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Original Article

In vitro and *In vivo* Antifungal Activity of *Alkanna tinctoria* against *Trichophyton rubrun* and *Trichophyton Mentagrophytes*

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Abstract

This study was conducted to investigate the antifungal properties of *Alkanna tinctoria* extracts against *Trichophyton rubrum* (PTCC5143), and *Trichophyton mentagrophytes* (PTCC 5054), and two archived clinical isolates of *T. rubrum* and *T. mentagrophyte*. Two reference strains, including *T. rubrum* (PTCC5143), and *T. mentagrophytes* (PTCC 5054) (drug-sensitive reference strains), were used, along with two archived clinical isolates of *T. rubrum* and *T. mentagrophytes*, which show resistance to terbinafine or fluconazole *in vitro* and clinic. A topical ointment was formulated using the dried extract of *A. tinctoria* (20%) roots, Vaseline, beeswax, and sesame oil. The ethanolic and aqueous extracts were prepared from the ointment and assessed for antifungal activity. *In vivo* antidermatophytic activity of *A. tinctoria* (20%) ointment was examined in six male Sprague Dawley rats infected by *T. mentagrophytes*. According to the results, both ethanolic and aqueous extracts showed growth inhibition against tested strains. Moreover, all strains were susceptible to amphotericin B (MIC≤0.5 µg/ml). The lowest fungistatic and fungicidal activities were exhibited by the MIC and MFC values of ethanol extract (both with 0.0125 mg/ml) against *T. mentagrophytes*, as well as the aqueous extracts against *T. rubrum*. The application of *A. tinctoria* (20%) ointment on the rats shortened the complete cure of *T. mentagrophytes* infection to 7 days compared to the 21-day complete cure using terbinafine. In the present study, *A. tinctoria* showed significant antifungal properties against clinical isolates of dermatophytes with resistance to azoles and terbinafine. Therefore, it can be considered a promising antifungal agent.

Keywords: Antifungal Properties, Dermatophyte Species, Dermatophytosis, Herbal, Herbs, Extract.

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1. Introduction

The incidence of fungal infections is becoming prevalent due to the increasing number of transplantation, HIV infection, immune-suppressive therapies, and individuals

with chronic diseases [1]. According to the World Health Organization, approximately 20% of the world's population suffers from dermatomycoses [1]. Regarding this, *Trichophyton rubrum* is the most common species causing tinea corporis followed by *Trichophyton mentagrophytes*, *Microsporum canis*, and *Microporum audouinii* [2]. The infection resulted from *T. rubrum* is chronic and in some cases lifelong [3]. The genotype VIII of *T. mentagrophytes* was identified as the main causative agent of the chronic recalcitrant dermatophytoses epidemic in India [4].

Synthetic antifungal drugs are developed with different chemical structures and different mechanisms. Most dermatomycoses are successfully treated with terbinafine [4]. Terbinafine inhibits squalene epoxidase resulting in the accumulation of squalene and depletion of ergosterol in the fungal cell membrane [2]. In various cases, due to resistance to this drug, the disease changes to a chronic or acute state and sometimes frequent relapses occur. On the other hand, in some cases, the need for long-term usage of antifungal drugs causes side effects imposing limitations on employing these compounds [5, 6]. Infections that involve resistant organisms put humans at risk.

The development of terbinafine resistance in clinical isolates of *T. rubrum* and *T. mentagrophytes* is rare [4, 7-14]. However, the spread of the drug-resistant strains may create problems and influence the medical treatment policy in the future. Therefore, performing research on finding plant extracts with strong antifungal activity and minimum adverse effects on the host can facilitate the treatment of a variety of fungal infections resistant to synthetic antifungal drugs. Currently, the efforts are focused on limiting the use of antifungal drugs, preventing infections, and discovering new antifungal compounds [15, 16]. The vast majority of utilized antifungal drugs are synthetic; nevertheless, it is estimated that at least one-third of all pharmaceutical products have plant-related origins [17, 18]. Given the importance of monitoring and treatment of fungal infections, the identification of effective compounds in addition to overcoming the drug resistance can reduce the additional costs of treatment.

Alkanna tinctoria (L.) Tausch (Boraginaceae) is a traditional medicinal plant used as food coloring, as well as for the treatment of macular eruptions, infectious diseases, sore throat, carbuncles, skin ulcers, and burns [19]. It has been reported that *A. tinctoria* root extracts are effective to heal wounds [20, 21]. Alkannin and shikonin have been regarded as the main active compound of *A. tinctoria* [20], of which alkannin derivatives show strong wound healing, anti-inflammatory, and antimicrobial properties [22].

Due to the emergence of drug-resistant pathogens, leading to increasing clinical challenges, new and more potent antifungals are required to eliminate the pathogen. This study was conducted to investigate the antifungal healing properties of *A. tinctoria* extracts for the first incidence of fungal skin infection caused by dermatophytes.

2. Materials and Methods

2.1. Fungal Strains

This study involved the use of two reference strains, including *T. rubrum* (PTCC5143), *T. mentagrophytes* (PTCC 5054), and two archived clinical isolates of *T. rubrum* and *T. mentagrophytes*, showing resistance to terbinafine or fluconazole *in vitro* and clinic. Reference strains were obtained from the Iranian Research Organization for Science and Technology, Shahriar, Tehran, Iran.

2.2. Plant Extraction

Initially, to conduct the extraction, 15 g of *A. tinctoria* root (Collected by Dr.Saghafi, Spring, Azarbaijan provience) was carefully washed under running tap water and allowed to dry for two days at room temperature (30°C). Afterward, it was pulverized into a fine powder and stored in sealed containers. The extracts were then prepared by soaking plant material in 150 ml of 67% ETOH, followed by being transferred in two separate bottles for the removal of dye under sonication (3 times) at room temperature for 72 h.

The extract was filtered through Whatman No. 1 filter paper, and all the supernatant and filtrates were then pooled up successively and concentrated under vacuum by a Rotary evaporator (Buchi® Rotavap R-210). In the next stage, a special mill was used to mix 20% w/w of dried extract into an ointment (i.e., a mixture of Vaseline, beeswax, and sesame oil) at 45°C for 72 hours in the dark. The final product was sterilized by gamma irradiation (25 kGy) to eliminate bacterial spores. Subsequently, 2 g of ointment was extracted using 150 ml of a solution of 67% ETOH in water for 72 hours at room temperature. The extract was removed from the ointment and the solvent was evaporated to obtain dry extract for *in vitro* antifungal susceptibility testing.

2.3. Preparation of Fungal Inocula

The inoculum suspensions from fresh cultures on potato dextrose agar (Merck, Germany) were prepared according to the Clinical and Laboratory Standards Institute guidelines [23]. This process was accomplished by covering the dermatophytes colonies with 1 ml of sterile saline solution plus 0.1% Tween 20 (as wetting agent). The yeast inocula were prepared by transferring several colonies of yeast into a sterile tube containing 1 ml of saline solution. The densities of these suspensions (i.e., yeast and mold) were subsequently adjusted to the optimal absorbance at 530 nm and further dilutions in RPMI 1640 medium were performed to obtain the final density.

2.4. Antifungal Assay

The ethanolic and aqueous extracts of the ointment containing *A. tinctoria* (L.) Tausch was assessed for antifungal activity against the two reference strains and two clinical isolates using the broth microdilution method [23]. The plant extracts were evaluated for the fungistatic effect by the minimum inhibitory concentration (MIC) and the fungicidal effect by the minimum fungicidal concentration (MFC). Amphotericin B was employed as a positive control in the susceptibility testing.

2.5. Determination of Minimum Inhibitory Concentration (MIC) of Plant Extracts

The serial dilution of control antifungals and plant extracts were prepared with concentrations of 0.0125 and 8 mg/ml, respectively. Afterward, 100 µl of each agent was dispensed in 96-well microplates. Growth and negative controls were included. The extracts were added with 100 µl of RPMI (Roswell Park Memorial Institute) 1640 medium and 100 µl of fungal inocula. Furthermore, a negative control was prepared using 25 µl of extract and 75 µl of RPMI 1640 medium. The plates were incubated at 35°C for 72 hours.

2.6. Determination of Minimum Fungicidal Concentration (MFC)

To obtain the MFC, 20 µl of the prepared extract was inoculated that showed no evidence of growth in the MIC determination assays onto PDA and spread using a sterile cotton swab. The lowest concentration with no growth observation was recorded as the MFC value. The tests were performed in triplicates. The standard strain of *Candida parapsilosis* ATCC 22019 was used as quality controls in every run.

2.7. In vivo Antidermatophytic Activity of A. Tinctoria Ointment

2.7.1. Ethical Consideration

The ethical approval for this study was obtained from the Animal Ethics Committee of Iran University of Medical Sciences, Tehran, Iran.

2.7.2. Animal Model

To carry out the study, six male Sprague Dawley rats, aged 6-8 weeks with a bodyweight of 300±10 g, were housed in the Animal House, Faculty of Medicine, Iran University of Medical Sciences, and were allowed to acclimate for a minimum of 5 days. These animals were kept in rooms maintained at 20–22°C and 70% humidity in a 12:12 light-dark cycle being fed with pelleted food and water. The samples were randomly divided into three groups, including the test (n=2), positive control (n=2) receiving terbinafine as the reference antifungal drug, and negative control (n=2) with no infection and no treatment, receiving distilled water.

2.7.3. Dermal Infection in Samples

The cases were randomly assigned to different groups. The hair from the back of each rat (test sites of 2 cm^2) was shaved, and the skin was slightly scraped by a single-use scalpel. Subsequently, 50 µl of *T. mentagrophytes* suspension was inoculated to a surface of about 2 cm^2 area within the shaved zone. The infection development was confirmed by an agar culture of scrapings from the area.

2.7.4. Treatment of Infected Samples

The positive group received treatment using the topical application of *A. tinctoria* (20%) ointment daily until the complete cure (21 days). The control groups were also considered in the study. The effect of the *A. tinctoria* (20%) ointment against *T. mentagrophytes* was assessed using the culturing skin scrapings and hair from the active border of the lesion of rats

on Sabouraud dextrose agar media to screen the growth colony of viable *T. mentagrophytes*. The cultures were incubated for 2 weeks at 28°C.

2.8. Statistical Analysis

The collected data were analyzed in SPSS software (version 19, SPSS Inc., Chicago) using ANOVA to calculate MIC and MFC for the tested isolates and determine the differences between the groups. According to the results, the p-value equal to or less than 0.05 was considered significant.

3. Results and Discussion

3.1. Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of Plant Extracts

According to the findings, the ethanolic extracts showed a growth inhibitory effect against the tested isolates. The MIC and MFC of plant extracts and control antifungals against the fungal strains are shown in Table 1. In this study, we found that all isolates were susceptible to amphotericin B (MIC≤0.5 µg/ml). In all cases, the MFC values were equal to MIC values. It was also revealed that the highest fungistatic and fungicidal activities were exhibited by the MIC and MFC values of ethanolic extract (both with 0.0125 mg/ml) against *T. mentagrophytes*. Moreover, the aqueous extract had minor fungistatic and fungicidal activities against four tasted isolates.

3.2. In vivo Antidermatophytic Activity

It was found that *A. tinctoria* (20%) ointment was an excellent remedy in a time-dependent manner, compared with terbinafine as the positive control ($P<0.05$). In addition, the application of *A. tinctoria* (20%) ointment shortened the length of complete cure of *T. mentagrophytes* infection to 7 days, in comparison to the 21-day complete cure using of terbinafine. In the control model, the hair culture was positive exhibiting 100% culture recovery between days 3 and 21.

In the present study, the fresh roots of *A. tinctoria* were utilized to extract the active compounds. This medicinal plant has been used for herbal remedies in Iran. The extraction of active compounds from fresh plant material is desirable due to the disintegration of metabolites during the drying process [24].

Table 1. Minimum inhibitory concentration and minimum fungicidal concentration of the plant extracts.

	Ethanolic Extract	**Eques extracts**	**Amphotericin B**
Test isolates	**MIC/MFC (mg/mL)**	**MIC/MFC (mg/mL)**	**MIC/MFC (µg/mL)**
T. rubrum PTCC5143	0.0625	2	0.031
T. mentagrophytes PTCC 5054	0.0125	2	0.25
Clinical isolate of T. rubrum	0.03	2	0.0125
Clinical isolate of T. mentagrophytes	0.03	4	0.5

MIC: Minimum inhibitory concentration, MFC: minimum fungicidal concentration

The obtained MIC values of amphotericin B were ≤0.5 μg/ml in all tested isolates, which is similar to those previously reported in the *in vitro* activity of amphotericin B [25-28]. Regarding the fungistatic and fungicidal properties, the antifungal activity of the ethanolic extract was 80 times stronger than that of aqueous extract. The negligible antifungal activity of the aqueous extract against tested isolates might be due to either a lower concentration of antifungal compounds in the extract or the unsuitability of water as a solvent for active compounds of the Alkanna roots [29, 30]. The reason for this inappropriateness can be related to the fact that most active antimicrobial ingredients of herbal medicine are soluble in ethanol or methanol.

It was revealed that the ethanolic extract of the plant showed strong inhibitory activity (MIC 0.0125 to 0.03 mg/ml) against 3 out of 4 tested fungal strains. It seems that the active component in this extract has a potential fungistatic/fungicidal property. It is possible that the active compounds in herbal medicine disrupt the fungal membrane via inhibition of the essential enzymes. The comparison of the growth inhibition effects of crude extracts and dilutions indicated that the inhibition activity of the extracts was concentration-independent [30]. In addition, in this study, the antimicrobial activity of plant extracts might be due to the synergistic effect of the minor portion of other compounds in the extracts.

Decreased susceptibility of two fluconazole- and terbinafine-resistant clinical isolates in the present study in exposure to the ethanolic and aqueous extracts of *A. tinctoria* highlighted the necessity of small and less frequent dose to achieve a favorable therapeutic outcome, which may lead to attenuated side effects. The ethanolic and aqueous extracts of *A. tinctoria* potentially inhibited the growth of tested dermatophytes in the *in vitro* stage. Therefore, the *in vivo* model for evaluating the therapeutic effect of *A. tinctoria* (20%) was investigated against *T. mentagrophytes* using the rat model. In this model, the significant efficacy of *A. tinctoria* (20 %) was observed against dermatophytosis induced in the rat.

The application of *A. tinctoria* (20%) significantly reduced skin redness, lesion severity, and dermatophyte occurrence. Its efficacy was confirmed by the recurrence of hair growth in the infected areas in the experimental groups, compared with that in the control group. The application of *A. tinctoria* (20%) ointment significantly shortened the length of the complete cure of *T. mentagrophytes* infection to 7 days ($P<0.05$), in comparison to the 21-day complete cure using of terbinafine.

4. Conclusion

In the present study, *A. tinctoria* showed significant antifungal property against clinical isolates of dermatophytes with resistance to azoles and terbinafine. Therefore, it can be considered a promising antifungal agent. In fact, the excellent properties of antifungal compounds are not only related to potent fungistatic/fungicidal effects, but also to minimum toxicity on normal human cells. Furthermore, screening the antifungal assay at a specific time interval (e.g., monthly) is

required to determine the stability of compounds in the extracts. It is also important to ensure that the antifungal potential will not degrade over time.

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Author's Contribution

MM.S designed the project. MM.S prepared the extracts and ointment. M.F and E.L performed antifungal susceptibility assay, wrote and critically revised the manuscript. F. B and M.V performed animal assay. In addition, E.L and M.F wrote and critically revised the manuscript. All of the authors approved the final version of the manuscript.

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