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FORMULATION OF AN ANTI-DERMATOPHYTE CREAM FROM HYDRO-ALCOHOLIC EXTRACT OF EUCALYPTUS *CAMALDULENSIS* LEAVES

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Abstract

Throughout the world, there has been an increasing incidence of fungal infections, and because of drug resistance and toxicity associated with long-term treatment with antifungal drugs, search for new drugs to treat fungal infections is ongoing. The aim of the present study was to formulate herbal antifungal cream containing hydro-alcoholic extract of *Eucalyptus camaldulensis* as an anti-dermatophytic preparation and evaluate its physicochemical properties and stability. Firstly, the minimum inhibitory concentration (MIC) of hydro-alcoholic extract of leaves of *E. camaldulensis* was determined against various dermatophyte species by *in vitro* tube dilution technique. To select the best cream formulation, one general formula of cleansing cream was considered and then corrected. The best base formula was chosen according to its monotonousness, straightness and external attractiveness. The formulation containing 1% of the plant extract was prepared and controlled by standard methods. Finally, a cream containing bees wax 10%, liquid paraffin 58.8%, hard paraffin 1.2%, spermaceti 5%, borax 1.5%, tween 80 1.5%, 0.15% methyl paraben, 0.05% propyl paraben, 0.15% lactic acid, 1% *Eucalyptus* extract and water was chosen as the best formulation. The final product was a w/o emulsion cream with suitable appearance and desirable physicochemical stability. Due to the stability of the extract in the cream formulation, it can be formulated for treatment of fungal skin infections.

Keywords:

Eucalyptus camaldulensis; Cream; Dermatophyte.

Introduction

Eucalyptus camaldulensis Dehnh. is a common and widespread tree along watercourses over much of mainland Australia. *The plant* is a perennial, single-stemmed, large-boled, medium-sized to tall tree to 30 m high (1), although some authors (2,3) record trees to 45 m. The phytochemical analysis of the crude extracts of the *Eucalyptus* spp. revealed the presence of saponin, saponin glycosides, steroid, cardiac glycoside,

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tannins, volatile oils, phenols and balsam (gum)(4). Qualitative phytochemical tests, thin layer chromatography (TLC) and TLC-autography of certain active extracts demonstrated the presence of common phytocompounds in the plant extracts including phenols, tannins and flavonoids as major active constituents. Some main compounds in the leaves of *E. Camaldulensis* include essential oil (1 to over 2%), betulinic acid, eucalyptic acid, eucalyptolic acid, oleanolic acid and ursolic acid (5). Crude methanolic extract of *E. Camaldulensis* has been reported to inhibit the growth of *Candida albicans* (4). Also, it has been shown that ethanolic leaves extract of *Eucalyptus camaldulensis* had marked fungicidal effect against clinical dermatophytic fungal isolates; *Microsporium gypseum* and *Trichophyton mentagrophytes* (6).

Dermatophytosis is caused by fungi in the genera *Microsporum, Trichophyton* and *Epidermophyton*. These organisms, called dermatophytes, are the pathogenic members of the keratinophilic (keratin digesting) soil fungi. *Microsporum* and *Trichophyton* are human and animal pathogens, while E*pidermophyton* is a human pathogen. They have the ability to invade keratinized tissues and cause dermatophytosis, the most common human contagious fungal disease (7, 8). The humid weather, overpopulation and poor hygienic conditions are conducive factors to the growth of dermatophytes. Even though it responds to treatment with conventional antifungal drugs, the diseases have a tendency to recur at the same or at different sites.

There is a growing movement to find new medicines, or rediscover old ways of treating illness and improving general health. Throughout the history of mankind, many infectious diseases have been treated with plant extracts. As *E. camaldulensis* has been shown to have antifungal effects (9, 10, 11), the aim of the present study was to formulate a cream with different concentrations of *E. camaldulensis* extract as antidermatophyte preparations, and evaluate its stability and physicochemical properties.

Materials and methods

Plant materials

Leaves of *E. camaldulensis* were collected from Ahvaz (Iran), and identified by Department of Pharmacognosy, Faculty of Pharmacy, Jundishapur University of Medical Sciences, Ahvaz, Iran. The collected material was air-dried at shade and room temperature. To avoid degradation, the air dried plant material was ground just prior to extraction.

Preparation of plant extract

Hydroalcoholic extract was prepared using the maceration method. The powdered leaves of the plant (500 g) were macerated in 1500 ml ethanol (80%, v/v) at the room temperature (25 °C) with occasional stirring for 3 days. After filtration with Whatman no.1 filter paper, the combined ethanolic extracts were evaporated *in vacuo* at 30 °C and stored in refrigerator for future use.

Preparation of fungal inoculum

The freeze-dried sealed glass ampoules of microorganisms used in this study were obtained from Persian Type Culture Collection (PTCC), the Iranian Research Organization for Science and Technology, Tehran, Iran. The microorganisms were: *Microsporum canis* PTCC no. 5069, *Microsporum gypseum* PTCC no. 5070, *Trichophyton rubrum* PTCC no. 5143, and *Trichophyton verrucosum* PTCC no. 5056. These microorganisms were activated on Saboraud Dextrose Broth (SDB) and then cultured on Saboraud Dextrose Agar (SDA) for 21 days at 22 °C to obtain adequate growth. Following the period of incubation, colonies were scraped with a sterile scalpel and macerated in 10 ml of sterile 0.05% Tween 80 solution (in sterile

distilled water). Fungal suspensions were adjusted to 10% absorbance at 580 nm using a spectrophotometer.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of *E. camaldulensis* was determined according to the method described by Evans and Richardson [9]. Various concentrations of the plant extract (0.04 to 0.10 g of dried extract per 100 ml of SDA with 0.01 g increments) were prepared, transferred to sterile Petri plates and were allowed to solidify. Triplicate plates were used for each concentration. Plates were inoculated with 0.2 µl fungal suspension and incubated at the room temperature for three weeks. Plates of *T. verrucosum* were incubated at 37 °C. Suitable controls were also included. SD broth with 0.2 µl of inoculum served as the positive control. Un-inoculated SD broth served as negative control. Following the incubation period, plates were examined for growth, and colony diameters were precisely measured and expressed in millimeter. The MIC was regarded as the lowest concentration of the extract that did not show any visible growth after 21 days of incubation.

Formulation design

Water in oil emulsion base was chosen for its emollient and detergency properties. The base was mainly composed of bees wax, liquid paraffin, spermaceti, borax and water. Then, different amounts of the ingredients were incorporated together and formulations (F_1 to F_6) were compared regarding their extent of oil phase, the viscosity of the product, and the amount of the emulsifier added in the final preparation.

Different formulations were evaluated regarding their appearance, particle size and phase homogeneity, emolliency and viscosity, and the best was chosen. Then, the required amount of the herbal extract was added to make a proper formula having the best antifungal activity. The composition and amounts of the ingredients are shown in Table 1. Regarding the effect of the formulations on selected dermatophyte species in culture media, the best percentage of the herbal extract was determined and the physicochemical stabilities were evaluated.

Homogeneity test

Five hundred mg of each sample was spread on a clean slide and observed using an optical microscope (×10 and ×40).

Creaming and coalescence

A 10 g sample of each formulation was placed in a beaker and stored at room temperature for 3 months. Their physical stability was determined after one week, one and three month storage.

Table 1. Amounts of ingredients (g) per 100 g of cream used in formulations 1 to 6

Ingredients	Formulation number						
	base	F_1	F_2	F_3	F_4	F_5	F_6
bees wax	8	9	10	10	10	10	10
liquid paraffin	63	61	60	58.8	58.8	58.8	58.8
paraffin	0	0	0	1.2	1.2	1.2	1.2
spermaceti	4	4	4	4	5	5	5
borax	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Parabens (total)	0	0.2	0.2	0.2	0.2	0.2	0.2
lactic acid	0	0	0	0	1 drop	2 drops	3 drops
Tween 80	0	0.15	0.15	0.15	0.15	0.15	0.15

Centrifugation
A 10 g portion of each formulation was placed in a centrifuge tube (1 cm diameter) and centrifuged at 2000 rpm for 5, 15, 30 and 60 min. Then the phase separation and solid sedimentation of the samples were evaluated.

Thermal cycle test
The portion were stored at 5 °C for 48 h and then at 25 °C for 48 h. The procedure was repeated 6 times and then their stability and appearance were evaluated.

Thermal change test
Three 20 g portion of each formulation (or base) were stored at 4-6 °C, 25 °C and 45-50 °C. After 24 h, one and three months, their stability and appearance were evaluated.

Freezing and thawing
Twenty gram portion of each formula were stored periodically at 45-50 °C and 4 °C for 48 h each. The procedure was repeated six times and then the samples were checked regarding their appearance and stability.

Determination of pH
A suspension of each portion in 1% potassium nitrate solution was prepared and its pH was determined. A magnetic stirrer was used to produce homogeneity. The pH was determined at 48 h, one and three weeks after preparation.

Viscosity determination
Using a Brookfield viscometer (model DV-I with No. 6 spindle) the rheologic behavior of the portion were studied. Each sample was placed in a container and spindle velocity was raised gradually to maximum extent. Then the viscosity was determined at 0.3, 0.6, 3, 6 and 60 rpm. If needed, student t-test (Microsoft excel software) was performed to compare test results with the control. $P<0.05$ was assumed as significant difference.

Antimicrobial preservative effectiveness determination
To evaluate the effectiveness of the formulation preservation, the single microbial challenge test was employed. The test was performed by adding 0.2 ml of 10^8 cfu/ml *Staphylococcus aureus* (PTCC No. 1189) and *Pseudomonas aeruginosa* (PTCC No. 1074) to each of the 20 ml pre-diluted sample of the formulation. Inoculated containers were kept at room temperature for 4 weeks. At appropriate time intervals (1, 7, 14, 21, and 28 days), aseptically, 1 ml portions from each sample was withdrawn and were subjected to the pour plate count procedure; and changes in microbial numbers at various intervals were recorded. (12)

Results and discussion

Dermatophytes live in the dead, top layer of skin cells in moist areas of the body, such as between the toes, the groin, and under the breasts. The fungal infections of dermatophytes may cause only a minor irritation. Although some types of fungal infections could be more serious. They can penetrate into the cells and cause itching, swelling, blistering and scaling. The dermatophytes, *Trichophyton*, *Epidermophyton* and *Microsporum canis* are commonly involved in such infections. However, their clinical differentiation is difficult.

The yield of the hydroalcoholic extract of *E. camaldulensis* was 2% w/w and pH of a 2% sample of the extract was 10.99±0.10%, 2.98±0.03% and 5.69±0.03, respectively. The results of growth inhibition and MIC determination are listed in Tables 2 and 3.

Creams are semisolid dosage forms intended mainly for external use and commonly consist of two immiscible

phases, an oily internal phase and an aqueous external phase. Due to emulsified nature of skin surface, drugs formulated as cream are more effectively interact with skin and more readily penetrate through biological membranes. Some of plant extracts with antifungal activity have been previously formulated as topical creams. It has been previously reported that formulation of *zataria multiflora* extract as topical cream may lead to enhancement of stability and acceptability of the active ingredient, while the antifungal activity remains considerable (13).

Table 2. Inhibitory effect of different concentrations of the herbal extract (g/100 ml) on fungal growth in SDA medium after one and two weeks.

Fungi	Extract Concentration (g/100 ml)	Growth Inhibition Zone Diameter (mm) (Mean±SD)	
		After 1 week	After 2 weeks
Trichophyton rubrum	Blank	3.00 ± 0.018	5.01 ± 0.030
	0.04	2.10 ± 0.021	3.00 ± 0.010
	0.05	1.80 ± 0.027	2.30 ± 0.020
	0.06	0.80 ± 0.020	1.00 ± 0.010
	0.07	0.50 ± 0.014	0.50 ± 0.01
	0.08	0	0
	0.09	0	0
	0.10	0	0
Trichophyton verrucosum	Blank	0.80 ± 0.032	1.20 ± 0.030
	0.04	0.50 ± 0.027	1.20 ± 0.030
	0.05	0.03 ± 0.035	0.60 ± 0.020
	0.06	0	0
	0.07	0	0
	0.08	0	0
	0.09	0	0
	0.10	0	0
Microsporum canis	Blank	3.80 ± 0.040	5.20 ± 0.030
	0.04	3.00 ± 0.036	4.50 ± 0.030
	0.05	2.8 ± 0.021	3.60 ± 0.010
	0.06	1.5 ± 0.033	3.60 ± 0.020
	0.07	0.80 ± 0.020	2.00 ± 0.010
	0.08	0.30 ± 0.014	0.80 ± 0.000
	0.09	0	0
	0.10	0	0
Microsporum gypseum	Blank	3.51 ± 0.037	5.00 ± 0.020
	0.04	2.80 ± 0.027	4.92 ± 0.020
	0.05	2.00 ± 0.033	3.50 ± 0.040
	0.06	1.53 ± 0.040	3.50 ± 0.030
	0.07	0.50 ± 0.032	2.00 ± 0.010
	0.08	0.20 ± 0.017	1.20 ± 0.010
	0.09	0	0
	0.10	0	0

The main problems with the preliminary base formula were the excess fat content which produced a greasy sense on usage, turbidity and its low consistency. Therefore, the formula was modified to overcome the problems. At first, the proportions of the oil phase components were changed. Solid paraffin was also added to produce better flow properties. The results showed that by increasing the amount of solid paraffin, the stiffness and consistency of the formula were increased. The best concentration was chosen to be 1.2 percent of paraffin. Lactic acid was added to adjust pH and borax was added in different quantities to enhance the stability of the formula. Microscopic inspection showed heterogeneous matrix of formulations F_1 to F_3, so due to undesirable appearance of these formulations, they did not undergo the physicochemical experiments and different concentrations of Tween 80 was utilized to enhance emulsion stability and prevent the changes in pH (Table 4). Although F_4 and F_5 had relatively good appearance and fluidity, they were not stable on thermal cycle and freeze-thawing tests (F_4) and three month storage stability test (F_4 and F_5).The results showed that F_6 had a good appearance and consistency and was stable during centrifugation and after 3 months of storage. Also, it had the best flow characteristics, so it was chosen as the best formula for delivery of *E. camaldulensis* extract. The results of the experiments mentioned in methods are listed in table 4.

Table 3. Minimum inhibitory concentrations (MICs) of the *E. camaldulensis* extract after 14 days.

MIC (mg/ ml)	Fungi
0.8	*Trichophytum rubrum*
0.6	*Trichophytum verrucosum*
0.9	*Microsporum canis*
0.9	*Microsporum gypseum*

Table 4. Results pH, fluidity, appearance, physicochemical and stability of formulations (n=5)
*Not determined

Property	Formulation number						
	base	F1	F2	F3	F4	F5	F6
Homogeneity test	Homogenous	Heterogeneous	Heterogeneous	Heterogeneous	Heterogeneous	Heterogeneous	Heterogeneous
Creaming and coalescence	*	*	*	*	stable	stable	stable
Centrifugation test	*	*	*	*	stable	stable	stable
Thermal cycle test	*	*	*	*	separated	stable	stable
Freezing and thawing	*	*	*	*	separated	stable	stable
pH (average)	*	*	*	8.0	6.0	5.7	5.4
long term stability	*	*	*	*	separated	separated	stable
fluidity	fluid	fluid	fluid	medium viscosity	medium viscosity	medium viscosity	medium viscosity
appearance	very soft and fatty	very soft and fatty	soft and fatty	fatty	clear, good flow	clear, good flow	clear, good flow

The minimum inhibitory concentration of the extract was 0.6 - 0.9 mg/ ml; therefore, one milligram per ml of the extract was incorporated with the base and its antifungal potency was determined again. According to the findings, the formulation met the USP criteria to prevent fungal growth.

Finally, since creams and other water containing dosage forms should be preserved from microbial contamination, a usual preservative mixture, methyl and propyl paraben (3:1 ratio) was added. Visual and microscopic inspection and stability determination showed a homogenous appearance during 3 month storage period and no creaming or coalescence occurred. There was no significant change in the appearance of the formula during centrifugation, thermal cycle and freeze and thawing tests. Also, there was no significant change ($p>0.05$) in the viscosity of the samples during 3 month storage period (Table 5). Antimicrobial preservation efficacy test showed that the preservative had the ability to preserve the formula completely. The amounts of ingredients used in the final formulation (F_6) are shown in Table 5. Therapeutic and biologic properties of *E. camaldulensis* have been extensively investigated. Among them, antidiabetic effect of decoction and analgesic activity of alcoholic macerated leaves have been reported (14). There are also several reports supporting antiasthmatic effect of *E. camaldulensis* inhalation (15). There are some reports supporting the idea that *E. camaldulensis* essential oil can inhibit growth of many microorganisms including *Bacillus subtilis* and *Pseudomonas aeroginosae* (16, 17). The results of the present study are in accordance with the previous records regarding antifungal activity of *E. camaldulensis.*

The culture medium, the technique of testing, botanical source and age of the plant, the state of plant material used (dried or fresh) and the isolation technique are some of factors which are implicated in the great variation of the activity (18, 19). The inhibitory effect may be due to the presence of phytochemical components such as volatile oils, saponins and phenols (18).

Some investigations have also demonstrated the inhibitory effect of *Eucalyptus* spp. against some fungal species. It has been shown that methanolic extract of *E. camaldulensis* leaves had antifungal activity against all known dermatophytes, among them, *trichophton mentagraphytes* was more susceptible (9).

In conclusion, due to the highly effective antidermatophyte activity of the extract of *E. camaldulensis*, it could be added in topical formulations in order to protect skin against damage caused by various dermatophytes. Nevertheless, evaluation of cutaneous permeation and *in vivo* efficacy of the formulation of plant extract are necessary in order to confirm their use for treatment of fungal skin infections.

Table5. The results of periodically viscosity monitoring of the final formulation (mean ± SD, n=3)

Shear rate (rpm)	Viscosity (cps)			
	After 2 days	After 1 week	After 1 month	After 3 months
0.3	389.00±0.05	399.00±0.05	394.00±0.04	389.00±0.11
0.6	138.00±0.07	140.00±0.10	142.00±0.15	142.00±0.80
3	8.01±0.01	8.22±0.14	9.11±0.58	9.23±0.88
6	2.71±0.14	2.78±0.63	2.83±0.25	2.95±0.16

60	0.04±0.01	0.04±0.00	0.04±0.01	0.04±0.02

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