In vivo anti-*Trichophyton* Activities of Seed Oil Obtained from *Caragana korshinskii* Kom.

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Key Words

Caragana korshinskii Kom. · *Trichophyton mentagrophytes* · Guinea pig model · Antifungal agent · Histopathology

Abstract

Aim: The objective of this study was to evaluate the effects of seed oil of *Caragana korshinskii* Kom. against *Trichophyton mentagrophytes* on an in vivo guinea pig model of dermatophytosis. **Methods:** The skin of albino guinea pigs was infected with *T. mentagrophytes*, and the animals were divided into five groups: negative control (NC group), positive control (PC group), vehicle control, CK50% group (received topical 50% seed oil of *C. korshinskii*), and CK100% group (received topical 100% seed oil of *C. korshinskii*). Evaluation of clinical efficacy was performed 72 h after the completion of a 10-day treatment regimen. Skin biopsy samples were processed for histopathological examination. **Results:** The infected untreated control guinea pigs showed patches of hair loss and ulcerated or scaly skin. Lower clinical scores indicate improved efficacy compared with NC. The lesion scores significantly declined in the CK50%, CK100%, and PC groups in comparison with the NC group. The CK50% group (45.31%) and the CK100% group (75%) showed clinical efficacy com-

pared with the PC group (78.13%). In addition, no fungal elements, inflammation, or tissue destruction was observed in any of the PAS-stained sections of the infected skin in the groups treated with CK100% or 1% terbinafine. **Conclusion:** Seed oil of *C. korshinskii* demonstrated high antifungal efficacy in experimental dermatophytosis.

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Introduction

Dermatophytes, a group of keratinophilic fungi thriving on the keratin substrate, are the etiological agents responsible for causing cutaneous infections. These fungi are universally distributed and may be categorized as geophilic, zoophilic, or anthropophilic based on their natural habitats [1]. Zoophilic dermatophytes such as *Microsporum canis*, *Trichophyton mentagrophytes*, and *Trichophyton verrucosum* are a group of closely related fungi that have the capacity to produce an infection [2, 3]. Fun-

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gal infections of the skin and nails are a common global problem. The high prevalence of superficial mycotic infections shows that 20–25% of the world population has skin mycoses, making these one of the most frequent forms of infection. In addition, it is considered to be the third most common skin disorder among children younger than 12 years and the second most common skin disorder in adults [4, 5]. Although control measures are available, they have limited effectiveness. The antifungal drugs most commonly used against these diseases include amphotericin B, ketoconazole, fluconazole, terbinafine, and flucytosine. Adverse side effects are associated with the use of available antifungal drugs, including nephrotoxicity, hepatotoxicity, and neurotoxicity [6]; their use is restricted in pregnant women and young people. The resistance of human pathogens to antifungal drugs and toxicity-related problems has resulted in the need for novel antimycotic agents with a broad spectrum of actions and fewer dose-limiting side effects.

In recent years, in order to identify and develop a novel antimicrobial agent, researchers have focused on finding novel antimicrobials from natural sources, including higher plants, microorganisms, insects, nematodes, and vertebrates. Plants are rich sources of beneficial secondary metabolites. A number of medicinal plants have been investigated extensively to achieve higher levels of human safety standards, since they display antimicrobial properties which can protect the human body against pathogens. Their essential oils and extracts have a wide array of biological activities, especially antimicrobial effects on different groups of pathogenic organisms [7–15].

Caragana korshinskii Kom. is a member of the Fabaceae family, *Caragana* genus and is native to arid and semiarid areas of China, such as Shaanxi, Gansu, and Inner Mongolia [16]. It is mainly cultivated for dune fixation, livestock forage, biological resources (fuel energy), fiber production, and so on [16–20]. It also has a long history of use in traditional Chinese medicine and has been used for the treatment of a wide range of ailments, including fevers, dizziness, headache, inflammation, and female disorders [21]. It has been reported that the seed oil of *C. korshinskii* has insecticidal effect and can be used to cure dermatitis, fester, and blain. Our previous studies have demonstrated that seed oil of *C. korshinskii* is composed of 71.84% unsaturated fatty acids [22]. Our team then evaluated the in vitro antifungal activity of seed oil against *M. canis*, *T. mentagrophytes* and *Trichophyton rubrum*, with MIC values of 64–512, 32–512, and 64–1,024 µg/ml, respectively, whereas the MIC values for terbinafine ranged from 0.5 to 1.0 µg/ml at 28°C for both 7 and

10 days of incubation [23]. There is no more information available regarding the utilization of the seed oil of *C. korshinskii* as a source of antidermatophytes. These aforementioned folklore usages and experimental results motivated us to study the application of the seed oil of *C. korshinskii* for the treatment of dermatophytosis in an experimental guinea pig model of dermatophytosis.

Materials and Methods

Plant Collection and Authentication

The seed of *C. korshinskii* was collected from Huoshaogou Mountains, Xining, Qinghai, China in November 2014 and was positively identified by Professor Xuefeng Lu (Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, China). The dried seeds were ground in a rotary mill and then sieved (20–30 mesh). The seed oil of *C. korshinskii* was extracted using supercritical CO₂ at 41°C and pressure of 35 MPa.

Antifungal Agent

Terbinafine powder was bought from (Hubei YuanCheng-SaiChuang Technology Co., Ltd., Hubei, China). Powdered terbinafine and 50% seed oil of *C. korshinskii* were dissolved in 2% carboxymethyl cellulose (CMC), which, as a solubilizer, can effectively improve the solubility and stability of the seed oil of *C. korshinskii*.

Preparation of Inocula

The *T. mentagrophytes* strain was obtained from Peking University (Beijing, China) as the infecting fungus. This particular strain was chosen because it is one of the major causative organisms associated with onychomycosis and can infect the skin, resulting in skin and hair root invasion. To prepare the inoculum used to challenge the guinea pigs, *T. mentagrophytes* were grown on Sabouraud dextrose agar slants at 37°C for 1 week. The conidia were scraped from the plates in normal saline, centrifuged, and washed twice in normal saline. The suspension was adjusted to a concentration of 1.0×10^7 conidia/ml. The working solution was prepared fresh in normal saline, and 0.1 ml was used to inoculate the guinea pigs [24–27].

In vivo Antifungal Assay

Male albino guinea pigs (Veterinary Research Institute, Lanzhou, China) with a body weight of 300–400 g were housed in the Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining and were allowed to acclimate for minimum of 5 days. The animals were kept in rooms maintained at 20–22°C and 70% humidity in a 12:12 light-dark cycle and fed pelleted food and water ad libitum [28].

The guinea pigs were divided into groups of 5 each. Dermatophytosis was induced by a slight modification of the procedure of Ghannoum and colleagues [13, 28, 29]. Briefly, the guinea pigs were anesthetized intramuscularly (0.2 ml per animal) with an anesthetic cocktail (acepromazine, ketamine, and xylazine, 1:3:3 v/v/v). An area of skin on the middle back of the guinea pigs was clipped and shaved, and a 2.5×2.5 cm (6.25 cm^2) area was abraded with sandpaper. A suspension (0.1 ml , 1.0×10^7 cells) of *T. mentagrophytes* conidia was applied to the marked area using a sterile

Fig. 1. Efficacy of seed oil of *C. korshinskii* Kom. compared with NC (untreated control; **a**), 2% CMC (solvent control; **b**) and PC (terbinafine-treated controls; **e**) in the treatment of dermatophytosis caused by *T. mentagrophytes* in guinea pigs. The CK50% (**c**) and CK100% (**d**) groups received 0.1 ml of 50% and 100% seed oil of *C. korshinskii* Kom., respectively.

pipette tip and rubbed thoroughly. The experimental animals were randomly divided into five groups, each of which was infected with *T. mentagrophytes*. One group was a negative control (NC) treated with 100 μ l of normal saline, and a second group was treated with 100 μ l of 2% CMC as solvent control. In addition, two groups were treated with 100 μ l of 50 or 100% seed oil of *C. korshinskii*. Finally, a positive control (PC) group received 100 μ l of 1.0% terbinafine. In this study, the formulations were applied topically to the infected area once a day beginning 72 h after the infection, and the treatments were continued for 1 week.

Clinical Evaluation of the Lesions

The lesions were clinically followed up daily by the same person (Heng Xu). The clinical evaluation consisted of a semiquantitative score where the inoculated areas were evaluated and compared with NC animals according to the methodology previously described [28]. Briefly, the infected 2.5×2.5 cm area on the back of each guinea pig was divided into four equal quadrants. Each 1.25×1.25 cm quadrant of this area was graduated as follows: 0 = no signs of infection; 1 = few slightly erythematous areas on the skin; 2 = well-defined redness, swelling with bristling hairs, bald patches, and scaly areas; 3 = large areas of marked redness, incrustation, scaling, bald patches, and ulcerated in places; 4 = partial damage to the integument and loss of hair, and 5 = extensive damage to the integument and complete loss of hair at the site of infection. The summated scores for the four sites on each animal (maximum possible score per animal = 20) were calculated and used in the clinical assessment of the efficacy of the different treatments and control regimens evaluated in this study. Percent efficacy was calculated using the following equation:

$$\text{Percent efficacy} = 100 - (T \times 100/C)$$

where T = total score of the treatment group and C = total score of the untreated control. The total score for any group denotes the average clinical score from the different animals in the same group.

Histopathology Analysis

For histopathological examination, skin biopsy samples were obtained from 1 animal per group at the end of the study. With a disposable sterile dermal biopsy punch (Beijing TianNuo-TianCheng Biotechnology Co., Ltd., Beijing, China), a piece of skin 5 mm in diameter was obtained from an anesthetized animal representing the group. Next, the tissue was fixed with 10% neutral buffered formalin (SJH0372; Shanghai RuJi Biological Technology Development Co., Ltd., Shanghai, China) embedded in paraffin and processed for histopathological examination. Fungal elements were detected by HE and PAS staining.

Fig. 2. Mean clinical score of seed oil of *C. korshinskii* Kom. in guinea pig dermatophytosis. PC: terbinafine-treated animals; 2% CMC: solvent control. The two other groups, CK50% and CK100%, received 50% and 100% seed oil of *C. korshinskii* in guinea pig dermatophytosis, respectively. Evaluation of clinical efficacy was performed 72 h after completion of a 7-day treatment regimen at the end of the study (day 13). Values represented are means $\pm$ SD.

Results

Clinical Evaluation of the Lesions

All animals showed clinical signs of infection on day 3. As expected, no significant differences were observed among groups on day 3 when the treatment was initiated. Figures 1 and 2 compare the infection and the appearance of guinea pig skin photographs taken on day 13 when the clinical efficacy of the control and various formulations tested was conducted. As can be seen, the NC and 2% CMC groups showed patches of hair loss and readily visible ulcerated or scaly skin (fig. 1a, b).

In contrast, the PC group showed normal hair growth with no signs of infection (fig. 1e), as did the animals treated with CK100% (fig. 1d). Similarly, improvements in the appearance of guinea pig skins with a little hair loss

Fig. 3. Histopathological evaluation of the skin with HE staining. **a** NC: untreated control. **b** Solvent control: 2% CMC-treated animals. **c** CK50%, which received 50% seed oil of *C. korshinskii* Kom. in guinea pig dermatophytosis. **d** CK100%, which received 100% seed oil of *C. korshinskii* Kom. in guinea pig dermatophytosis. **e** PC: 1% terbinafine-treated animals.

and mild ulceration were noted in the animals treated with CK50% (fig. 1c).

Figure 2 shows the comparative clinical efficacy scores of each tested formulation compared with the infected untreated control. Lower clinical scores indicate improved efficacy compared with the untreated controls. The lesion scores significantly declined in the CK50% (8.75 ± 1.09 ; $p < 0.001$), CK100% (4 ± 1.25 ; $p < 0.001$), and PC (3.5 ± 0.5 ; $p < 0.001$) groups in comparison with the NC group (16 ± 1). In this regard, the CK50% group demonstrated a significant improvement in comparison with the NC group. However, the lesion score in the 2% CMC group (15.5 ± 0.866 ; $p = 0.7049$) did not show any difference compared with NC. The lesion score in the 2% CMC group ($p < 0.001$) was significantly higher than that of the PC group. However, the lesion score in the CK50% group ($p = 0.027$) showed a significant difference compared with PC. The lesion score in the CK100% group ($p = 0.539$) was nonsignificantly higher than that of the PC group. In this sense, the CK50% group ($p = 0.0024$) showed significant differences compared with the

CK100% group. The CK50% and CK100% groups showed a similar improvement against dermatophytosis caused by *T. mentagrophytes* compared with the PC group. The clinical assessment of the efficacy of each tested formulation was compared with the infected untreated control. Compared with the untreated control, significant clinical efficacy was demonstrated by the PC and CK100% formulations (78.13 and 75%, respectively). In addition, 2% CMC as vehicle-treated control did not demonstrate clinical efficacy (3.13). In addition, the CK50% group (45.31 vs. 78.13%) and the CK100% group (75 vs. 78.13%) showed clinical efficacy compared with the PC group. It appears that the antifungal activity of the seed oil of *C. korshinskii* is dose dependent.

Histopathology

Histological analysis of skin biopsies of the infected areas revealed the presence of fungal elements in the skin sections from NC guinea pigs infected with *T. mentagrophytes*, indicating successful infection (fig. 3a, 4a). Many fungal elements, occasional inflammation, severe acan-

Fig. 4. Histopathological evaluation of the skin with PAS staining. **a** NC: untreated control. **b** Solvent control: 2% CMC-treated animals. **c** CK50%, which received 50% seed oil of *C. korshinskii* Kom. in guinea pig dermatophytosis. **d** CK100%, which received 100% seed oil of *C. korshinskii* Kom. in guinea pig dermatophytosis. **e** PC: 1% terbinafine-treated animals.

thosis, and tissue destruction were observed in the PAS- and HE-stained sections of the infected skin in the NC and 2% CMC groups (fig. 4a, b), while only occasional fungal elements, mild inflammation, or tissue destruction was observed in the CK50% group (fig. 4c). Similarly, no fungal elements or any evidence of tissue destruction and mild acanthosis were detected in the skin sections obtained from the guinea pigs treated with the CK100% formulations (fig. 3d, 4d) and the PC group (fig. 3e, 4e).

Discussion

The dermatophytes are a group of fungi that can invade the keratinized tissues of humans and animals. These fungi are universally distributed and may be categorized as geophilic, zoophilic, or anthropophilic based on their natural habitats. In this study, *T. mentagrophytes* was selected as the infecting fungus because it is one of the major causative organisms associated with dermatophytosis. *T. mentagrophytes* exhibited a maximum degree

of erythema and infiltration at the inoculation site in experimentally inoculated guinea pigs between the 9th and 14th day of the development of delayed hypersensitivity; after that there was regression, and 4 weeks later the lesions were healed, which suggests the participation of the immune response in the resolution of lesions [11, 13]. The host antifungal response against dermatophytosis is considered to involve the antidermatophyte activity of iron-unsaturated transferrin in serum, the activation of phagocytes, neutrophils, and macrophages in the infected areas of the skin that display direct killing activity against *Trichophyton* spp. in vitro, and the development of cell-mediated immunity correlated with delayed hypersensitivity [30–33]. Interferon- γ , interleukin-2, and the granulocyte-macrophage colony-stimulating factor are produced by peripheral blood mononuclear cells in response to stimulation by *Trichophyton* antigens [34]. The different activities of *Caragana* have been the subject of several studies [35–37]. For example, according to these reports, the seeds of *Caragana microphylla* showed the same analgesic activity as the roots and had an anti-in-

flammatory effect on ear edema in mice induced by xylol, the extract of *Caragana tangutica* exerted an anti-inflammatory effect by inhibiting the protein expression of cyclooxygenase-2 and inhibiting the vascular permeability factor which caused the increase of fluid extravasation from blood into the tissues, and the CHCl₃-soluble fraction of the MeOH extract prepared from the whole plant of *Caragana jubata* (pall.) Poir. exhibited potentially antifungal activities against three *Candida* species (*Candida albicans*, *Candida krusei*, and *Candida parapsilosis*). The improvement of skin lesions following the topical application of the seed oil of *C. korshinskii* may be related to the various above-mentioned activities.

Ergosterol is the major sterol component of the dermatophyte cell membrane and is responsible for maintaining cell function and integrity [38]. The primary mechanism of action by which azole antifungal drugs inhibit dermatophyte cell growth is the disruption of normal sterol biosynthetic pathways, leading to a reduction in ergosterol biosynthesis [39]. A previous study by our group showed that after incubation of the seed oil of *C. korshinskii* against *T. mentagrophytes*, a reduction of ergosterol content was observed [23]. The seed oil of *C. korshinskii* therefore induces considerable impairment of the biosynthesis of ergosterol. The activity of the seed oil of *C. korshinskii* acting on dermatophytes agrees with the mechanism of action proposed: cytoplasmic membrane lesion. There is no more information available regarding the biological activity of *C. korshinskii* apart from the above-mentioned research.

Evaluation of the efficacy of various formulations of the seed oil of *C. korshinskii* using a dermatophytosis guinea pig model that has utility in evaluating the antifungal efficacy of new antifungal agents or new formulations of known drugs demonstrated that 50 and 100% seed oil of *C. korshinskii* formulations possess significant clinical efficacy against dermatophytic infection caused by *T. mentagrophytes*. These data are in agreement with a previous study by our group that showed that the in vitro efficacy of the seed oil of *C. korshinskii* used alone against *T. mentagrophytes* with MIC₅₀ and MIC₉₀ ranged from 128 to 1,024 µg/ml. We used terbinafine as the reference drug in vivo as it is topically prescribed in China for the treatment of various types of dermatophytosis. The seed oil of *C. korshinskii* at a dose of 100% showed an acceptable efficacy in comparison with the licensed compound terbinafine, and skin redness and lesion severity scores were significantly reduced and continued to decline in contrast to the progressive infection observed in the untreated animals throughout the 13-day observation period.

Conclusion

We found the seed oil of *C. korshinskii* to have antifungal activity in vivo, and it exerted a significant clinical efficacy against *T. mentagrophytes* dermatophytosis in a guinea pig model when applied topically. Compared with terbinafine, *C. korshinskii* is cheaper, easily available, and already widely used by the residents living in Qinghai, China for many years. However, to develop a *C. korshinskii*-based formulation for the treatment or control of dermatophytosis-related complications, more research will be needed to identify the active ingredients in *C. korshinskii* as well as the mechanism that mediates the action of *C. korshinskii* in vitro and in vivo.

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Statement of Ethics

We hereby assure that all experiments involving animals have been carried out according to the ethical standards set by the University of the Chinese Academy of Sciences. The care and maintenance of the animals was in accordance with the licensing guidelines of the University of the Chinese Academy of Sciences. The institutional committee has approved the protocol used for the animal experiments.

Disclosure Statement

The authors have no conflicts of interest to declare.

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