact
		Ointment/g	Lotion/ml			
BSAD	1% v/v	0.90	0.70	No adverse side effects.	24 to 60	Renewable, biodegradable, absence of residual toxicity.
Dactarin	2% w/w	2.80	–	Occasionally produced gastrointenstinal side effects such as nausea, vomiting, and diarrhoea.	35	Non-renewable, non-biodegradable, and residual toxicity.
Nizral	2% w/w	3.75	3.17	Adverse reactions observed were mainly burning and irritation. The drug may block testosterone synthesis, lower serum testosterone, cause gynaecomastia, and loss of lipibo and potency.	24	Non-renewable, non-biodegradable, and residual toxicity.
Tenaderm	1% w/v	1.06	1.30	Adverse reactions observed were fever, nausea, vomiting, diarrhoea, and skin rash, rarely produced irritation.	24	Non-renewable, non-biodegradable, and residual toxicity.
Batrafine	1% w/v	1.50	1.60	Adverse reactions observed were fever, nausea, vomiting, diarrhoea, and skin rash	24	Non-renewable, non-biodegradable, and residual toxicity.

drugs for dermatophytoses in guinea pig model may not be the same as in human dermatophytoses. As such, following the clinical trials conducted by Shahi *et al.*[4] the present study was conducted for the topical testing of the oil of *E. citriodora* in the form of ointment 'BSAD' on human patients perhaps for the first time.

Although the undiluted oil of *E. globulus* has been shown to have some side effects when administered orally[13], in the present investigation the oil of *E. citriodora* interestingly did not show any irritation or adverse side effects up to 5% concentration on human skin. The ointment 'BSAD' effectively cured dermatophytoses at concentration of 1%.

Reactions of drugs vary with partients, being dependent on individual age, sex, and their immune system. Therefore, if the drugs respond favourably in 50% patients, it can be used as an alternative to synthetic drugs.

Hence, the oil of *E. citriodora*, owing to its strong antifungal activity, inhibiting heavy doses of inocula, with long shelf life, having fungicidal properties, and with no irritation on human skin, can be used successfully in the form of broad spectrum antimycotic drug for the control of fungal infection in human beings. The commercial viability of the oil can be determined after successful multicentre clinical trials, which is in progress.

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