

ANTIMYCOTICACTIVITY OF AQUEOUS EXTRACT OF TAMARIXRAMOSISSIMA LEDEBBARKON IN VITRO AND IN VIVO GUINEA PIG MODEL OF DERMATOPHYTOSIS

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ABSTRACT

The decoction of the bark of different species of tamarisk is used as a remedy for the treatment of ringworm in sheep in Kurdish ethno medicine (Iran). The aim of this study was to evaluate the antifungal effects of the aqueous extract of *Tamarix ramosissima* Ledeb. (Tamaricaceae) bark against *Epidermophyton floccosum* and *Trichophyton verrucosum*. The antifungal activity of aqueous extracts of both dried and burned barks were evaluated against *E. floccosum* and *T. verrucosum* by disc diffusion test and determination of minimum inhibitory concentrations (MICs). The dorsum of guinea pigs was infected with *T. verrucosum* conidia and the animals topically treated as follows: negative control (NC) received normal saline solution plus distilled water; positive control (PC) received 1% terbinafine, while three other groups, namely TR10%, TR20% and TR40%, received 10, 20 and 40% aqueous extract of the bark of *T. ramosissima*, respectively. Both aqueous extracts of bark and burned bark of *T. ramosissima* exhibited significant inhibitory effects against *E. floccosum* and *T. verrucosum*. MICs of aqueous extracts of bark and burned bark were 15 and 30 mg/ml against both *T. verrucosum* and *E. floccosum*, respectively. Lesions were improved in TR20%, TR40% and PC groups compared with NC group. The clinical efficacy in TR10%, TR20%, TR40%, and terbinafine groups were 15.84, 47.4, 78.96, 73.7% compared with NC group, respectively. Phytochemical analysis of the bark indicate the presence of flavonoids, alkaloids, condensed tannins, coumarins, saponins, triterpenes, steroids, cardiac glycosides and anthocyanines. Our findings support the historical use of *T. ramosissima* for the treatment of tinea, and also indicate its potential as an antifungal phytomedicine.

KEYWORDS: *Tamarix ramosissima*, Antifungal, in vitro, Guinea pig model, dermatophytosis

INTRODUCTION

Epidermophyton floccosum and *Trichophyton verrucosum* are two keratinophilic and keratinolytic ascomycetous molds that lead to important fungal infections (dermatophytosis) in

the animals. *E. floccosum* is an anthropophilic dermatophyte which can be found worldwide while *T. verrucosum* is a zoophilic dermatophyte which causes ringworm in domestic and wild ruminants (plus pseudo ruminants) and human.¹ The prevention of ringworm in cattle by means of

vaccination appears possible but the results are not satisfactory.² Despite the existence of the synthetic antifungal drugs, there is interest in the use of medicinal plants in the treatment of fungal infections because the synthetic antifungal drugs are potentially toxic and cause many side effects on the host. *Tamarix* (tamarisk, salt cedar) is the largest genus in the family Tamaricaceae and includes at least 50 species with global distribution. In this context, *Tamarix ramosissima* Ledeb. (TR) is a perennial shrub to small tree that grows in Iran. The tamarisk bark has been used to cure jaundice, diarrhea, hepatosplenic problems, pulmonary congestion, and stomachache. Tamarisk has been considered as a holy plant in archeological scriptures found in Iran especially in historical Sistan. Its poultice has been used to cure wounds, leukoderma, ocular problems, and bleeding.³ In orthodox medicine, *T. ramosissima* has been investigated for its antimicrobial and antioxidative activities.⁴ The decoction of the tamarisk bark is

employed for the treatment of ringworm in sheep in Kurdish ethnomedicine (Iran). In the present study, we evaluated the antifungal activities of *T. ramosissima* against an anthropophilic (*E. floccosum*) and a zoophilic (*T. verrucosum*) dermatophyte in both in vitro and *T. verrucosum*-inoculated guinea pig model of *Tinea corporis*.

Experimental

Plant material

The stems of tamarisk shrub (Figure 1), grown in village of Hussein-Abad (34° 33' N, 47° 24' E and 4406 ft above sea level) in Kermanshah province (Iran), were collected in late winter 2011. This feral plant species, *Tamarix ramosissima* Ledeb. (TR) was authenticated by Dr. Nastaran Jalilian, a botanist of Research Center of Agriculture and Natural Resources of Kermanshah Province, Kermanshah (Iran).



Figure 1
Photos of bark (left) and whole plant of Tamarix ramosissima (right).

To prepare aqueous decoction, the dried bark of tamarisk was ground and boiled in sterile distilled water (0.1%w/v) for 4 h. After cooling, the aqueous extract was separated by centrifuging of the resulting mixture at 3000 rpm at 4 °C for 15 min and dried using a rotary evaporator at 40 °C. Also, the bark chips were completely flamed in a porcelain plate and the resulting ash used to obtain an aqueous decoction of burned bark (0.1%w/v) as described above for the dried bark. The dried aqueous extract was tested for the presence of phytochemical components according to the methods mentioned in the literature.⁵

In vitro antifungal assay

T. verrucosum (ATCC 10694) was purchased from IROST (www.irost.org), while the clinical isolate of *E. floccosum* was prepared from a patient with tinea cruris. The isolates of *T. verrucosum* and *E. floccosum* were grown on Sabouraud Dextrose Agar

(SDA, Merck, Germany) containing 0.05% chloramphenicol and 0.4% cyclohexamide at 37 ± 2 °C for 2 weeks. The inocula were prepared by spectrophotometric method to give a final concentration of 1.0×10^6 CFU/ml in normal saline solution (NS). The in vitro antifungal activity of the extracts was assayed by disc diffusion test and determination of minimum inhibitory concentrations according to the methods mentioned in the literature.⁵ Briefly, the resulting inocula of aforementioned fungi were swabbed in the amount of 1 ml/cm² over the surface of SDA plates. The sterile paper discs uniformly loaded with 20 µl of the given concentrations of TR extract (100, 200, 300, 400, and 500 mg/ml), corresponding solvent of TR extract (10 % DMSO as negative control) and terbinafine solution (100 mg/ml as positive control), were placed on the agar plates at equal intervals. All the tests were carried out in triplicate and the diameter of the inhibition zone around the

discs was measured daily for 4 weeks. Serial dilution method was used for the determination of minimal inhibitory concentration (MIC). Therefore, five doses of each extract (10, 15, 20, 25, 30 mg/ml) were added to SDA (100 ml) at 45 °C, these being mixed rapidly and poured into four 9.5 cm² petridishes. After the agar was cooled down to room temperature, 1 ml of the fungal suspension was inoculated per each cm² of petridishes. The MIC was taken as the lowest concentration of plant extract to inhibit growth of the fungus after 2 weeks. Each experiment was repeated two times. Then, each extract that showed highest antifungal activity against *T. verrucosum* and *E. floccosum* was employed for in vivo experiments.

In vivo antifungal assay

In the in vivo test, male albino guinea pigs (mean body weight 350 g, n = 25) were anaesthetized by intramuscular administration of ketamine and xylazine cocktail and their middle back was clipped and shaved, and a 2.5 cm × 2.5 cm (6.25 cm²) area was abraded with sandpaper. A suspension (0.1 ml, 1.0 × 10⁷ cells) of *T. verrucosum* conidia was applied to the marked area using a sterile pipette-tip and rubbed thoroughly. Animals were divided into five groups (n=5 in each group) according to Table 1. Seventy two hours after inducing infection, all formulations were applied topically to the infected area once a day for 1 week. The Medical Ethical Committee of Kermanshah University of Medical Sciences (Iran) reviewed and approved (reference number 228) this study.

Table 1
Characteristics of treatment groups (n=5) in a guinea pig model of dermatophytosis

Group	Treatment
NC	The negative control animals received a vehicle (100 µl of normal saline solution plus 0.1 ml of distilled water)
TR10%	Animals received 0.1ml of 10% aqueous extract solution of tamarisk bark
TR20%	Animals received 0.1ml of 20% aqueous extract solution of tamarisk bark
TR40%	Animals received 0.1ml of 40% aqueous extract solution of tamarisk bark
PC	The positive control animals received 0.1 ml of 1% terbinafine solution

The inoculated areas of treated animals were compared with negative control animals on 21 days after treatment according to the method previously described.⁶ Briefly, the infected area on the back of each guinea pig was divided into four equal quadrants. Each quadrant (1.25 cm × 1.25 cm) was scored as follows: 0= no signs of infection, hair was fully re-grown; 1= few slightly erythematous areas on the skin, no scaling; 2= well-defined redness, swelling with bristling hairs, bald patches, little scaling; 3= large areas of marked redness, incrustation, little scaling, hair started to re-grow,

bald patches, ulcerated in places; 4= partial damage to the integument, loss of hair; and 5= extensive damage to the integument and complete loss of hair at the site of infection. The cumulated scores for quadrants on each animal (maximum possible score per animal was 20) were calculated and employed for the clinical assessment of the efficacy of the different treatment regimens used in this study as described previously.⁶ Accordingly, percent efficacy was calculated using the following equation:

$$\text{Efficacy (\%)} = 100 - (T \times 100 / C)$$

Where T and C stand for total score of treatment group and total score of untreated control, respectively. The total score for any group denotes the average clinical score of different animals in the same group. The severity of dermatophytosis also was numerically scored according to method described by Ghannoum et al.⁷ as follows: 0= none; 1= insignificant; 2= slight; 3= moderate; and 4= severe.

STATISTICAL ANALYSES

The results are expressed as the mean ± SEM. All statistical analyses were performed using SPSS version 16.0 software (SPSS Inc, Chicago, IL, USA). Student's t-test was performed to determine any significant difference between different extracts and their concentrations for in vitro antifungal assays. Comparison of in vivo antifungal activities was carried out using one-way analysis of variance (ANOVA) and Tukey's HSD test. P < 0.05 was considered statistically significant.

RESULTS

Phytochemical screening

Phytochemical analysis of aqueous extract of tamarisk bark revealed the presence of the

following secondary metabolites (Table 2). Hydrolysable tannins, cyanogenic glycosides and both free and glycosylated anthraquinones were not detected in this study.

Table 2
Phytochemical analysis of aqueous bark extract of Tamarix ramosissima Ledeb.

Component	Qualitative results
Flavonoids	+++
Alkaloids	+++
Condensed tannin	+++
Coumarins	+++
Saponins	++
Triterpenes	++
Steroids	++
Cardiac glycosides	++
Anthocyanines	+

Note: High (+++), moderate (++) , trace (+) and negative (-) results reflect degree of color development compared with the respective blank for each component.

Antifungal activity

In disc diffusion test, TR aqueous extract in concentration of 500 mg/ml has significantly higher anti-Trichophyton activity than 10% terbinafine ($p < 0.05$, Table 3). At doses of 400 and 500 mg/ml, the ash extract showed significantly higher anti-Trichophyton activity than the bark extract ($p < 0.05$, Table 3). In the concentration of 400 mg/ml, bark extract of TR showed higher anti-Epidermophyton activity than that of 10% terbinafine (100 mg/ml) while in the concentration 500 mg/ml both bark and ash extracts showed higher anti-Epidermophyton activities compared with 10% terbinafine ($p < 0.05$, Table 3). Bark ash extract showed significant higher anti-Epidermophyton activity in comparison to bark

extract in doses 100 and 200 mg/ml. However, in higher doses of 400 and 500 mg/ml, bark extract was more efficient against *E. floccosum* ($p < 0.05$, Table 3). In serial dilution method, the aqueous extracts of bark and burned bark showed MICs as 15 and 30 mg/ml against both *T. verrucosum* and *E. floccosum*, respectively. The NC group showed patches of hair loss and readily visible ulcerated or scaly skin on day 30 post inoculation (Figure 2). PC and TR40% groups showed normal hair growth, with no signs of infection on day 30 post inoculation. TR10% and TR20% groups showed small patches of hair loss and scaly skin one month after inoculation and they showed moderate improvement in skin compared with NC on day 30 after inoculation (Figure 2).

Table 3
In vitro antifungal activity of Tamarix ramosissima Ledeb. Extracts against Trichophyton verrucosum and Epidermophyton floccosum (determined by diameter of inhibition zone)

	Dose (mg/ml)					Terbinafine (100)
	100	200	300	400	500	
Trichophyton verrucosum						
Bark	3.33(0.33) ^a	5.00(0.00) ^b	11.16(0.60) ^c	14.00(0.57) ^{dA}	18.33(0.33) ^{eA}	12.66(0.33) ^d
Bark ash	NI	NI	10.00(0.00) ^a	23.80(0.16) ^{bB}	25.50(0.28) ^{cB}	12.66(0.33) ^d
Epidermophyton floccosum						
Bark	NI	3.66(0.66) ^{aA}	10.33(0.33) ^{bA}	19.00(0.00) ^{cA}	23.33 (0.33) ^{dA}	14.66(0.44) ^f
Bark ash	NI	13.50(0.50) ^{acB}	13.66(0.33) ^{acB}	14.16(0.16) ^{acB}	17.33 (0.33) ^{bB}	14.66(0.44) ^c

Note: Values are mean \pm SEM (mm) of tri-plate test of the experiment. NI = no inhibition zone. In rows, values with different lowercase letters are significantly different while in columns related to each fungus, values with different uppercase letters are significantly different ($p < 0.05$).

Lower clinical lesion scores indicate improved efficacy of terbinafine compared with untreated controls (Table 4). The clinical lesion scores in TR20%, TR40% and PC groups were lower than that of NC group ($p < 0.05$, Table 4). The animals treated with 10, 20 and 40% aqueous extracts of TR exhibited clinical efficacies of 15.84, 47.4 and 78.96% respectively compared with terbinafine

(1%) that had a clinical efficacy of 73.7%. The severity of lesion scores in TR-treated groups and PC group were lower than that of NC (Table 4). TR20% and TR40% groups showed similar improvement against dermatophytosis caused by *T. verrucosum* compared with PC group ($p > 0.05$, Table 4).

Table 4
The effect of aqueous extract of Tamarix ramosissima Ledeb. bark on lesions of guinea pig model of dermatophytosis caused by Trichophytonverrucosum

Score (arbitrary unit)	Group				
	NC	TR10%	TR20%	TR40%	PC
Clinical lesion ($P_{ANOVA}=0.00$)	3.80(0.37) ^a	3.20(0.37) ^{ab}	2.00(0.31) ^{bc}	0.80(0.37) ^c	1.00(0.44) ^c
Severity of lesion ($P_{ANOVA}=0.00$)	3.20(0.48) ^a	3.00(0.31) ^a	1.20(0.37) ^b	0.40(0.24) ^b	0.60(0.40) ^b

Note: NC, the animals received a vehicle; PC, the animals received reference drug terbinafine 1% and three other groups: TR10%, TR20% and TR40% which received 10, 20 and 40% aqueous extract of T. ramosissimaLedeb. Evaluation of clinical efficacy was performed 21 days after completion of a 7-day treatment regimen. Values represent mean \pm SEM. In rows, values with different lowercase letters are significantly different ($p < 0.05$).

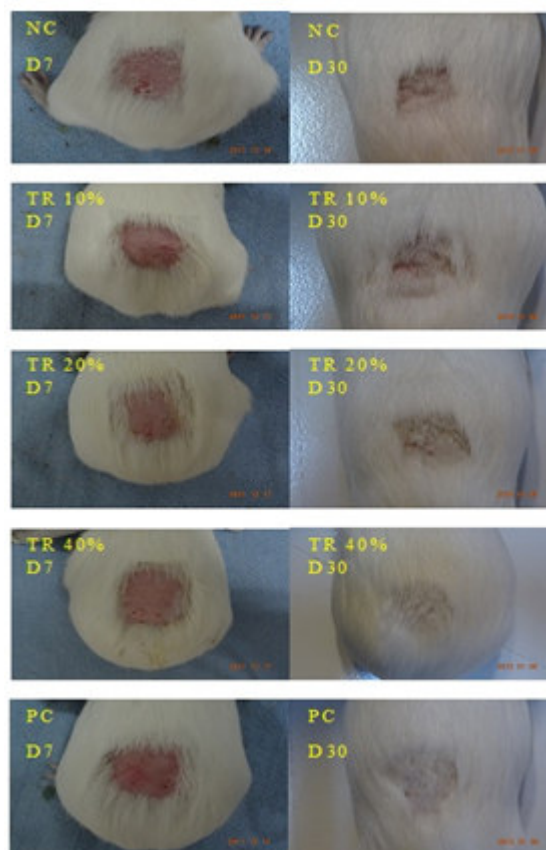


Figure 2
Clinical score of Efficacy of aqueous extract of Tamarix ramosissima Ledeb in a guinea pig model of dermatophytosis

Efficacy of aqueous extract of *Tamarix ramosissima* Ledeb. versus untreated (NC) and terbinafine-treated (PC) controls in a guinea pig model of dermatophytosis caused by *Trichophytonverrucosum* after one week of

treatment (D7) and one month post-infection (D30). TR10%, TR20%, TR40% groups received 0.1 ml of 10, 20 and 40 % aqueous extract of *Tamarix ramosissima*.

DISCUSSION

In the present study, the aqueous extract prepared from burning of TR bark showed antidermatophytic activity. To the best of our knowledge, there have been no previous experimental reports about the antifungal activity of TR bark ash. The ash resulting from burning of this plant has a therapeutic effect useful for wounds, ulcers and various burns. Tamarix is reported to be rich in polyphenolic compounds such as flavonoids, phenolic acids, tannins and coumarins^{8,9} Flavonoids were surprisingly found in tamarisk bark in the present study as well as in other tamarisk species.⁴ A known flavanoid compound, tamarixetin, has been isolated from *T. ramosissima* which showed significant DNA damaging activity in mutant yeast bioassay.³ Tamarisk, because of its tannin-derived astringency is moderately antibacterial and antifungal. According to qualitative results of present study, TR has condensed tannins while it was free of hydrolysable tannins. Tannins inhibit cell wall formation in fungi leading to the death of the organisms.¹⁰ The bark tannins of this plant are used as astringent while galls possess medicinally important tannins.¹¹ It seems that TR is a good source of alkaloids. The antifungal activity of alkaloids against dermatophytes has been reported.¹² The aqueous extract of TR bark contained triterpenoids. In this regards, other triterpenoids like beta-sitosterol isolated from the aerial part of plants from the genus *Tamarix*.¹³ The triterpenoids isolated from other plants have shown antifungal activities.¹⁴ The phytochemical screening of TR showed a conspicuous absence of cyanogenic glycosides and free or glycosylated anthraquinones. Other metabolites and bioactive compounds were identified such as cardiac glycosides, steroids and anthocyanines. Cardiac glycosides and steroids also were found in *T. aphylla*.¹⁵ The antifungal properties of plant steroids as lipophilic compounds have been reported.¹⁶ Schaefer¹⁷ showed that anthocyanins reduce fungal growth in fruits. In the present study, coumarins also have been found in bark of *T. ramosissima* in high value. Parmar¹⁸ have isolated trigocoumarin from *T. troupitii*. Antifungal activities of coumarin isolated from other herbal resources have been reported.¹⁹ The saponin content of TR was also considerable in our study. The antifungal activities of saponins are the subject of many studies.²⁰ The improvement of skin lesions after topical application of aqueous extract of TR may be mediated through its immuno-boosting and anti-inflammatory effects beside its antidermatophytic activity. The severity of dermatophytosis depends

on the type of agent, environmental factors and immunologic status of host.²¹ Chronic dermatophytic infections develop when conditions of the local environment or virulence factors of the fungus outweigh the capabilities of cell-mediated immunity, or when a patient does not develop cell-mediated immunity to fungal antigens.²¹ The improvement of skin lesion in TR-treated groups may be due to anti-inflammatory effects of tamarisk.²² Researchers²³ have been shown that alcoholic extract derived from the leaves of *T. dioica* has considerable antifungal effects against *Microsporum canis*. Antimycotic activity of the crude n-butanolic extract of *T. gallica* L. aerial parts also has been reported.⁴ Several researches have proved antioxidant and antimicrobial activities of *Tamarix* species such as TR and *T. hispida*.³ Antimicrobial activity of *T. gallica* has also been reported.²⁴ On the other hand, researchers²⁵ have reported that essential oil of *T. boveana* exhibited an interesting antibacterial activity but no antifungal activity was detected. In the present study, the aqueous extract of TR at dose 40% showed higher efficacy in comparison with terbinafine (antifungal drug). The lower clinical lesion score and severity of lesion score in guinea pigs treated with dose of 40% TR compared to guinea pigs treated with terbinafine obviously show that TR is a potentially valuable antifungal remedy. The qualitative phytochemical profile of TR also shows that this plant can contain a series of possible antifungal components.

CONCLUSION

The findings of this study provide good evidence of the antifungal activity of *T. ramosissima* and hence lend support for the folkloric use of the plant. However, additional studies are required to develop it for actual use for the treatment of mycotic diseases.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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