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In vitro Evaluation of Antidermatophytic Activity of Egyptian Bee Propolis in Combination with Plant Essential Oils in Sheep Hoof Plate: An Experimental Model

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Bee propolis ethanolic extract with some plant essential oils was investigated for its antidermatophytic properties. The tested plant essential oils included jasmine, clove, lemon, Arabian jasmine, mint, rosa, olive and basil. The antidermatophytic activity has been compared to Naftifine-HCl and Clotrimazole used for dermatophyte treatment. Experimental model has been tested using sheep hoof plate for the *in vitro* tests to stimulate human nails. Mint, clove and basil with 4 mg/m*l* of bee propolis have a comparable efficacy to those of Naftifine-HCl and Clotrimazole. There is a great necessity for new effective low price and safe antidermatophyte agents to avoid recurrent infection. Propolis synergistic could be of great importance with essential oils of plants in dermatophyte therapy.

KEYWORDS: Antifungal drugs, Bee propolis, Dermatophytes, Plant essential oils

The available antifungal agents have a limited effectiveness and lack specificity against the dermatophytes. Dermatophytes are attacking skin, nails, hair and foot. It is well known that there are three fungal genera responsible for the dermatophytic infection namely *Trichophyton*, *Microsporum* and *Epidermophyton*. Foot and nail mycoses are resistant to antifungal agents and there are recurrent infections. As Williams (1993) indicated that fungal infection of the nails include primary nail pathogens, which invade healthy nail plate, and those, which invade secondarily in subjects with pre-existing nail disease. Fungal nail infections onychomycoses are caused by three groups of pathogens: dermatophytic fungi such as *T. rubrum*, yeasts such as *Candida albicans* and non-dermatophytes such as *Scopulariopsis brevicaulis*, *Aspergillus* species or *Hendersonula toruloidea* and by various combinations of these three groups (Moharrem, 1999). In Egypt, onychomycosis represented 2% of human dermatophytic disease (Mahmoud, 1991).

Recently, the number of the antidermatophytic medications for systemic and topical treatment has been multiplied. The available antifungal agents include allylamine, azoles and others have severe side effects and inefficient. Dermatologists often attribute treatment failures to short therapies and lacking the drug specificity. Actually the penetration of antifungal drugs is very poor in respect to nails. Many researchs used different keratinous substrates in order to evaluate the efficiency of antidermatophytic agents, Ceshin-Roques *et al.* (1991) used pigskin and Malecky and McClausland (1982) used ovine hoof. However, Hemidy *et al.* (1994) found that sheep hooves seem to possess quite similar physico-chemical properties to those of human nails, although they are thicker about 1.3 mm and harder. Benfields and Clissold (1988) studied the antifungal efficiency of 1% (w/v) solution of Caryophyllene oxide, sulconazole, and ciclopriroxolamine against dermatophytes strains and found that sulconazole and ciclopiroxolamine have the ability to penetrate human nails in onychomycosis model. The aim of this work was to develop new and safe antidermatophytic agents that might be more effective for treatment with no side effects. Therefore, some plant essential oils have been screened for its antidermatophytic activity with and without bee propolis.

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Material and Methods

Essential oils and antifungal agents. Essential oils were obtained from industrial oil company in Egypt, however Naftifine-HCl (Exoderil) was obtained from October Pharma S.A.E., Cairo under license of Pfizer Inc. USA, and Clotrimazole (Lotrimin) was obtained from Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Tanta University, Egypt.

Propolis. Propolis (bee glue) was collected from Tanta area, Egypt during March 2002.

Propolis extraction and sample preparation. One gram of propolis was cut into small pieces and extracted at room temperature with 50 m*l* of 70% ethanol (twice after 24 hours). Then the alcoholic extract was evaporated

under vacuum at 50°C until dryness.

Fungal strains. *Epidermatophyton floccosum, Trichophyton rubrum, Microsporum canis* and *Candida albicans* were isolated from about 10 patients with dermatophytosis of Tinea cruris, Tinea unguium, and Tinea corporis as diagnosed by dermatologist of Tanta University hospitals, Tanta, Egypt. These organisms were isolated on Sabouraud dextrose agar amended with 0.05% of each of cycloheximide and chloramphenicol (Al-Doory, 1980). The isolated cultures were incubated up to 3 weeks at 28°C during which the growing fungi were identified and purified.

Antifungal assay on agar medium. To evaluate the effect of essential oil and bee propolis on dermatophyte growth, these were sterilized by Millipore filter (0.22 μm) and mixed with cooled autoclaved Sabouraud dextrose medium at concentration at 0.5, 1.0, and 5.0%. Tween 80 was added to the medium at concentration of 1% for emulsification. Also, bee propolis was added to the medium containing the essential oil to give final concentration of 4 mg/ml. The Petri dishes with control plates containing media without and with oils were inoculated with mycelial discs (10 mm diameter) of each of the tested organisms and incubated at 28°C for the specified period of time. The inhibition percentage was calculated using the formula of Vincent (1927):

$$I = \frac{C - T}{C} \times 100$$

Where I = percent of inhibition of fungal growth, C = fungal growth of check and T = fungal growth of treatment.

***In vitro* determination of penetration and absorption of essential oils with bee propolis into sheep hoof (an experimental model).** Sheep hoofs were cut into plates of 2.5 cm length and 1.3 cm width and 0.5 cm (thickness) and then sterilized by 75% ethanol and washed in sterile dist.water. Then, in sterile Petri-dish, culture agar blocks of the dermatophyte fungus under investigation of 1.6 cm were cut and placeded above sterile filter paper and the hoof plates were placed on the culture block, where the external hoof surface is in contact with the dermatophyte. The whole plates were transferred into a larger petri-dish containing sterile water to prevent dehydration (Fig. 5). Daily adding of a Sabouraud broth at the side and the base of culture block kept the dermatophyte moist. The essential oils with bee propolis and the commercial antifungal drugs were added with a micropipette directly onto the hoof internal surface on daily basis for 14 days.

Strategy of dermatophyte treatments. Two protocols were tried, simultaneous treatment and post-treatment. In simultaneous treatment an essential oils with bee propolis were added into the hoof at the same time. However in the post-treatment the antifungal agents were applied after 7 days of fungal agar block implantation on the external face of hoof plate in order to get a similar environment to clinical infection. Post treatment is more similar to the topical therapy in practice (Cohen and Richard, 1994). Growth controls without antifungal treatments were set up. The percentage of growth inhibition exerted by tested agents was evaluated.

Results

Antifungal activity of essential oils with or without bee propolis in agar medium. In search for new antifungal agents with a lower toxicity redundant, essential oils with bee propolis as a natural components have been tested for antifungal activity against dermatophytic fungi and *C. albicans*. Current antifungal treatment has had disappointingly low success rate and high prices. Evaluation of the inhibitory effect of jasmine, clove, Arabian jasmine, mint, rosa, olive at 0.5, 1.0 and 5.0% showed inhibitory effects to the tested dermatophytic fungi (*T. rubrum, M. canis, E. floccosum*) and *C. albicans*. Largely incorporating 4 mg/ml of bee propolis to the all tested essential oils enhanced the inhibitory effect of the tested essential oils.

Figure 1(A) shows that clove and olive oils have the highest percentage of growth inhibition for *T. rubrum* at

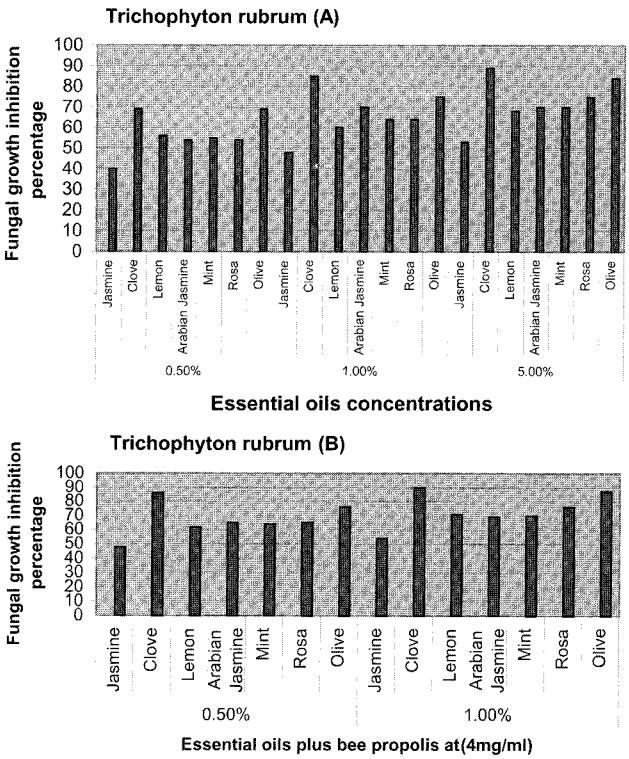

Fig. 1. Effect of plant essential oils on *Trichophyton rubrum* growth at different concentrations for two weeks at 28°C without bee propolis **(A)** and with bee propolis **(B)**.

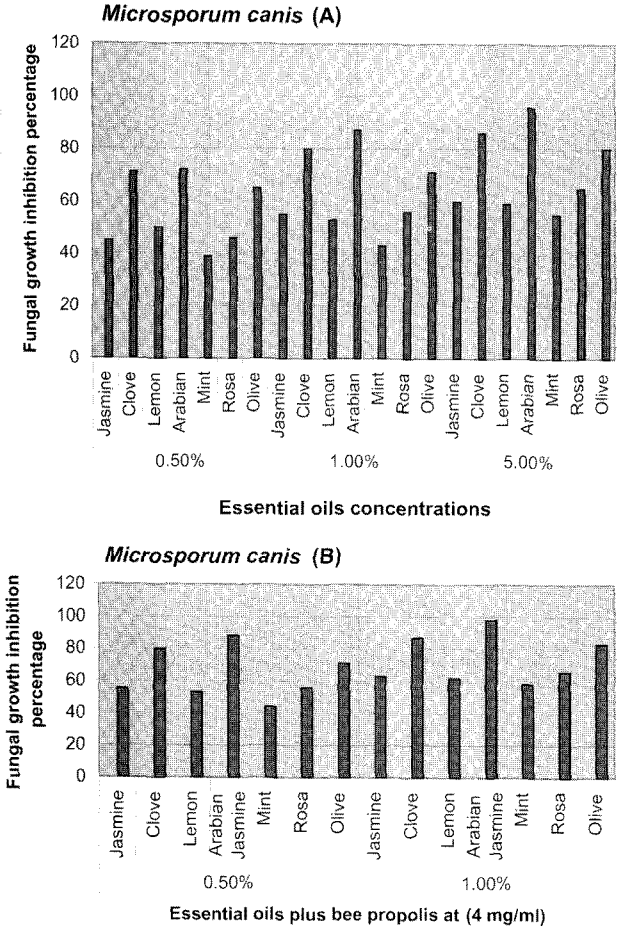

Fig. 2. Effect of essential oils on *Microsporum canis* growth without bee propolis **(A)**, and with bee propolis **(B)** for two weeks at 28°C.

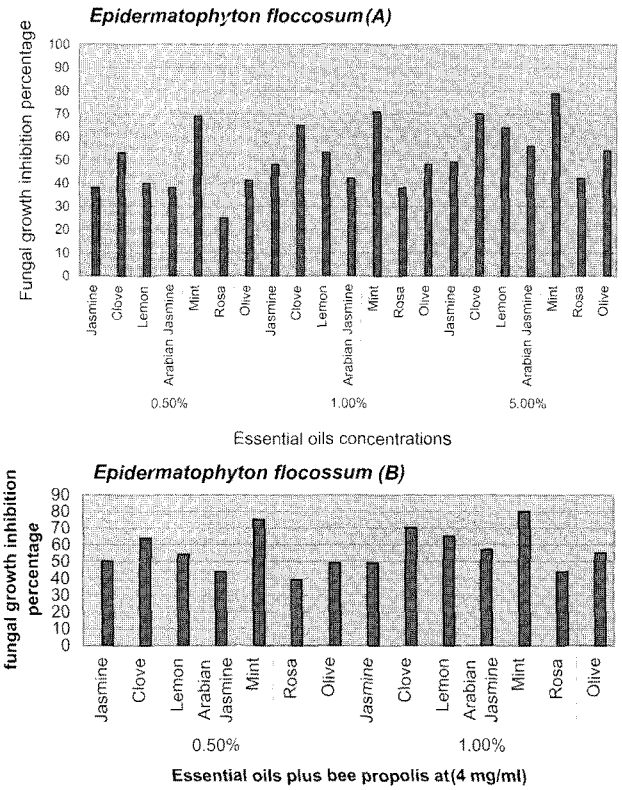

Fig. 3. Effect of essential oils on *Epidermatophyton floccosum* growth without bee propolis **(A)**, and with bee propolis **(B)** for two weeks at 28°C.

different tested oil concentrations; clove oils performed 70, 85 and 90% of growth inhibition for *T. rubrum* at 0.5, 1 and 5% of oils respectively.

Adding the bee propolis to the different oils greatly enhance the antidermatophytic activity, where clove oil performed 90% growth inhibition for *T. rubrum* at 1% concentration which was produced at 5% concentration of clove oil only (Fig. 1B). In case of *M. canis* arabian jasmine oil proved to be the most effective one a mong eight tested oils, where it produced nearly 100% inhibition at 5% (Fig. 2A).

The addition of bee propolis enhanced the antifungal activity of Arabian jasmine oil against *M. canis*; the antifungal activity of 5% of Arabian jasmine oil alone was comparable with that of 1% Arabian jasmine oil in combination with bee propolis (Fig. 2B).

Epidermatophyton flocossum was largely inhibited with mint essential oil followed by clove oil (Fig. 3A, B). Mint as an essential oil produced 80% *E. floccosum* growth inhibition at 5%. The addition of 4 mg/m*l* of bee propolis to that oil produced nearly the same inhibition percentage at 1%. The antimycotic activity of the essential oils with bee propolis was also screened against *C. albicans* a clinically important infectant. Clove oil followed by mint and jasmine performed the strongest growth inhibition of *C. albicans* (Fig. 4A, B). Also, bee propolis enhanced the antifungal efficacy of plant essential oils tested.

Sheep hoof onychomycosis experimental model. Results obtained from onychomycosis experimental model using sheep hoof as testing material infected with the tested dermatophytes indicated a treatment ability for the tested essential oils with bee propolis (Table 1). The mixture of essential oil (1%) and bee propolis (4 mg/m*l*) proved to be effective in eliminating the dermatophyte infection. In post treatment of infected sheep hoof with essential oils plus bee propolis, rosa, clove showed highest activity against *C. albicans*, *E. floccosum*, *T. Rubrum* and *M. canis*, respectively. Essential oil (mint, clove and basil) at different concentrations (0.5, 1.0, 5 and 10% with 4 mg/m*l* of bee propolis) was tested for antifungal activity against dermatophytic fungi and *C. albicans* (Tables 2, 3 and 4). Mint, clove and basil have been chosen to be tested in sheep hoof experimental model because they gave a pronounced inhibition effect on petri dishes testing. Mint oil (10%) with bee propolis performed a good inhibition of *C. albicans* and a considerable inhibition for *T. rubrum* and *M. canis*. However, *E. floccosum* is the least affected one during the dermatophyte post treatment (Table 2). On

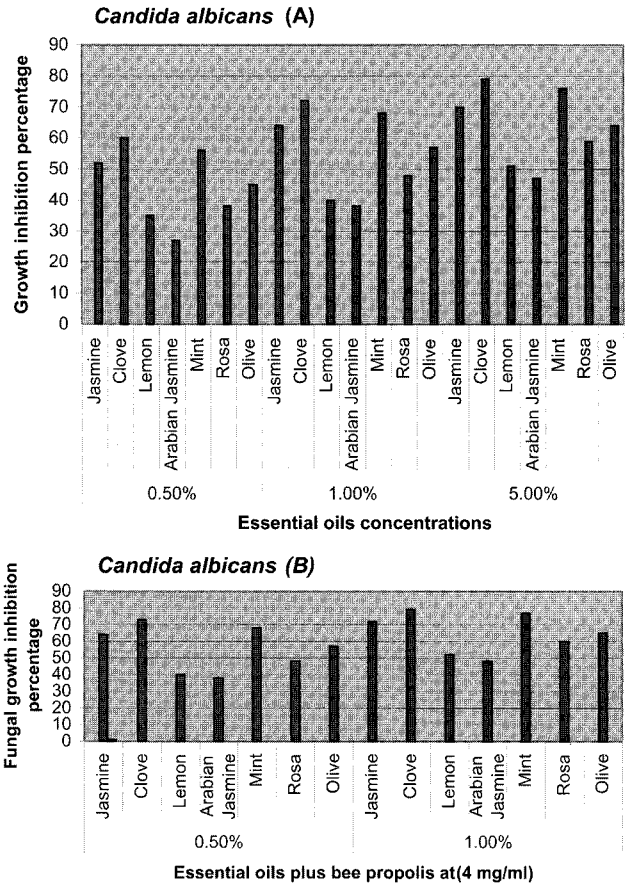

Fig. 4. Effect of essential oils on *Candida albicans* growth without bee propolis **(A)**, and with bee propolis **(B)** for two weeks at 28°C.

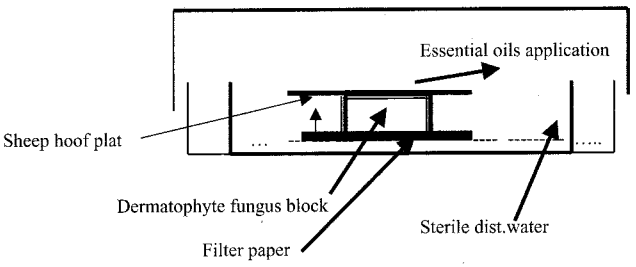

Fig. 5. Experimental Model for *in vitro* evaluation of antidermatophytic activity of bee propolis and essential oils.

the other hand, clove oil (10%) with bee propolis in post fungal treatment proved to be excellent antifungal agents for against the four tested fungi where it gave 80% growth inhibition (Table 3). Additionally, basil oil (10%) with bee propolis exhibited 65~78% of growth inhibition of the four fungi in case of the post treatment which is the more real to the fungal infection (Table 4). Comparing the efficiency of the essential oils (clove, mint and basil with bee propolis) with commercially available antidermatophytic drugs like Naftifine-HCl (Table 5) and Clotrimazole (Table 6) indicated that 10% of the essential oil with bee propolis more or less has the same efficiency of the commercial antidermatophytic agents (with 5% concentration).

Discussion

Plant essential oils are not real oils. They are the subtle, aromatic and volatile liquids extracted from the leaves

Table 1. Dermatophyte development on implanted sheep hoof treated with some essential plant oils and bee propolis at (4 mg/ml) after two weeks

Treatments at (1%)	Dermatophyte development (%)			
	Candida albicans	*Epidermatophyton floccosum*	*Trichophyton rubrum*	*Microsporum canis*
Simultaneous treatment				
Jasmine	23±2.0[a]	40±3.2	39±3.0	30±2.2
Clove	42±3.5	37±3.0	53±4.5	42±3.4
Lemon	17±1.0	25±2.1	42±3.4	28±1.2
Arabian Jasmine	19±1.6	38±3.2	40±3.2	28±2.1
Mint	28±2.0	46±4.5	35±3.0	52±4.6
Rosa	42±4.0	28±1.6	43±4.1	38±2.8
Olive	38±4.1	18±2.1	28±2.5	17±1.0
Basil	33±2.0	45±3.5	48±4.0	61±5.3
Post treatment				
Jasmine	26±2.0	47±3.5	42±3.5	34±3.1
Clove	45±4.0	35±3.4	70±6.5	40±4.0
Lemon	20±2.0	29±2.0	46±4.1	31±2.5
Arabian Jasmine	23±1.6	45±4.0	45±3.2	32±2.6
Mint	30±3.0	87±7.6	42±3.2	63±5.0
Rosa	50±4.2	25±2.0	46±4.5	41±3.2
Olive	42±3.2	23±2.0	32±3.1	23±2.0
Basil	41±3.2	51±4.2	51±4.6	62±5.2

[a]Mean±SD (n = 3).

Table 2. Percentage of dermatophyte development on implanted sheep hoof treated with bee propolis at (4 mg/m*l*) and with different concentrations of Mint, after two weeks

Treatments	Microorganisms	Mint (%)			
		0.5	1.0	5.0	10
Simultaneous treatment	*C. albicans*	36±3.0[a]	30±3.0	28±2.0	18±1.0
	E. floccosum	50±4.1	45±4.0	47±3.1	32±3.0
	T. rubrum	38±3.2	30±3.2	35±2.1	28±2.1
	M. canis	61±4.5	56±4.8	54±4.2	45±3.6
Post treatment	*C. albicans*	43±3.0	34±3.2	30±3.0	27±1.6
	E. floccosum	93±7.8	85±7.9	89±7.0	78±6.3
	T. rubrum	54±4.2	46±4.5	44±3.5	38±2.6
	M. canis	79±6.8	70±6.4	65±5.2	45±3.2

[a]Mean±SD (n = 3).

Table 3. Percentage of dermatophyte development on implanted sheep hoof treated with bee propolis at (4 mg/m*l*) and different concentrations of Clove, after two weeks

Treatments	Microorganisms	Clove oil (%)			
		0.5	1.0	5.0	10
Simultaneous treatment	*C. albicans*	58±4.5[a]	47±4.2	25±2.1	18±2.0
	E. floccosum	52±4.9	45±3.9	30±2.7	23±2.0
	T. rubrum	69±5.2	60±5.7	35±3.5	18±1.0
	M. canis	55±3.8	43±4.1	20±2.8	14±1.2
Post treatment	*C. albicans*	57±4.2	48±4.0	35±3.2	20±2.2
	E. floccosum	53±3.8	40±3.8	27±2.7	18±1.9
	T. rubrum	88±6.8	75±6.8	28±2.1	18±1.7
	M. canis	67±4.	56±4.1	35±3.1	22±2.8

[a]Mean±SD (n = 3).

Table 4. Percentage of dermatophyte development on implanted sheep hoof treated with bee propolis at (4 mg/m*l*) and different concentrations of Basil, after two weeks

Treatments	Microorganisms	Basil (%)			
		0.5	1.0	5.0	10
Simultaneous treatment	*C. albicans*	46±3.0[a]	34±3.0	25±2.0	18±1.0
	E. floccosum	52±4.1	45±4.0	37±3.1	22±3.0
	T. rubrum	68±3.2	50±3.2	45±2.1	28±2.1
	M. canis	71±4.5	61±4.8	44±4.2	35±3.6
Post treatment	*C. albicans*	53±3.0	41±3.2	32±3.0	22±1.6
	E. floccosum	63±7.8	52±7.9	41±3.0	28±6.3
	T. rubrum	53±4.2	49±4.5	34±3.5	28±2.6
	M. canis	79±6.8	60±6.4	45±5.2	35±3.2

[a]Mean±SD (n = 3).

stems, seeds bark, flowers and roots of various plants through distillation. Essential oils are soluble in the lipids in the skin and in most cases easily penetrate it and are absorbed into the bloodstream. They have advantages over the antidermatophytic agents, because essential oils have no side effects. Ahmed and Agnihotri (1977) reported antifungal activity of volatile oils obtained from several plants including, *Mentha piperata* against *Alternaria brassicae*, *Colletotrichum papaya* and *Helminthosporium* spp. Deshnukh *et al.* (1986) also studied the mycotoxicity of some essential oils against *Trichophyton equinum*, *T. mentagrophytes*, *T. rubrum*, *T. terrestre*, *M. gypseum* and *Keratinomyces ayelloi*. El-Naghy *et al.* (1992) tested the inhibitory effects of some natural oils and fatty acids on the growth of some of the dermatophytes and they revealed high fungistatic effects of clove and peppermint oils. *Eucalyptus melliodora* (yellow box) leaves were found to have a cineole level of 71.2% the remainder consisting mainly of terpenes such as pinene and limonene. Analysis of the red gum *E. blakelyi* revealed a wide range of constituents, the highest levels were for cymene (30%), cryptone (11.7%) and pinene. Investigation revealed that cymene is used in perfumery and as a solvent, while cryptone possesses marked germicidal properties and had been used in the manufacture of disinfectants. Plant oils can be applied and used as liquid, sprays, crystals, gels and pellets, and by impregnating material. Other variations are also used, which means the safe use of those oils. Inouye,

Table 5. Percentage of dermatophyte development on implanted sheep hoof treated with different concentrations of Naftifine-HCl, after two weeks

Treatments	Microorganisms	Naftifine-HCl (%)		
		0.1	1.0	5.0
Simultaneous treatment	*C. albicans*	45±3.2	50±5.0	85±7.4
	E. floccosum	35±2.6	24±2.1	10±1.0
	T. rubrum	48±3.4	31±3.0	12±1.3
	M. canis	61±5.1	42±3.5	21±2.0
Post treatment	*C. albicans*	50±4.3	62±5.1	75±6.2
	E. floccosum	28±1.9	18±1.0	11±1.0
	T. rubrum	37±3.2	20±2.0	14±1.3
	M. canis	45±4.0	30±3.0	22±2.0

[a]Mean±SD (n = 3).

Table 6. Percentage of dermatophyte development on implanted sheep hoof treated with different concentrations of Clotrimazole, after two weeks

	Microorganisms	Clotrimazole (%)		
		0.1	1.0	5.0
Simultaneous treatment	*C. albicans*	45±3.2[a]	30±3.0	18±1.1
	E. floccosum	42±3.6	22±2.0	10±1.0
	T. rubrum	38±3.2	27±2.5	12±0.9
	M. canis	51±5.0	32±3.0	21±1.8
Post treatment	*C. albicans*	40±3.8	32±3.1	20±2.0
	E. floccosum	38±2.8	28±2.6	11±0.9
	T. rubrum	39±3.2	20±2.1	14±1.3
	M. canis	47±4.3	30±3.0	22±2.0

[a]Mean±SD (n = 3).

et al. (2000) studied the inhibitory effect of essential oils on apical growth of *Aspergillus fumigatus* by vapour contact and they concluded that suppression of the fungus apical growth by vapour contact was ascribed to the direct disposition of essential oils on fungal mycelia, to gether with an indirect effect via the agar medium absorption. It has been reported that the most of plant essential oils contains phenols mostly, particularly thymol (Sainsbury and Safowora, 1971) and that these are probably responsible for its reported antimicrobial action. In addition to the phenolic nature of some essential oils there are some others contain terpenoid and aldehyde class of compounds (Cowan *et al.*, 1999). So, the degree of antidermatophytic activity of mint, clove and basil might return to its contents of aldehyde or phenols and/or terpenoid class present. Studying the mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil) revealed that minimum bactericidal/fungicidal concentrations of tea tree oil inhibited respiration and increased the permeability of organisms plasma membranes as indicated by uptake of propidium iodide. It is worth to be mentioned out that adding bee propolis to essential oils greatly enhanced their activity towards dermatophytes. The actual composition of propolis varies according to the area of collection. In general terms, it is composed of 50% resins and vegetables balsams, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% other substances including organic detritus (Callejo *et al.*, 2001). Propolis has been shown to be a non-specific immunostimulators.

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