Iranian Journal of Pharmaceutical Sciences
Spring 2007: 3(2): 77-84
www.ijps.ir

Original Article

Characterization of an Anti-Dermatophyte Cream from *Zataria multiflora* Boiss.

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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 this study was to evaluate the physicochemical properties and stability of creams containing different concentrations of *Zataria multiflora* extract as anti-dermatophyte preparations. First, the minimum inhibitory concentration (MIC) of methanolic extract of aerial parts of *Z. multiflora* was assayed against various dermatophytes by *in vitro* tube dilution technique (MIC=0.5%). 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. Formulations containing 1, 2 or 3% of the plant extracts were prepared. Finally, a cream containing 10% bees wax, 58.8% liquid paraffin, 1.2% hard paraffin, 5% spermaceti, 1% borax, 1.4% tween 80, 0.15% methyl paraben, 0.15% lactic acid, 0.05% propyl paraben, 2% *Zataria* extract and water was chosen as the best formulation. The final product was a W/O cream with suitable appearance and desirable physicochemical stability.

Keywords: Cream; Dermatophyte; MIC; *Zataria multiflora*.
Received: December 7, 2006; *Accepted:* March 2, 2007.

1. Introduction

The Labiatae family has several members with a significant content of essential oils, which have commercial and medical values. This family is generally known for its multiple pharmacological effects such as analgesic, anti-inflammatory [1], antioxidant [2], hepatoprotective [3] and hypoglycemic actions [4]. Labiatae is widespread throughout the world as well as Iran [5-9].

Zataria multiflora Boiss. is a plant belonging to the Labiatae family that is geographically distributed only in Iran, Pakistan and Afghanistan. This plant with the vernacular name of "Avishan-e Shirazi" (in Iran) has several traditional uses such as antiseptic, anesthetic, antispasmodic and laxative [6]. It is used for the treatment of

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premenstrual pain, chronic catharsis, asthma, jaundice, sore throat, neuralgia and edema [6]. It is also prescribed for the treatment of premature labor pain [10, 11]. The aerial parts and leaves of *Z. multiflora* are reputed to be an effective remedy for treating pains and gastrointestinal disorders [12, 13]. Also, the extracts of the aerial parts of *Z. multiflora* show anti-inflammatory effects against acute and chronic inflammations in mice and rats [14]. Antibacterial and antifungal effects of *Z. multiflora* Boiss. have also been reported [15, 16].

Dermatophytes are the major cause of superficial mycoses and remain a public health problem. They have the ability to invade keratinized tissues and cause dermatophytosis, the most common human contagious fungal disease [17, 18]. The humid weather, overpopulation and poor hygienic conditions are conductive 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 *Z. multiflora* has been shown to have antifungal effects [15, 16], the aim of this study was to prepare creams with different concentrations of *Z. multiflora* extract as anti-dermatophyte preparations, and evaluate the physicochemical properties and stability of the formulations.

2. Materials and methods

2.1. Plant materials

Aerial parts of *Z. multiflora* Boiss. were collected in July 2005, from the Kolahghazi Mountains (Isfahan Province, Iran). The plant was identified by Department of Botany, Faculty of Agriculture, Shahid Chamran University, Ahwaz, Iran, and a voucher specimen was deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ahwaz Joundishapour University of Medical Sciences, Ahwaz, Iran. The collected material was air-dried at shade and the room temperature. To avoid degradation, the air dried plant material was ground just before extraction.

2.2. Preparation of plant extract

Hydroalcoholic extract was prepared using the maceration method. Therefore, the powdered plants (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. The yield of the hydroalcoholic extract of *Z. multiflora* was 2% w/w.

2.3. 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 are: *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

Table 1. Amounts of the main ingredients used for making 100 g of the primary general base formulation.

Ingredient	Amount (g)
Bees wax	8
Liquid paraffin	63
Spermaceti	4
Borax	1.5
Water	23.5

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.

2.4. Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of *Z. multiflora* was determined according to the method described by Evans and Richardson [9]. Various concentrations of the plant extract (0.5, 0.25, 0.1, and 0.05 g of dried extract per 100 ml of SDA) were prepared, transferred to sterile Petri plates and were allowed to solidify. Triplicate plates were used for each dilution. Each plate was inoculated with 0.2 µl fungal suspension and incubated at the room temperature for seven to fourteen days. 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. Uninoculated 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

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)**	**Average Growth Inhibition (mm)**	
		After 1 week	**After 2 weeks**
Trichophytum rubrum	Blank	3.1±0.05	4.9±0.04
	0.05	2.9±0.04	4.5±0.16
	0.1	1.9±0.04	3.9±0.04
	0.25	0.8±0.03	2.4±0.02
	0.5	0	0
	0.75	0	0.5±0.06
	1	0	0.4±0.00
Trichophytum verrucosum	Blank	0.8±0.07	0.3±0.01
	0.05	0.7±0.00	0
	0.1	0.5±0.03	0
	0.25	0	6.4±0.05
	0.5	0	5.5±0.04
	0.75	0	5.2±0.04
	1	0	1.9±0.01
Microsporum canis	Blank	3.7±0.02	0
	0.05	2.9±0.04	6.7±0.07
	0.1	2.5±0.10	5.3±0.02
	0.25	0	4.8±0.04
	0.5	0	1.4±0.03
	0.75	0	0
	1	0	4.9±0.04
Microsporum gypseum	Blank	3.6±0.10	4.5±0.16
	0.05	2.9±0.04	3.9±0.04
	0.1	1.8±0.02	2.4±0.02
	0.25	0	0
	0.5	0	0.5±0.06
	0.75	0	0.4±0.00
	1	0	0.3±0.01

Table 3. Minimum inhibitory concentration of the *Z. multiflora* extract after 14 days.

Fungi	MIC (g/100 ml)
Trichophytum rubrum	0.5
Trichophytum verrucosum	0.25
Microsporum canis	0.5
Microsporum gypseum	0.5

regarded as the lowest concentration of the extract that did not show any viable growth after 21 days of incubation.

2.5. 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_{25}) 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 one was chosen. Then, the required amount of the herbal extract was added to make a proper formula having the best antifungal effect. The composition and amounts of the ingredients are shown in Table 1. Regarding the effect of the formulations on selected dermatophytes in culture media, the best percentage of the herbal extract was determined and the physicochemical stability of the formulations were evaluated.

2.6. Homogeneity test

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

2.7. Creaming and coalescence

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

2.8. Centrifugation

A 10 g sample 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.

2.9. Thermal cycle test

The samples 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.

2.10. Thermal change test

Three 20 g samples 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.

2.11. Freezing and thawing

Twenty gram samples 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.

2.12. Determination of pH

A suspension of each sample 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.

2.13. Viscosity determination

Using a Brookfield viscometer (model DV-I with No. 6 spindle) the rheologic behavior of the samples 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.

2.14. Chemical controls

Table 4. Amounts of ingredients (g) used in formulations 1 to 12 and the results of their pH and stability considerations (n=5).

Ingredients	**Formulation number**												
	Base	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}	F_{11}	F_{12}
Bees wax	8	8	9	10	10	10	10	10	10	10	10	10	10
Liquid paraffin	63	62	61	60	59.4	59.1	58.8	58.8	58.8	58.8	58.8	58.8	58.8
Paraffin	-	-	-	-	0.6	0.9	1.2	1.5	1.2	1.2	1.2	1.2	1.2
Spermaceti	4	4	4	4	4	4	4	4	4	4	4	4	4
Borax	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.2	1.0	0.9	0.7	1
Tween 80	-	-	-	-	-	-	-	-	-	-	-	-	1
pH (average)	-	-	-	-	-	-	-	8.25	8.22	8.15	8.10	8.05	7.50
Fluidity	Fluid	Fluid	Fluid	Intermediate	Fluid	Fluid	Intermediate	Thick	Thick	Intermediate	Intermediate	Intermediate	Intermediate
Stability	Unstable	Unstable	Unstable	Unstable	Unstable	Unstable	Unstable	Unstable	Unstable	Unstable	Unstable	Unstable	Unstable

*Distilled water was added to all of the formulations to make 100 ml volume.

Table 5. Amounts of ingredients (g) used in formulations 13 to 25 and the results of their pH and stability considerations (n=5).

Ingredients	**Formulation number**												
	F_{13}	F_{14}	F_{15}	F_{16}	F_{17}	F_{18}	F_{19}	F_{20}	F_{21}	F_{22}	F_{23}	F_{24}	F_{25}
Bees wax	10	10	10	10	10	10	10	10	10	10	10	10	10
Liquid paraffin	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8	58.8
Paraffin	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.5	1.2	1.2	1.2	1.2	1.2
Spermaceti	4	4	4	4	4	3	4.5	4.5	4.5	5	5	6	7
Borax	1.0	1.0	0.9	0.9	0.9	1.0	1.0	1.0	0.9	1.0	0.9	1.0	1.0
Tween 80	1.2	1.4	1.0	1.2	1.4	1.4	1.4	1.2	1.0	1.4	1.0	1.4	1.4
pH (average)	-	-	-	-	-	-	-	8.25	8.22	8.15	8.10	8.05	7.50
Fluidity	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate	Ideal	Ideal	Soft	Soft	Soft
Stability	Unstable	Stable	Stable	Unstable	Unstable	Unstable	Unstable	Unstable	Stable	Stable	Stable	Stable	Stable

*Distilled water was added to all of the formulations to make 100 ml volume.

Table 6. Results from viscosity monitoring of the final formulation after 3 months storage.

Shear rate	Viscosity (cps)			
(rpm)	After 2 days	After 1 week	After 1 month	After 3 months
0.3	381.00±0.05	400.00±0.01	391.40±0.02	389.20±0.00
0.6	139.80±0.02	140.10±0.01	143.20±0.00	141.20±0.58
3	7.90±0.01	8.10±0.01	9.10±0.00	3.00±0.00
6	2.58±0.00	2.78±0.00	2.14±0.01	3.00±0.00
60	0.04±0.00	0.04±0.00	0.04±0.00	0.03±0.00

The extent of volatile and non-volatile composition and water content of the formulations were determined using standard methods.

2.15. Antimicrobial preservative effectiveness determination

To evaluate the effectiveness of the formulation preservation, the single microbial challenge test prescribed in USP30 was employed. The test was performed by adding 0.1 ml of 10^8 cfu/ml *Staphylococcus aureus* (PTCC No. 1189) and *Pseudomonas aeruginosa* (PTCC No. 1074) to each of the 20 ml prediluted sample of the formulation. Inoculated containers were kept at 22.5±2.5 °C for 28 days. At appropriate time intervals as specified in the USP(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.

3. Results and discussion

Many skin diseases such as tinea and ringworm caused by dermatophytes exist in tropical and semitropical areas. In general, these fungi 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. These fungal infections cause only a minor irritation. Other 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 herbal extract total ash, insoluble ash percent and pH of a 2% sample of the extract were 11.75±0.07%, 2.89±0.06% and 5.76±0.06, respectively. The results from MIC determination are listed in Tables 2 and 3.

One of the main problems with the preliminary base formula was the excess fat content which produced a greasy sense on usage. In addition, it was relatively turbid

Table 7. The final composition and amount of ingredients used to make *Zataria* anti-dermatophyte cream.

	Compound	Amount (%)
Oil phase	1 Bees wax	10
	2 Liquid paraffin	58.8
	3 Paraffin (solid)	1.2
	4 Spermaceti	5
	5 Propyl paraben	0.05
Water phase	6 Borax	1
	7 Tween 80	1.4
	8 Methyl paraben	0.15
	9 Lactic acid	0.15
	10 *Z. multiflora* extract	1-3
	11 Water q.s.	100

and its low consistency and rigid nature were the other problems with that. Therefore, the formula was modified to overcome the problems. At first, the proportions of the oily phase components were changed and three formulations were made (formulations F_1 to F_3; Table 4). F_1 and F_2 were very dilute and had low consistency, but the third was more consistent. These 3 formulations were rejected due to their greasy appearance. In the second step, solid paraffin was used to produce better flow properties (F_4 to F_7 formulations, Table 4). 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. Centrifugation and thermal tests showed that all of the formulations (F_4 to F_7) were unstable and a phase separation phenomenon occurred.

To enhance the stability of the formula, borax was added in different quantities (F_8 to F_{11}; Table 4). F_8 was very solid and had a turbid and stiff appearance. F_9 had better flow properties but was broken after centrifugation. F_{10} had a good appearance and consistency and was stable during centrifugation and after 2 weeks of storage. For F_{11} formula, the amount of the borax was too low to produce a stable emulsion.

To adequate stability and prevent the changes in pH, tween 80 was added in formulations F_{12} to F_{17} (Tables 4 and 5). All of the mentioned formulations had a proper consistency, while only F_{14} and F_{15} had relatively stable emulsion characteristics during centrifugation and thermal cycle tests. Due to undesirable appearance of the formulations F_{12} to F_{17}, different amounts of spermaceti were added to soften and brighten the product [19]. F_{18} to F_{25} (Table 5) were prepared using 3 to 7% of this material. The results showed that F_{21} and F_{22} had the best flow characteristics, of which F_{22} was more homogenous, clear and bright; so it was chosen as the best base formula for incorporation with the *Z. multiflora* extract.

As mentioned before, the minimum inhibitory concentration of the extract was 0.5%; therefore, three different percentages of the extract were incorporated with the base and their antifungal potency were determined. According to the findings, 2% formulation had enough requirements to prevent the 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. Control experiments and stability determination showed a homogenous appearance during 3 month storage period and no creaming or coalescence occurred. Also, there was no significant change in the appearance of the samples and the base during centrifugation, thermal cycle and freeze and thawing tests. Finally there was no significant change in the viscosity of the samples in 3 month storage period (Table 6). Antimicrobial preservation efficacy test showed that the preservative used had the ability to preserve the formula completely.

The amounts of ingredients used in the final formulation are listed in Table 7. Due to the good antidermatophyte activity of the herbal extract, 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 formulations added with plant extract are necessary in order to confirm their use for skin protection.

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