Topical Treatment of Dermatophytic Lesion on Mice (*Mus musculus*) Model

Bindu Sharma · Padma Kumar · Suresh Chandra Joshi

Received: 12 August 2009 / Accepted: 8 October 2010 / Published online: 28 January 2011
© Association of Microbiologists of India 2011

Abstract Antidermatophytic potential of three weed plants viz. *Tridax procumbens* L., *Capparis decidua* (forsk) Edgew and *Lantana camara* L. were explored and experimentally induced dermatophytic lesion was topically treated in mice. Microbroth dilution method was carried out for determination of MIC and MFC of different extracts of selected plants. In animal studies, mice were experimentally inoculated with *Trichophyton mentagrophytes* and infected animals were topically treated with 5 mg/g terbinafine and two concentrations, i.e., 5 and 10 mg/g of test extract ointment. Complete recovery from the infection was observed on 12th day of treatment for reference drug terbinafine (5 mg/g) and 10 mg/g concentration of test extract ointment whereas 5 mg/g concentration of test extract ointment showed complete cure on 16th day of treatment. Fungal burden was also calculated by culturing skin scrapings from infected animals of different groups. Test extract ointment successfully treated induced dermatophytosis in mice without any disease recurrence incidences, thereby indicating efficacy of test extract as an excellent topical antifungal agent for the cure of dermatophytosis.

Keywords Dermatophytosis · Flavonoids · *Trichophyton mentagrophytes*

Introduction

Dermatophytosis is one of the most infectious diseases of humans, caused by invasion of stratum corneum by dermatophytic fungi viz. *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Microsporum* spp, *Epidermophyton* spp [1] and remains a common public health problem, especially in tropical countries such as India [2]. *Trichophyton mentagrophytes* is considered as a zoophilic and anthrophilic dermatophyte [3–6] which causes hair, skin and nail infections in humans.

Disease incidences are very common with *T. mentagrophytes* in human beings as well as in animals. Animals can harbour dermatophytes and transmit the disease to man and other animals [7] either by direct contact with the infected host through fomites containing infected material or by exposure to environments contaminated with fungal spores [8].

Despite the availability of new systemic antifungal therapies, dermatophytic infections are difficult to eradicate completely, with recurrence reported in up to 25–40% of cases [9]. Many antifungal synthetic drugs viz. imidazoles, butanafine and terbinafine are effective in the treatment of dermatophytosis [10] but disease recurrence, resistant dermatophytic strains and adverse effects are some drawbacks associated with popular antifungals [11]. In the present scenario plants and their products have gained more importance as a possible source of alternative and effective drugs. Because of the long history of plants in the treatment of different human ailments, most of the herbal drugs are believed to be safer than the synthetic drugs with no side effects. Plants remain as an untapped reservoir of potentially useful chemical compounds not only as drugs but also as unique templates that could serve as a starting point for synthetic analogues [12].

B. Sharma (✉) · P. Kumar
Laboratory of Plant Tissue Culture and Secondary Metabolites,
Department of Botany, University of Rajasthan, 84/52,
Mansarovar, Jaipur 302020, India
e-mail: sharmabindu111@gmail.com

S. C. Joshi
Laboratory of Reproductive Toxicology, Department of
Zoology, University of Rajasthan, Jaipur 302004, India

Therefore in the present study antidermatophytic potential of flavonoids of three plants viz. *Capparis decidua* (Forsk) Edgew (capparaceae), *Lantana camara* L (verbenaceae) and *Tridax procumbens* L (asteraceae) against *Trichophyton mentagrophytes* (Robin) Blanchard was determined in vitro and most active extract was selected for the topical treatment of experimental dermatophytic lesion in mice model.

Materials and Methods

Flavonoid Extraction

Different parts of selected plants were taken for flavonoid extraction following the well established method [13]. Each extract was dried in vacuo and stored at 4°C in air tight vials for further use.

In vitro Antifungal Activity

Test dermatophyte *T. mentagrophytes* (MTCC No. 7687) was procured from IMTECH, Chandigarh (India) and maintained on Sabouraud medium at $27 \pm 2^\circ\text{C}$. Minimum inhibitory concentration (MIC) was determined for each extract by 'Microbroth Dilution' method [14]. Stock solution (10 mg/ml) of each extract was prepared in acetone (inactive against test dermatophyte). Each extract was twofold serially diluted in 96 well microtiter plates, thereafter fungal suspension (inoculum size 10^6 CFU/ml) was added to each well and incubated at $27 \pm 2^\circ\text{C}$ for 7 days. The MIC values were taken as the lowest concentration of the extracts in the well of the microtiter plate that showed no turbidity after incubation. The turbidity of the wells in the microtiter plate was interpreted as visible growth of microorganisms. The minimum fungicidal concentration (MFC) was determined by subculturing the content of each well (Table 1).

Infection on Mice Model

Cultures of *T. mentagrophytes* were subcultured on Sabouraud dextrose agar media and incubated at $27 \pm 2^\circ\text{C}$. After 7 days a suspension of conidia was obtained by washing the surface of the plate with sterile distilled water. Conidial suspension was tenfold serially diluted in Sabouraud dextrose broth to bring inoculum size to 1×10^6 cfu/ml and conidial viability was checked by subculture [15].

Male swiss albino mice (*Mus musculus*) of approximately 8 weeks old and 25–30 g weight were used for the present investigation. Mice were first immunosuppressed by subcutaneous injection of 500 µg of estradiol valerate.

Table 1 Determination of minimum inhibitory concentration and minimum fungicidal concentration of different extracts of selected three plants against *T. mentagrophytes*

Plant	Plant part	Extract	<i>T. mentagrophytes</i>	
			MIC (mg/ml)	MFC (mg/ml)
<i>C. decidua</i>	Root	E ₁	–	–
		E ₂	0.625	1.25
	Stem	E ₁	–	–
		E ₂	0.625	1.25
	Fruit	E ₁	–	–
		E ₂	–	–
<i>L. camara</i>	Root	E ₁	–	–
		E ₂	0.078	0.078
	Stem	E ₁	–	–
		E ₂	–	–
	Leaf	E ₁	–	–
		E ₂	0.625	0.625
	Flower	E ₁	–	–
		E ₂	–	–
<i>T. procumbens</i>	Root	E ₁	0.312	1.25
		E ₂	0.625	0.625
	Stem	E ₁	–	–
		E ₂	–	–
	Leaf	E ₁	–	–
		E ₂	0.625	0.625
	Flower	E ₁	–	–
		E ₂	–	–

E₁ free flavonoids, E₂ bound flavonoids, – inactive

Estradiol pretreatment is known to inhibit innate and acquired immune defenses [16]. After 3 days of immunosuppression flanks of mice were shaved with an electric razor and the exposed area lightly abraded with a sterile scalpel blade. Hundred microliter of standard inoculum size (1×10^6 cfu/ml) was applied on the shaved site and was gently rubbed with the flat part of a sterile blade [17].

Experimental Design

Six experimental groups (A, B, C, D, E and F) were made containing five animals each. Animals of group B, C, D, E and F were immunosuppressed before inoculating with *T. mentagrophytes*, while animals of group A were inoculated without immunosuppression. Group A and B were remain untreated to see the lesion severity, time course of disease and considered as control groups.

The lesions were visually examined daily throughout the experiment to determine the severity and recovery of lesion. On seventh day of infection skin lesions were scored as follows: 0 (absence of lesion), 1 (appearance of erythema at infected site or new hair growth on the bald

exposed area), 2 (moderate erythema spreading over entire infected site), 3 (intense erythema with abrasions, swelling and scaling), 4 (severely erythematous lesion with crusting spreading over the entire exposed area). The average lesion scores were calculated for each group by dividing the sum of the lesion scored by the number of animals in the group.

Most active plant extract (bound flavonoids of roots of *L. camara*) in the study was selected for further animal studies by considering lowest MIC/MFC values against test dermatophyte. Ointment for topical treatment was prepared by mixing 50 and 100 mg of test extract in 10 g inert petroleum jelly to make 5 and 10 mg/g concentrations of test extract. Terbinafine (5 mg) was used as reference drug. On eighth day of infection all animals of group C, D and E were topically treated by applying 0.2 g of the reference drug (5 mg/g), test ointment 5 and 10 mg/g, respectively, once in a day at the infected site. Treatment continued until complete recovery was observed. To find out any effect of petroleum jelly on lesion, 0.2 g of petroleum jelly was applied once in a day at the infected site of the animals of group of F.

Infected site of each animal was carefully examined daily throughout the treatment period to determine % recovery of infected site and treatment scores were given to animals as follows: 0 (not cured), 1 (25% cured), 2 (50% cured), 3 (75% cured), 4 (100% cured). The average treatment scores for an experimental group were determined by dividing the treatment score by number of animals.

Fungal burden was calculated by culturing infected skin tissues. Five skin samples (skin scrapings, hair stubs) were collected on 15th day of infection (control group A and B) and 20th day of treatment (treated groups C, D and E) from the infected site of each animal of different groups. Each sample was cultured on SDA medium at $27 \pm 2^\circ\text{C}$ for 7 days and samples showed fungal growth were considered as culture positive. Fungal burden was assessed with scores ranging from 0 to 5 based on the number of culture positive skin samples of each animal. The average fungal burden of a group was determined by dividing the sum of the burden scores by number of animals [18].

Results

In Vitro Antifungal Activity

It is evident from the results obtained (Table 1) that out of 22 extracts of different parts of selected three plants viz. *C. decidua*, *L. camara* and *T. procumbens*, seven extracts showed their antidermatophytic activity against test dermatophyte (*T. mentagrophytes*). Four extracts were found to be fungicidal where same values of MIC and MFC were

recorded; while three extracts were recorded as fungistatic (MFC values were higher than their MIC values).

For animal studies most active plant extract (bound flavonoids from roots of *L. camara* with the lowest MIC and MFC values 0.078 mg/ml) was selected.

Induction of Dermatophytic Infection on Mice

The severity of skin lesions in all groups of animals was assessed everyday after inoculation of test dermatophyte. Time course of changes in mean lesion score is shown in Fig. 1. Infection of mice model resulted lesion in 100% of the animals. First signs of infection were observed on second and third day of inoculation and lesion reached at its maximum at the infected site on eighth and 10th day of inoculation in all animals of group B (immunosuppressed infected animal group) and A (infected animal group without immunosuppression), respectively. Lesion scores started decreasing as a result of spontaneous healing after 20 and 25 days of inoculation, whereas complete spontaneous recovery was observed on 26th and 32nd day of inoculation in group A and B, respectively. Disease recurrence was recorded in 40% animals of both the groups during the observational period of 6 months.

Treatment of Induced Dermatophytosis on Mice by Test Compound

Recovery of skin lesions of animals of treated groups (C, D and E) was assessed by giving them treatment scores depending upon % resolution of infected site (Fig. 2). First signs of recovery was seen on second day of treatment in animals of group C (group treated with terbinafine 5 mg/g ointment), and E (test extract ointment 10 mg/g), while group D (test extract ointment 5 mg/g) showed recovery signs on third day of treatment. On 12th day of treatment 100% animals of group C and E were completely cured and new hairs were observed on the bald exposed area of infected site. In case of group D 100% recovery (i.e. treatment score 4) of dermatophytic infection was observed on 16th day of treatment in all animals of the group. Course of infection in animals of group F (treated with petroleum jelly) was found similar as in untreated groups (A and B) and lesion became more severe with time indicating ineffectiveness of petroleum jelly.

Skin irritation was observed in 50% animals of the group treated with terbinafine whereas such side effects were not observed in both the concentrations (5 and 10 mg/g) of test ointment. During observational period of 6 months, 20% animals of 'standard drug treated group' showed disease recurrence even after complete recovery from the infection, whereas no such recurrence was observed in test extract treated groups (D and E).

Fig. 1 Time course changes in skin lesion severity. 0 (absence of lesion), 1 (appearance of erythema at infected site or new hair growth on the bald exposed area), 2 (moderate erythema spreading over entire infected site), 3 (intense erythema with abrasions, swelling and scaling), 4 (severely erythematous lesion with crusting spreading over the entire exposed area)

Fig. 2 Treatment scores of animal groups topically treated by standard and test ointments. 0 (not cured), 1 (25% cured), 2 (50% cured), 3 (75% cured), 4 (100% cured)

Table 2 Determination of fungal burden (FB) of animal groups

Animal group	Days	Score of fungal burden FB $\pm$ SEM
A	15 ^a	3.2 $\pm$ 0.490
B	15 ^a	3.6 $\pm$ 0.400
C	20 ^b	0
D	20 ^b	0
E	20 ^b	0
F	15 ^a	3.90 $\pm$ 0.200

A animal group inoculated without immunosuppression, B animal group immunosuppressed before inoculation, C animal group treated with terbinafine cream (5 mg), D animal group treated with test ointment (5 mg), E animal group treated with test ointment (10 mg), F animal group treated with petroleum jelly

$\pm$ SEM standard error mean

^a Days after inoculation

^b Days after treatment

The fungal burden (FB) of infected loci in animals of untreated groups (A and B) and group F (treated with petroleum jelly) was recorded relatively high, i.e., 3.2 ± 0.49 , 3.6 ± 0.04 and 3.90 ± 0.20 , respectively. Fungal burden was calculated 0 for group C, D and E on 20th day of treatment, thus proved complete eradication of the fungal mycelia/conidia from the infected loci (Table 2).

Discussion

The clinical course of *T. mentagrophytes* infection in mice model was in agreement to other studies [15]. Present study shows that immunosuppression is not essentially required for inducing dermatophytic infection like other studies [17] as infection could also induced without immunosuppressing experimental animals but skin/epidermal abrasion was required for establishing infection. Recurrence and relapse incidences are very common with dermatophytic infections [19] in the animals of control groups (A and B) were observed even after complete spontaneous recovery.

For comparing potency of test drug terbinafine was taken as reference drug because it is highly active, very popular antidermatophytic agent and frequently prescribed by dermatologists to the patients of dermatophytosis. It is worth mentioning that efficacy of 10 mg/g concentration of test ointment was found to be same as that of reference drug with 5 mg/g concentration whereas, significant results were also obtained for 5 mg/g concentration of test ointment.

Animals experimentally infected with *T. mentagrophytes* had been used as models of dermatophytosis to assess disease severity and therapeutic effects of drugs [20,

21]. Synthetic antimycotic agents are being frequently investigated to evaluate therapeutic efficacy in animal models [22, 23] but systematic studies dealing with herbal products to find out their therapeutic potential to cure dermatological infections are still lacking. Few reports are available which evaluated pre-clinical antidermatophytic potential of ointments from natural products [12, 24].

Test extract was obtained from *L. camara* which is a notorious and poisonous weed [25] imposing serious threat to native vegetation and biodiversity [26]. Though *L. camara* has been screened for its antimicrobial activities [27–29] but work with dermatophytes has not been done as yet. It is the first study which evaluates in vitro as well as in vivo (mice model) antidermatophytic potency of *L. camara*.

Findings of the present work are indicative of the therapeutic activity of bound flavonoids of roots of *L. camara* suggesting its uses as raw material by pharmaceutical industries for development of antidermatophytic drug in the prevailing conditions where dermatophytes are becoming resistant against popular antifungals [30, 31]. Present study offers a high possibility of complete cure of dermatophytosis and/or ability to maintain its effect for long duration. Thus test extract is viewed as a promising and excellent topical antifungal agent which can be explored for future promising drug against notorious dermatophytes.

Acknowledgments Authors are thankful to Head, Department of Botany and Department of Zoology, University of Rajasthan for providing all necessary facilities. Financial support of UGC, Delhi is gratefully acknowledged.

References

- Weitzman I, Summerbell RC (1995) The dermatophytes. Clin Microbiol Rev 8:240–259
- Venugopal PV, Venugopal TV (1994) Antidermatophytic activity of neem (*Azadirachta indica*) leaves in vitro. Indian J Pharmacol 26:141–143
- Treiber A, Pittermann W, Schuppe HC (2001) Efficacy testing of antimycotic prophylactics in an animal model. Int J Hyg Environ Health 204(4):1–5
- Donald GF, Brown G (1964) *T. mentagrophytes* and *T. mentagrophytes* var *quinckeanum* infections of South Australian mice. Aust J Dermatol 7:135–140
- Bilek J, Baranova Z, Kozak M, Fialkovicova M, Weissova T, Sesztakova E (2005) *Trichophyton mentagrophytes* var *quinckeanum* as a cause of zoophilic dermatomycosis in a human family. Bratisl Lek Listy 106(12):383–385
- Garcia-Sanchez MS, Pereiro MM, Toribio J (1997) Favus due to *Trichophyton mentagrophytes* var *quinckeanum*. Dermatology 194(2):177–179
- Mantovani A (1978) The role of animals in the epidemiology of the mycoses. Mycopathologia 65(1):61–66
- Moriello KA (1990) Management of dermatophyte infection in catteries and multiple cat households. Adv Clin Dermatol 20(6):1456–1474
- Hay RJ (2001) The future of onychomycosis therapy may involve a combination of approaches. Br J Dermatol 145:3–8
- Watanabe S (1999) Present state and future directions of topical antifungals. Jpn J Med Mycol 40:151–155
- Artis WM, Odle BM, Jones HE (1981) Griseofulvin resistant dermatophytosis correlates with in vitro resistance. Arch Dermatol 117(1):16–19
- Tiwari TN, Chansauria JPN, Dubey NK (2003) Antimycotic potency of some essential oils in the treatment of induced dermatomycosis of an experimental animal. Pharm Biol 41(5):351–356
- Subramanian SS, Nagarjan S (1969) Flavonoids of the seeds of *Crotalaria retusa* and *Crotalaria striata*. Curr Sci 38:65
- Basri DF, Fan SH (2005) The potential of aqueous and acetone extracts of galls of *Quercus infectoria* as antibacterial agents. Indian J Pharmacol 37:26–29
- Roderick JH, Raquel AA, Collins JC (1983) Experimental dermatophytosis: the clinical and histopathologic features of a mouse model using *Trichophyton quinckeanum* (mouse favus). J Invest Dermatol 81:270–274
- Carlsten H, Holmdahl R, Tarkowski A (1991) Analysis of the genetic encoding of oestradiol suppression of delayed type hypersensitivity in (NZB × NZW) F₁ mice. Immunology 73:186–190
- Odds F, Ausma J, Gerven FV, Woestenborgh SF, Meerpoel L, Heeres J (2004) In vitro and in vivo activities of the novel azole antifungal agent R 12 6638. Antimicrob Agents Chemother 48(2): 388–391
- Uchida K, Tanaka T, Yamaguchi H (2003) Achievement of complete mycological cure by topical antifungal agent NND-502 in guinea pig model of tinea pedis. Microbiol Immunol 47(2):143–146
- Julian LJ, Luis P, Pedro I, Emilo PT (2005) Dermatophytosis with concurrent lesion in different locations. Prognostic and therapeutic significance. Enferm Infecc Microbiol Clin 23(4):191–193
- Arruda MSP, Gilioli S, Moreno FRV (2001) Experimental dermatophytosis in hamsters inoculated with *T. mentagrophytes* in cheek pouch. Rev Inst Med Trop Sao Paulo 43(1):29–32
- Ghannoum MA, Hossain MA, Long L, Mohamed S, Reyes S, Mukarjee PK (2004) Evaluation of antifungal efficacy in an optimized animal model of *T. mentagrophytes* dermatophytosis. J Chemother 16(2):139–144
- Hnilica KA, Medleau L (2002) Evaluation of topically applied enilconazole for the treatment of dermatophytosis in a persian cattery. Vet Dermatol 13(1):23–28
- Saunte DM, Hasselby JP, Brillawska A, Frimodt MN, Svejgaard EL, Linnemann D (2008) Experimental guinea pig model of dermatophytosis: a simple and useful tool for the evaluation of new diagnostic and antifungals. Med Mycol 46:303–313
- Jain N, Sharma M (2003) Broad spectrum antimycotic drug for the treatment of ringworm infection in human beings. Curr Sci 85(1):30–34
- McGaw LJ, Eloff JN (2005) Screening of 16 poisonous plants for antibacterial, anthelmintic and cytotoxic activity in vitro. S Afr J Bot 71(3–4):302–306
- Sharma GP, Raghubanshi AS, Singh JS (2005) *Lantana* invasion: an overview. Weed Biol Manag 5(4):157–165
- Hernandez T, Canales M, Avila JG, Duran A, Caballero J, de Vivar AR (2003) Ethnobotany and antibacterial activity of some plants used in traditional medicine of Zapotitlan de las Salinas, Pubelo (Mexico). J Ethnopharmacol 88:181–188
- Saleh M, Kamel A, Li X, Swaray J (1999) Antibacterial triterpenoids isolated from *Lantana camara*. Pharm Biol 37(1):63–66
- Rajakaruna N, Cory SH, Towers GHN (2002) Antimicrobial activity of plants collected from serpentine outcrops in Sri Lanka. Pharm Biol 40(3):235–244

30. Mukharjee PK, Leidich SD, Isham N, Leitner I, Ryder NS, Ghannoum MA (2003) Clinical *Trichophyton rubrum* strain exhibiting primary resistance to terbinafine. Antimicrob Agents Chemother 47(1):82–86
31. Favre B, Ghannoum MA, Ryder NS (2004) Biochemical characterization of terbinafine resistant *Trichophyton rubrum* isolates. Med Mycol 42(6):525–529