Antifungal studies of some essential oils at various pH levels for betterment of antifungal drug response

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Antifungal evaluation of the stored essential oil of some *Eucalyptus* species, viz. *E. amygdalina*, *E. citriodora*, *E. dalrympleana* and *E. laveopinea* was tested against dermatophyte, viz. *Epidermophyton floccosum*, *Microsporum gypseum*, *M. nanum*, *Trichophyton mentagrophytes*, *T. rubrum* and *T. violaceum*. The efficacy of the oils increased for inhibiting the mycelial growth of test orgainsms by adjusting the pH of the media to 4, 7 and 9 from their normal pH of 5.6. The enhancement in the efficacy of various oils recorded as percentage changed in minimum fungistatic and minimum fungicidal concentrations. The minimum fungistatic and fungicidal concentrations decreased in the range from 12.5 to 80.0% and 11.1 to 75.0%, respectively against all the test organisms, except for the oil of *E. citriodora* which showed an increase of 25% in the minimum fungistatic concentration against *T. rubrum* and *T. mentagrophytes*. These oils at normal and alkaline pH inhibited heavy doses of inoculum potential. Moreover, oils did not show any adverse effect on mammalian skin up to 5% concentration at normal and alkaline pH.

SUPERFICIAL ringworm infections occur more frequently than subcutaneous and systemic mycoses and remain a therapeutic problem in tropical and subtropical countries, despite the availability of a number of antifungal ointments, paints, lotions and powders. Sources of these agents are largely nonrenewable petroproducts that are nonbiodegradable and cause adverse effects and residual toxicity[1]. Recently, some products of higher plant origin have been shown to be effective source of chemotherapeutic agents and provide renewable sources of useful antifungals of biodegradable nature which are devoid of side effects[1-3]. These findings prompted the exploration of other plant products that could be exploited as antifungals. In our preliminary screening, the essential oils extracted from fresh leaves of the *Eucalyptus* spp. and tested at 0.4 µl/ml were found to possess antifungal activity against dermatophytes[4], viz. *Microsporum gypseum* (Bodin) Guiart et Grigorakis, *Trichophyton mentagrophytes* (Robin) Blanchard and *T. rubrum* (Castellani) Sabouraud. The present communication describes detailed antifungal studies of the four effective stored essential oils of *Eucalyptus* spp., viz. *E. amygdalina* Labill, *E. citriodora* Hook, *E. dalrympleana* Maiden and *E. laveopinea* Baker with special attention to pH adjustment against test organisms, viz. *Epidermophyton floccosum* (Hartz) Langeron et Milochevitch, *Microsporum gypseum* (Bodin) Guiart et Grigorakis, *M. nanum* Fuentes, *Trichophyton mentagrophytes*, *T. rubrum* and *T. violaceum* Bodin which cause dermatophytoses in animals and human beings.

The oils were extracted from fresh leaves of four *Eucalyptus* spp. by hydrodistillation using Clevenger's apparatus[5] and tested at 1.0 µl/ml for their antifungal evaluation after storage for 10 years at 27 ± 5°C against the test organisms, viz. *E. floccosum*, *M. gypseum*, *M. nanum*, *T. mentagrophytes*, *T. rubrum* and *T. violaceum* by poisoned food technique[6] with slight modification[1].

The nature of toxicity, i.e. fungistatic/fungicidal along with the minimum effective concentrations (MECs) of the oils were ascertained by the method of Garber and Houston[7] with slight modification[1].

To study the effect of pH adjustment on the toxicity of the oil, the technique of Dikshit *et al.*[8] with slight modification[1], has been followed using citrate phosphate buffer[9] for pH 4 and 7, and boric acid–borax buffer[10] for pH 9. Two sets of culture media were prepared separately for control and treatments. In the test sets of pH 4, 7 and 9, requisite amount of the oil samples was mixed in acetone (2% of the required quantity of the medium) and then added into the sterilized Sabouraud dextrose agar (SDA) medium of respective pH levels. In controls of each buffered medium, the same volume of sterilized water (in place of the oil) and acetone was mixed in appropriate amounts.

Mycelial discs of 5 mm diameter, cut out from the periphery of 7-day-old culture of the test organisms were aseptically inoculated upside down on the surface of the SDA medium in plates. Inoculated petri plates were incubated at 27 ± 1°C and observations were recorded on the seventh day. Percentage of mycelial growth inhibition on different pH levels was calculated using eq. [I] of Shahi[1].

$$I = \frac{(c-t)\times 100}{c},$$

where I is % growth inhibition at adjusted pH, c is colony diameter at adjusted pH in controls, and t is colony diameter at adjusted pH in treatment.

The nature of toxicity, i.e. fungistatic/fungicidal of the oils at adjusted pH levels in treatments as well as in controls, was determined by reinoculating the inhibited discs on SDA medium (pH ± 5.6) in petri plates[1]. Fungal growth on the seventh day indicated fungistatic activity while its absence denoted fungicidal nature. Percentage of changed minimum static concentration (CMSC) as well as changed minimum cidal concentration (CMCC) of the oils at adjusted pH levels 4, 7 and 9 was calculated following the equations of Shahi[1].

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$$Cs\,(\%) = \frac{(S-s)\times 100}{S},$$

where Cs is % change in minimum static concentrations, S is minimum static concentration at normal pH, and s is minimum static concentration at adjusted pH.

$$Cc\,(\%) = \frac{(C-c)\times 100}{C},$$

where Cc is % change in minimum cidal concentrations, C is minimum cidal concentration at normal pH, and c minimum cidal concentration at adjusted pH.

The effect of inoculum density at normal and alkaline pH (increased progressively up to 30 discs in multiples of 5 and each of 5 mm diameter) of various test organisms on toxicity of the oils, was determined following the procedure outlined by Dikshit and Dixit[11] using the agar-free medium as adopted by Pandey *et al.*[12].

To see the irritant activity, if any, of the oils by their topical application on human skin at normal and alkaline pH 9, requisite quantity of the oil sample was mixed in PEG 400 LR and then the pH was adjusted using NaOH drop by drop with constant stirring[1]. The experiment was carried out following the patch test method of Roxburgh and Borrie[13].

All the four oils at 1.0 µl/ml inhibited 100% mycelial growth for all the organisms tested. The minimum static concentrations (MSCs) of oil from *E. amygdalina* ranged from 0.5 to 0.9 µl/ml, *E. citriodora* from 0.4 to 0.9 µl/ml and *E. laveopinea* from 0.3 to 0.4 µl/ml depending upon the test organisms while that of *E. dalrympleana* exhibited 0.3 µl/ml as the MSC for all the test organisms (Table 1) at the normal pH of the oils.

The minimum cidal concentrations of *E. citriodora* ranged from 0.8 to 1.0 µl/ml, *E. dalrympleana* and *E. laveopinea* from 0.5 to 0.8 µl/ml, depending upon the test organisms. The minimum cidal concentration of *E. amygdalina* could not be observed as it remained static at the concentrations tested (Table 2).

The MSCs of *E. amygdalina* and *E. citriodora* ranged from 0.5 to 0.9 µl/ml and 0.4 to 0.9 µl/ml, respectively at normal pH 5.6 which changed to 0.2 to 0.7 µl/ml at adjusted pH 4 and 7 against different test organisms (Table 3). In other words, the decrease in MSC ranged from 12.5 to 50.0%. However, the oil of *E. citriodora* showed an increase of MSC by 25% against *T. mentagrophytes* and *T. rubrum* at pH 7. Similarly, the MSCs of the oils of *E. laveopinea*, ranging from 0.3 to 0.5 µl/ml and of *E. dalrympleana*, at 0.3 µl/ml at normal pH 5.6 changed to 0.1 to 0.2 µl/ml. These differences in MSCs are statistically significant (Table 1). Their calculated decreased MSCs were found to be in the range 33.3–75.0%, 50.0–80.0% and 33.3–66.6%, respectively against various dermatophyte at adjusted pH levels 4 and 7. The MSCs at pH 9 could not be considered due to absence of mycelial growth in controls. Further, it was confirmed that higher pH (pH 9) checked the growth of the fungi but did not kill it, i.e. the effect was fungistatic (Table 4).

The minimum fungicidal concentrations of *E. citriodora*, *E. dalrympleana* and *E. laveopinea* ranged between 0.5 and 1.0 µl/ml at normal pH, changed to 0.2–0.9 µl/ml at pH 4, 7 and 9 (Table 2), thereby percentage of decreased minimum cidal concentration ranged between 11.1 and 75.0 at the pH of 4, 7 and 9 with different test organisms. Unlike the aforementioned oils, the effect of the oil of *E. amygdalina* remained static at the normal pH 5.6 as well as at pH 7. However, at altered pH 4 and 9 their minimum cidal concentrations were found to be variable between 0.6 and 0.8 µl/ml with different test organisms. On comparing the MSCs at normal pH 5.6, the essential oils of all the species of *Eucalyptus* withstood heavy doses of inocula, exhibiting 100% mycelial growth inhibition at their respective MSCs of various test organisms at normal and alkaline pH 9, and the oils did not show any irritation or adverse effect on mammalian skin up to 5% concentration at normal and alkaline pH 9.

Table 1. Minimum static concentration (µl/ml) with no mycelial growth on the essential oil of *Eucalyptus* spp. at normal and adjusted pH levels

	Essential oils of *Eucalyptus* spp. tested at various pH levels											
	E. amygdalina			*E. citriodora*			*E. dalrympleana*			*E. laveopinea*		
Fungi[a]	4	5.6[b]	7	4	5.6[b]	7	4	5.6[b]	7	4	5.6[b]	7
Ef	0.4	0.5	0.4	0.6	0.9	0.7	0.1	0.3	0.2	0.2	0.3	0.15
Mg	0.5	0.9	0.7	0.7	0.9	0.5	0.1	0.3	0.1	0.1	0.3	0.15
Mn	0.4	0.6	0.5	0.7	0.8	0.7	0.1	0.3	0.2	0.1	0.4	0.15
Tm	0.5	0.8	0.7	0.2	0.4	0.5	0.1	0.3	0.1	0.1	0.4	0.1
Tr	0.5	0.8	0.7	0.2	0.4	0.5	0.1	0.3	0.2	0.1	0.3	0.15
Tv	0.5	0.9	0.7	0.7	0.9	0.5	0.1	0.3	0.1	0.2	0.4	0.1
P value[c]	5.9	6.3		7.7	3.6		0.0	8.9		7.5	7.5	

[a]*Ef*, *Epidermophyton floccosum*; *Mg*, *Microsporum gypseum*; *Mn*, *Microsporum nanum*; *Tm*, *Trichophyton mentagrophyteis*; *Tr*, *Trichophyton rubrum*; *Tv*, *Trichophyton violaceum*.
[b]Normal pH of the medium.
[c]T test for pH differences.
Tabulated value at $p = 0.05$ is 2.23 for 6 d.f.

Very few antifungal substances are available in the market when compared to antibacterial ones and they are also relatively unsatisfactory in controlling the lesions produced by fungal organisms[13]. Stock[14] emphasized the need of powerful and specific antimycotic agents on an increasing scale to combat fungal infections. Discovery of essential oils exhibiting narrow or wide range of antifungal activity, as suggested in the present investigation, may prove useful in the development of effective antidermatophytic substances. Most workers in India have so far studied antifungal activity of candidate substances employing qualitative assay techniques[15,16] and have not described the nature of toxic action (fungistatic/fungicidal) encountered with such materials.

In the earlier study of the *Eucalyptus* spp. fresh oil was used by Dikshit[4]. Unlike the essential oil of *Adenocalymma allicea* where its fungitoxicity expired after 21 days[17], the present study was conducted with oils stored for 10 years which were quite effective against the tested dermatophytes, in contrast to plant pathogenic and other fungi, viz. *Alternaria alternata*, *Aspergillus flavus* and *Penicillium italicum* (Shahi, unpublished). Therefore, the fungitoxic stability of the oil has been found to be organism-dependent. Hence, fresh oils can easily be used for developing an ointment without any fear of expiry.

In the present investigation, the minimum static/cidal concentrations of all the oils were determined except the oil of *E. amygdalina* which remained static at all the tested concentrations (Table 2). Thus, the oils may be strictly fungicidal or fungistatic or their nature may be dose-dependent. Further, the variations in minimum fungistatic concentration among the various *Eucalyptus* spp. against the test organisms are probably due to variations in the cineole contents (27–78%), the major constituent of essential oils as suggested by Singh *et al.*[18].

During pH adjustment studies, absence of growth in control sets (at pH 9) makes this part of the experiment apparently insignificant. But reinoculating the inhibited discs of control and test sets revealed the expression of differential activity, i.e. pH 9 is inhibitory in controls with fungistatic action while pH 9 along with required concentrations of the oils exhibited fungicidal nature. As such, it is significant to extend the test further instead of discarding it at a preliminary level. In an earlier study, the toxicity of the oils of *Cedrus deodara* and *Mentha arvensis* increased at adjusted pH levels against the plant pathogen *Helminthosporium oryzae*[8].

An ideal drug for topical application should not cause any irritation or burning effect on human skin. Therefore, the oils were tested for their irritant activity, if any, on

Table 2. Percentage of changed minimum static concentration of the essential oil of *Eucalyptus* spp. at adjusted pH levels

	Essential oils of *Eucalyptus* spp. tested at various pH levels											
	E. amygdalina			*E. citriodora*			*E. dalrympleana*			*E. laveopinea*		
Fungi[a]	4	7	9	4	7	9	4	7	9	4	7	9
Ef	20.0	20.0	–	33.3	22.2	–	66.6	33.3	–	33.3	50.0	–
Mg	44.4	22.2	–	22.2	44.4	–	66.6	66.6	–	66.6	50.0	–
Mn	33.3	16.0	–	12.5	12.5	–	66.6	33.3	–	75.0	62.5	–
Tm	37.5	12.5	–	50.0	– 25	–	66.6	66.6	–	75.0	75.0	–
Tr	37.5	12.5	–	50.0	– 25	–	66.6	33.3	–	66.6	50.5	–
Tv	44.4	22.2	–	22.2	44.4	–	66.6	66.6	–	50.0	75.0	–

[a]*Ef, Epidermophyton floccosum*; *Mg, Microsporum gypseum*; *Mn, Microsporum nanum*; *Tm, Trichophyton mentagrophyteis*; *Tr, Trichophyton rubrum*; *Tv, Trichophyton violaceum*.
–, Not considered.

Table 3. Minimum cidal concentration (µl/ml) of the essential oil of *Eucalyptus* spp. at normal and adjusted pH levels

	Essential oils of *Eucalyptus* spp. tested at various pH levels															
	E. amygadalina				*E. citriodora*				*E. dalrympleana*				*E. laveopinea*			
Fungi[a]	4	5.6[b]	7	9	4	5.6[b]	7	9	4	5.6[b]	7	9	4	5.6[b]	7	9
Ef	–	–	–	0.8	0.9	–	–	0.7	0.8	–	–	0.4	–	–	–	0.3
Mg	0.7	–	–	0.8	0.8	–	–	0.6	0.8	–	–	0.4	0.5	–	–	0.3
Mn	–	–	–	0.8	0.8	0.9	–	0.6	0.4	0.5	–	0.5	–	–	–	0.3
Tm	0.7	–	–	0.7	0.3	0.8	–	0.2	0.6	0.8	–	0.4	0.2	0.8	0.6	0.3
Tr	–	–	–	0.6	0.6	1.0	0.7	0.4	0.6	0.8	0.7	0.4	0.4	0.5	0.3	0.3
Tv	0.7	–	–	0.7	0.8	–	–	0.6	0.4	0.8	–	0.4	0.2	0.8	–	0.3

[a]*Ef, Epidermophyton floccosum*; *Mg, Microsporum gypseum*; *Mn, Microsporum nanum*; *Tm, Trichophyton mentagrophyteis*; *Tr, Trichophyton rubrum*; *Tv, Trichophyton violaceum*.
[b]Normal pH of the medium.
–, Remained static.

Table 4. Percentage of changed minimum cidal concentration of the essential oil of *Eucalyptus* spp. at adjusted pH levels

	Essential oils of *Eucalyptus* spp. tested at various pH levels								
	E. citriodora			*E. dalrympleana*			*E. laveopinea*		
Fungi[a]	4	7	9	4	7	9	4	7	9
Ef	–	–	–	–	–	–	–	–	–
Mg	–	–	–	–	–	–	–	–	–
Mn	11.1	–	33.3	20.2	–	0.0	–		
Tm	62.5	–	75.0	25.0	–	50.0	75.0	25.0	62.5
Tr	40.0	30.0	60.0	25.0	12.5	50.0	20.0	40.0	40.0
Tv	–	–	–	50.0	–	50.0	75.0	–	62.5

[a]*Ef, Epidermophyton floccosum*; *Mg, Microsporum gypseum*; *Mn, Microsporum nanum*; *Tm, Trichophyton mentagrophyteis*; *Tr, Trichophyton rubrum*; *Tv, Trichophyton violaceum*.
–, Remained static.

mammalian skin at their normal and alkaline pH. Since the oils showed increased antifungal activity at pH 9 during *in vitro* investigation, they were tested for their irritant activity at pH 9. The oils did not produce any irritation or adverse effect up to 5% concentrations. It can be concluded that ointments prepared from the oils may be most effective at pH 9 without causing any irritation on the human skin. Therefore, the present study clearly demonstrates that the oils of *Eucalyptus* spp. hold good promise as antidermatophytic agents which could be used in therapeutic remedy against dermatophytoses on account of their various fungitoxic properties, viz. antifungal, long shelf life, can withstand heavy inoculum density, efficacy at various pH levels, wide range of activity and absence of any adverse effect. These results can be interpreted with caution. Although the *in vitro* susceptibility testing shows promising activity against commonly encountered dermatophytes, its clinical usefulness should be established by further studies. Hence, the oils can be easily used as ointments for better results to the control of fungal infection in human beings.

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Two-dimensional NMR spectroscopic study of fibroblast and fibrosarcoma cell lines

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Two-dimensional NMR spectroscopy has been used to study a fibroblast (MRC-5) and human fibrosarcoma cell lines (HFS-9 and HT-1080). The cell lines were graded based on their tumorigenic characteristics in nude mice, and the synthetic phase fraction of their cell cycle. Analysis of the spectra from cells suggests an increase in the levels of lipids, metabolites and resonance patterns attributed to fucosylated antigens as a function of increasing tumorigenicity. The paper discusses the use of one- and two-dimensional ^{1}H NMR techniques in the gradation of fibrosarcoma cells and in differentiating them from the normal homologue, namely fibroblast cells.

SOFT tissue tumours/sarcomas are a heterogenous group of tumours that arise as soft tissue masses and usually exhibit the differentiated features of adult soft tissue, although in some cases there is no clearly defined normal tissue homologue. Soft tissue includes smooth and striated muscle, fat, fibrous tissue and the vessels that serve these tissues. These tumours can occur anywhere in the body and at any age although the distribution varies according to the histological type. Benign tumours are at least 100

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