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Research Article

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Evaluating antifungal effects of *Myrtus communis* nano-essence VS Terbinafine 1% Topical cream in guinea pig infected by *Microsporumcanis*

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ABSTRACT

Dermatophytosis, caused by Microsporum canis, is a superficial fungal infection that invade human and animals' skin. It can be treated by topical and oral antifungal drugs. Nano-particles produced by chitosan have appeared as promising vehicles for anti fungal delivery. They are able to enhance absorption by increasing cellular permeability. Myrtus communis possess several pharmacologic, biological and medical activities including antiviral, antibacterial, anti-inflammatory, antioxidant, wound healing effects.

In this study, 24 male guinea pigs were infected by M. Canis (by traumatisation method) and treatment was done using Terbinafine 1% cream vs Chitosan nanoparticles containing M. Communis essence. Treatment duration was 40 days and it was started since 5 days post inoculation. Fourier Transform Infrared Spectrometer and Surface Electron Microscopy were used to evaluate nanoessence. The average of clinical score was calculated for each group. Microscopic examination and fungal culture of plucked hairs and scraped scales were also observed.

Nanoparticles were around 150-200 nm. MIC ranges of Myrtus communis nano-essence were 4.2±0.2 µg/ml. The score in nano-essence group began to reduce in comparison with Terbinafine group at day 10. This healing trend continued until day 40 of the treatment. Nano-essence comparing to non treatment group treated the infection significantly ($p<0.05$). Comparing Terbinafine and the nano-essence group with each other revealed a significant difference on days 10-25 ($p<0.05$). Three consecutive culture results for all animals were negative on days 30, 37 and 44in treatment and negative control groups.

Nanoparticles in the range of around 200 nm enhanced absorption through hair follicles. Both Terbinafine and Nano group showed a healing trend, however the pace of healing was higher in nano group. The average of clinical score in nano VS Terbinafine group was not significantly different at the beginning of the experiment (4.5 ± 0.2 VS 4.2±0.2) while this difference became significant through days 10-25. When the antifungal effects of nano-essence start, it is more potential for obviation of M.canis than Terbinafine. Nano-essence comes in to action with a delay.

The findings reveal that the nano-essence treatment group showed improvement in clinical symptoms faster than the Terbinafine treatment group while its efficacy starts with a delay.

Key words: *nano-essence, Myrtus communis, Terbinafine, Microsporum canis and guinea- pig.*

INTRODUCTION

Dermatophytosis is a superficial fungal infection caused by dermatophytes that invade the keratinized tissue of human and animals. Dermatophytosis in animals mainly caused by *Microsporumcanis,*Trichophytons, Epidermophytons[1]. However, *Microsporum canis (M. canis)* is the most common cause of dermatophytosis in animals and human being[2,3,4].

Dermatophyte infections are treated with a variety of topical and oral antifungal drugs. Topical lotions and shampoos used concurrently to decrease shedding of fungi and spores, or to help treat kerion[5]. Terbinafine is an allylamine derivative with antifungal activity and can be administered both orally or topically[6].

Nano-particles have appeared as promising vehicles because of several attractive properties such as an increased surface-to-volume ratio, which offers high potential for macromolecule association and capacity to improve drug absorbtion[7]. Another advantage of administrating nanoparticles on the skin is reduction of epithelial resistance to drug transport, or the ability to carry the drug across the epithelium[8]. Chitosan (CS), is one of the main agents in the production of nanoparticles, and it is able to enhance absorption by increasing cellular permeability[13,14,15,16].

Myrtus communis possess several pharmacologic, biological and medical activities including antiviral, antibacterial, anticandida, antimutagenic, antihemorrhagic, analgesic, anti-inflammatory, antioxidant, wound healing and anti-hyperglycemic[9,10]. Although the chemical compositions of this herb varies according to the geography in which the plant grows, all of the species share the main components including α-Pinene, 1,8-Cineole, Linalool and Limonene[9,10,11,12].

The purpose of this study is to use *Myrtus communis* nano-essence to treat dermatophytosis caused by *M.canis* under experimental conditions.

MATERIALS AND METHODS

In this study, 24 male guinea pigs (350-450 g) were obtained from Pasture institute (Tehran, Iran). All of the animals were kept in separate polycarbonate cages under controlled condition (12 hours light period, relative humidity 50±3%, and temperature 25±1°C). The animals were put in an optimized condition and fed with basic diet for 1 week to adopt to the situation. *Myrtus communis* essence was purchased from Barij Essence Pharmaceutical Company (Kashan, Iran) and 5 ml nano-essence was sufficient to produce 1 litre of nano-essence. To confirm the reliability of the product, Fourier Transform Infrared Spectrometer and Surface Electron Microscopy (SEM) were used (Figure 1 and 2). The Terbinafine hdydrochloride topical cream 1% used in this study was purchased from Tehran Chemi Pharmaceutical Company (Tehran, Iran).*M.canis* standard isolate (PTCC 5069) and 4 field isolates were used to measure the minimum inhibitory concentration (MIC), and infection was caused by the standard isolate.Clinical and Laboratory Standards Institute (CLSI) broth microdilution M38-A protocol was used to determine MIC in vitro. Through the use of RPMI1640 medium, a $0.5\text{-}5\times10^4$ cells/ml suspension was obtained[17,18,19].An area of 2 × 2 cm on the back of each animal was clipped and gently scraped with the edge of a sterile scalpel[20,21]. Such gentle skin traumatization makes the animal more susceptible to skin infection. A suspension adjusted to a 0.5 McFarland turbidity standard ($1\text{-}5\times10^6$ CFU/ml NaCl 0.9%) of *M.canis* was prepared and used to inoculate scratched skin area. It was administered on the mentioned area using a Pasteur pipette. The entire area was occluded with Vaseline® in order to keep the area closed just for 24 hours[22,23]. Experimental animals were divided into 4 groups (n=6 in each) randomly including:

Non-treated (NT): inoculation was done and NaCl 0.9% was used as placebo;

Negative control (NC): no inoculation and NaCl 0.9% was used as placebo;

Nano-essence (Nano): inoculation and administration of nano-essence;

Terbinafine hydrochloride treatment (Terbi): inoculation and administration of Terbinafine cream.

Treatment was started on day 5 after inoculation, when clinical features of *M.canis*infection became most evident. Based on previous researches, we started topical treatment every 12 hours on the 5th day with both nano-essence and Terbinafine cream 1%. During the 40-day treatment, the nano-essence was sprayed by a sprinkler on and around the infected area and Terbinafine cream was applied on and around the infected area, too. Changes in lesion area, erythema, ulceration and alopecia were monitored and recorded every 5 days.Therapeutic effects of various treatments were evaluated by clinical lesion scoring and fungal culture. Changes in lesion scores were divided into 6 grades which are as follows:

0 – No signs of infection; hair fully re-grew.
1– Skin was calm; half-length long hair; no scaling.
2 – Hair re-grew on entire lesion surface; little scaling.
3 – No redness; little scaling; hair started to re-grow; few bald patches.
4– Slightly erythematous skin; loss of hair; evident scaling.
5 – Extensive skin damage; redness; crusting, ulceration, loss of hair[24].

Microscopic examination and fungal culture of plucked hairs and scraped scales were observed on days 30, 37 and 44, respectively.

Data analysis

Kruskal-Wallis Test was used to analyse lesion scores in SPSS statistical package.

RESULTS

Chitosan nanoparticles containing *M.communis* were prepared by Zist Shimi Azma Roshd Company. SEM image shows that nanoparticles were spherical and their size was 150-200nm.

Fig.1: Surface electron microscopy(SEM) of chitosan nanoparticles containing *myrtus communis* essence

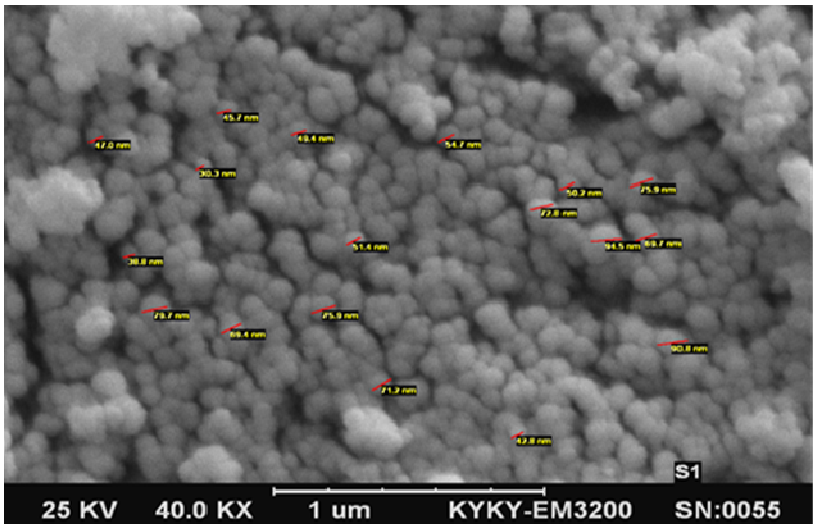
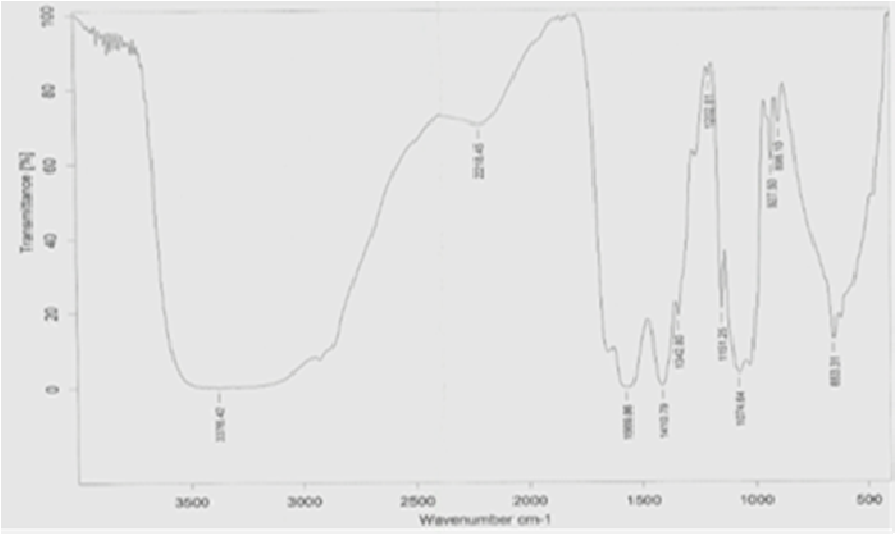


Fig.2: Loaded Fourier Transform Infrareded Spectrometer(FTIRs) of chitosan nanoparticles containing *myrtus communis* essence



MIC ranges of *Myrtus communis* nano-essence were 4.2±0.2 µg/ml.All the groups except the negative control found to be infectedwhen the treatment was going to begin (5 days after inoculation). Clinical lesion score on day 5 was 4.3±0.1 in each group, except the negative control group. The score was not significantly different among groups at the beginning of the experiment (Fig.5). One guinea pig in Terbinafine group presented a fluctuating response to the treatment, so it was excluded from statistical analysis.

The score in nano-essence group began to reduce in comparison with Terbinafine group at day 10. This decreasing trend continued until day 40 of the treatment. Nano-essence and NT groups showed statistically significant difference on days 15, 20, 25, 30, 35 and 40 ($p<0.05$). A statistically significant difference was observed between the Terbinafine group and NT group. Comparing Terbinafine and the nano-essence group with each other revealed a significant difference on days 10, 15, 20, 25 ($p<0.05$). The cure trend can be seen in(fig.5).

Fig.3: Terbinafine intergroup clinical lesion scores

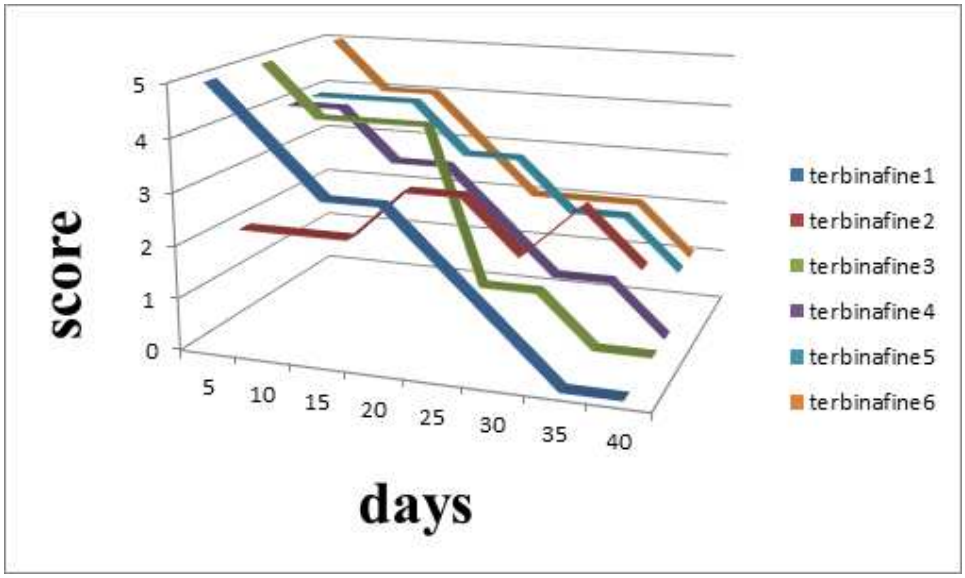


Fig.4: Nano-essence intergroup clinical lesion scores. Nano1-6 represents animal numbers.Note the score at which most of animalswere when treatment started and dramatic decrease at the days 15 and 20

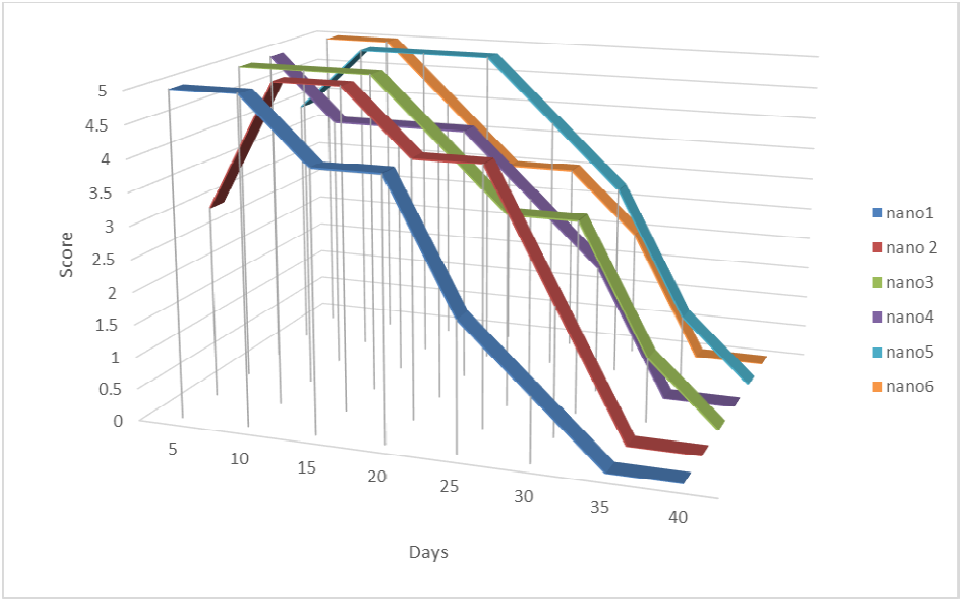


Fig.5: Clinical score average in different groups. Scores decreased from day 15 to day 40 in Nano-Essence. NT,non-treatment; NC, negative control; Nano, nano-essence; Terbi, Terbinafine

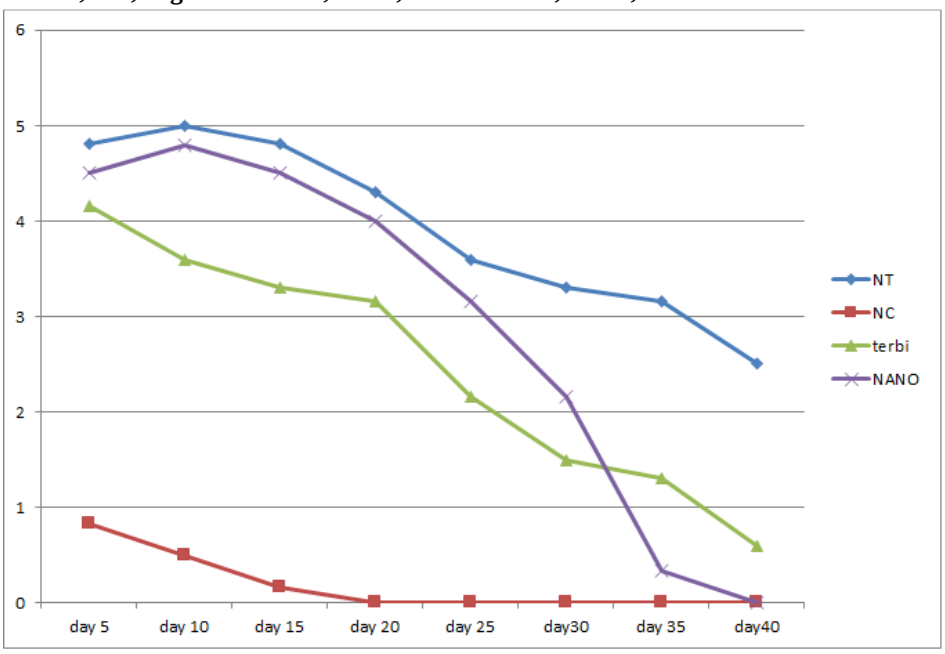


Fig.6: Time manner gross finding in different groups infected with *M.canis*. NT,non treatment. NC, negativecontrol; Nano, nano-essence; Terbi, Terbinafine

Three consecutive culture results for all animals was negative on days 30, 37 and 44 in treatment groups and negative control group (table -1). The treatment was ceased, when the second approve was achieved.

Table -1: Number and percentage of culture positive animals in every group

	Culture Positive		
Group	30day	37 day	44 day
NT	6/6(100%)	6/6(100%)	5/6(83.3%)
NC	0/6(0%)	0/6(0%)	0/6(0%)
Nano	0/6(0%)	0/6(0%)	0/6(0%)
Terbi	0/6(0%)	0/6(0%)	0/6(0%)

DISCUSSION

Topical agents are very important in the treatment of dermatophytosis. Topical therapy reduces the spread risk of infectious spores in the environment so reducing the probability of disease transmission to humans and other animals.

Minimum inhibitory concentration of nano-essence (4.2±0.2 µg/ml) was evaluated to understand the amount of nano-essence needed through the experiment.

Chitosan was used as nano particle carrier of *Myrtus commonis* nano-essence. Chitosan – a biocompatible ingredient in pharmaceutical sciences facilitates the delivery and improve efficacy of the drugs through the skin[25]. It produces a positively charged nanoparticles which enhances its attachment to the keratinized tissue. This layer of skin is negatively charged because of phospholipid layer. Another advantage of nanoparticles used in this study is their size[26,27,28]. It is approved that nanoparticles in the range of around 200 nm enhanced absorption through hair follicles Which is an interesting way of drug absorption through the skin for scientists. It should not be neglected that the spherical shape of these nanoparticles guarantee their highest surface-to-volume ratio [29,30].

All guinea pigs in this study were completely infected (except negative control group) and treatment was began on the 5th day post inoculation. Treatment duration was 40 days (BD) and all animals except the NT group were clinically treated at the end of the study as seen in (fig.6).

Both Terbinafine and Nano group showed a healing trend, however it was seen that the pace of healing was higher in nano group, since a sharper slope was obvious in diagrams (Fig.3 and 4). Treatment in Nano group did not present notable plateau phase, while there was almost 2-3 plateau phase by treating with Terbinafine. It suggests that nano-essence formulation would result in more predictable healing trend, however it was faster than Terbinafine.

The average of clinical score in nano *VS* Terbi group was not significantly different at the beginning of the experiment (4.5 ± 0.2 *VS* 4.2±0.2). According to the results obtained (fig.5) on days 5 to 10 clinical lesions in nano-essence show small increase. The percentage of the score reduction through days 15 to 20 in nano-essence and Terbinafine group was 8.4% and 3.4%, respectively. As we investigated this noteworthy phenomenon that from 10 to 20 days after treatment drastic reduction in the average score treatment groups can be seen. Through days 25 to 30, a 20% and 12% reduction of scores were occurred in nano-essence and Terbinafine group, respectively. It shows that when the antifungal effects of nano-essence starts, it is more potential for obviation of *M.canis* than Terbinafine. The rate of healing in nano-essence group through days 30-35 was 36% but in the Terbinafine group scores was only 4%. This means that Nano-essence come in to action with a delay (fig.5). The reason must be the nanoparticles characteristics which is accumulated in follicles and lipophilic tissue and also the slow release character of chitosan nanoparticles[31]. So in the initial days the concentration of *Myrtus communis* essence on the infected skin was not enough to threat *M.canis*. But after 10 days the amount of essence which is released from nanoparticles is enough to kill *M.canis* and nanoparticles efficacy appears. To sum up on days 5 to 10 clinical lesions score in nano-essence group showed small increase but from day 10 to 40, it was lowered more quickly than Terbinafine group. The efficacy of nano-essence prepared in this study was clinically like Terbinafine (Fig.6).

This declining trend in the average of clinical scores from day 30 after the infection was followed by 3 consecutive negative cultures in 100% of the animals in both nano-essence and Terbinafine groups, as opposed to 100% positive culture observed on days 30 and 37, 83.3% on day 44 in the positive control group(Table 1).

The findings reveal that the nano-essence treatment group showed improvement in clinical symptoms faster than the Terbinafine treatment group while its efficacy starts with a delay. Generalization of results in animals and human patients needs further clinical trials.

CONCLUSION

It is concluded that nano-essence of *M. communis* has efficient antifungal effects against *M. Canis* and it can be a treatment candidate for dermatophytosis but generalization of results in animals and human needs further clinical trials.

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