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Antifungal Activity of the Essential oil of *Thymus vulgaris* L. and Thymol on Experimentally Induced Dermatomycoses

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The *in vivo* evaluation of the toxicological and antifungal activity of the essential oil of *Thymus vulgaris* L. and its main component thymol was made on 2-month-old male Wistar rats. We examined the therapeutic potency against experimentally induced dermatomycoses in rats, using the most frequent dermatomycetes, *Trichophyton mentagrophytes, T. rubrum,* and *T. tonsurans.* The therapeutic efficacy of a 1% solution of the essential oil of *Thymus vulgaris* and thymol as well as the commercial preparation bifonazole was evaluated. During the 37-day observation period the oil-treated animals were cured.

Keywords *Thymus vulgaris*; *Trichophyton* spp; antifungal activity; dermatomycetes; dermatomycoses; thymol

INTRODUCTION

Among the animal and human pathogens, the dermatomycetes are the main causes of dermatomycoses (infections of the hair, skin, and nails), superficial infections that are not life threatening but are chronic and cause considerable morbidity (Bell-Syer, Hart, Crawford, Tyrrel, & Torgerson, 1998). The fungi invade into keratinized tissues and hair follicles, and cause patchy alopecia, scale, and subsequent inflammation (Scott, Miller, & Griffin, 2001). Dermatomycoses are sometimes difficult to eradicate using a drug treatment. Besides, commercial antifungal agents can have adverse effects such as gastrointestinal disturbances, hepatotoxicity, and leucopenia and these primarily occur with systemic administration. However some of these antifungal agents are still used orally, ketoconazole is one of the commonly used antifungal drugs administrated orally for the treatment of both superficial and deep infections caused by *Trichophyton* species. The unpleasant side effects of this drug include nausea, abdominal pain, and itching, and its toxicity limits its therapeutic use in many cases (Shin & Lim, 2004). Therefore, the development of more effective and less toxic antifungal agents is required for the treatment of dermatomycosis (Silva et al., 2005). Various plant materials are believed to have antifungal activity, and many essential oils have been reported to have antifungal activities with no side effects on humans and animals (Sokmen, Jones, & Erturk, 1999). Essential oils play a great role in these investigations. Previous in vitro and in vivo investigations of the antifungal activity of the essential oils of some medicinal and aromatic plants, *Origanum vulgare* subsp. *hirtum*, *Mentha spicata*, *Lavandula angustifolia*, and *Salvia fruticosa*, suggested that they could be used as effective antifungal agents (Adam, Sivropoulu, Kokkini, Lanaras, & Arsenakis, 1998).

The purpose of this study was to investigate the essential oil of *Thymus vulgaris* and thymol for potential antifungal activity in vivo. The selection of plants for evaluation was based on traditional usage for treatment of infection diseases (Crespo, Jimenz, Gomis, & Navaro, 1990; Hitokoto, Morozumi, Wauke, Sakal, & Kubata, 1980; Janssen, Chis, Scheffer, J., & Baerhein Svendsen, 1986; Panizzi, Flamini, Cioni, & Moreli, 1993; Sokmen et al., 1999). However, there are only limited data in the literature on the antifungal activity of essential oils toward human fungal pathogens in vivo.

In this work the antifungal activity of thyme (*Thymus vulgaris*) essential oil and its main component thymol was examined against widely spread pathogenic fungal strains *Trichophyton mentagrophytes*, *T. rubrum*, and *T. tonsurans*, which cause superficial skin infections in humans.

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MATERIALS AND METHODS

Plant Material

Thymus vulgaris L. plants were collected during the summer (July) in 2006 at the experimental field of the Institute for Medicinal Plant Research "Josif Pančić" in Pančevo (Serbia). The species was identified at the Institute of Botany, Faculty of Biology of the University of Belgrade, where a voucher specimen is deposited (17432).

Isolation and Identification of the Essential Oil

The aerial part of plants was dried in air flow (25°C), powdered, and stored until further use. The essential oil was isolated by hydrodistillation in a Clavenger type apparatus for 2 h. The composition of essential oil was investigated using analytical GC/FID (gas chromatography/flame ionization detector) and GC/MS (mass spectrometry) techniques. For this purpose, HP 5890 series II gas chromatograph, equipped with split–splitless injector, fused silica capillary column (25 m × 0.32 mm), coated with cross-linked methyl silicone gum (0.5-μm film thickness), and FID was employed. Essential oil solutions in ethanol (1%) were injected in the split mode (1:30). The injector was heated to 250°C, FID at 300°C, whereas column temperature was linearly programmed from 40 to 280°C (4°C/min).

The GC/MS analysis was done on a HP-GCD equipped with a split–splitless injector, fused silica capillary column (50 m × 0.2 mm) PONA, coated with cross-linked methyl silicone gum (0.5-μm film thickness) and a mass-selective detector (MSD). The chromatographic conditions were as above. The transfer line (MSD) was heated to 280°C. The electron microscopy image simulation (EMS) spectra (70 eV) were acquired in scan mode in *m*/*e* range 40–300.

The identification of individual constituents was done by comparison of their retention times with those of analytical standards, and by computer searching, matching the mass spectral data with those held in the Wiley/NBS library of mass spectra. For quantification purposes area percent reports obtained by FID were used.

Bioassay

Animals

Locally bred, 2-month-old male Wistar rats weighting about 250 g were used. The rats were maintained in propylene cages, separately, at room conditions (temperature of 22 ± 2°C; relative humidity ~60%) in a 12-h light–dark cycle. They were given pelleted diet (Veterinary Institute, Subotica, Serbia) and tap water ad libidum. Protocols for animal use followed the Public Health Service Policy on Human Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee.

Analysis of Nonharmful Effects

To determine the nonharmful concentrations of the essential oil and thymol, we used 25 locally bred male Wistar rats (170–240 g). The rats were maintained under the same condition as described previously. Rats used for tests were randomly divided into five groups according to concentration of applied compounds investigated. A 0.5 mL of prepared stock solution of the essential oil and thymol diluted in ethanol (0.01–1%, vol/vol) was injected intraperitoneally. The ointment is considered as nonharmful if all the five animals in a group survive 48 h after application (*Pharmacopeia Jugoslavica*, 1984). Concentrations that are nonharmful (0.1%) to the feet of animals were used for further investigation.

In Vivo Fungitoxicity Assay

The in vivo investigation of the antifungal activity of *Thymus vulgaris* essential oil and thymol was made according to Adam et al. (1998). Three dermatomycetes were used: *T. mentagrophytes*, *T. rubrum*, and *T. tonsurans.* The organisms were isolated from patients at the Center for Preventive Medicine, MMA, in Belgrade.

The cultures were maintained on Sabouroud dextrose agar (SDA) containing 40 g glucose, 10 g agar, and 10 g peptone in 1 L of distilled water. The cultures were stored at 4°C and subcultured once a month (Booth, 1971).

Locally bred, 2-month-old male Wistar rats were divided into four groups for the five animals; untreated animals served as a control, treated animals with *Thymus vulgaris* oil, thymol, and bifonazole.

On the back of each animal, 4 cm^2 areas were cleaned and depilated. The fungal inoculum was prepared from 7-day-old cultures of *T. mentagrophytes*, *T. rubrum*, and *T. tonsurans.* The conidia of all fungal species tested separately were suspended in sterilized physiological saline containing 0.1% Tween 80. Following filtration through four layers of sterile gauze to remove hyphal fragments and agar flicks, the final conidial suspension was adjusted to 10^7 conidia/mL for use as the inoculum. The conidia were counted using a hemocytometer (STOCK/15170-173, VRW Scientific, Arlington Heights, Illinois, USA) under a microscope (Type 020-518.500DMLS, Leica, Solms, Germany) (Inouye, Uchida, & Yamaguchi, 2001). The inoculum was applied on the back of the animals immediately after depilation and left for 3 days. The establishment of active infection was confirmed on day 4 by isolation of the pathogens from skin scales cultured from infected loci on SDA plates containing 100 units/mL penicillin and streptomycin. Infections were also confirmed by visual examination of the animals on days 8–10. In the animals in which active infections were confirmed, treatment was initiated on day 20 postinoculation and continued until complete recovery from infection was achieved. The ointments contained 0.1% (vol/vol) of thyme essential oil and the thymol, separately, mixed in petroleum jelly. The commercial fungicide bifonazole was used as a control. The treatments were applied once daily, and the infected areas were scored visually for inflammation and scaling. Clinical assessment of inoculates skin area was performed using a modified lesion score from 0 to 4 as indicated: score 0, no

visible lesion; score 1, few slightly erythematous lesions on the skin; score 2, well-defined vesicles; score 3, large areas of marked redness incrustation, scaling, blade patches, ulcerated in places; score 4, mycotic foci well developed with ulceration in addition to a score 3 lesion (Petranyi, Meingassner, & Meith, 1987). The presence of the pathogens was confirmed by cultivation of skin scales from infected loci on SDA plates containing 100 units/mL penicillin and streptomycin each day.

RESULTS

The dominant components of *Thymus vulgaris* essential oil were thymol (48.92%) and *p*-cymene (18.99%) (Table 1). The essential oil of thyme and thymol were tested for their nonharmful effects as 0.01 and 1% (vol/vol) solutions in ethanol and petroleum jelly, separately. There was nonharmful activity for the 0.1% solutions of thyme oil and thymol and for further investigation these solutions were used. The chosen essential oil and its component, thymol, and concentration are in accordance with literature data; thyme oil and thymol are traditionally used in herbal medicine as an antiseptic and/or antimicrobial to help treat minor wounds and sores (Blumenthal, Goldberg, Brinkmann, 2000; Bradley, 2006). Standardized amounts of thyme oil are found in topical ointments in 0.6–1.2% volatile oil and 0.5% thymol. In vitro and in vivo studies of thyme oil and thymol have also demonstrated antioxidative and anti-inflammatory effects including inhibition of prostaglandin synthesis (Basch, Ulbricht, Hammernes, Bevins, & Sollars, 2004). Previous investigation indicated that thyme oil may serve as a protective agent to the damaged skin tissues (due to experimentally induced burn wounds) by decreasing the NO level (Dursun, Liman, Ozyazgan, Gunes, & Saraymen, 2003). Thyme oil promotes hair growth in patients with alopecia and induced significant reduction in area of alopecia in treated group compared with controls (Hay, Jamieson, & Ormerod, 1998).

TABLE 1
Composition of the Essential oil of *Thymus vulgaris*

Components	%	RI[a]
α-Thujene	1.17	307
α-Pinene	1.21	319
Camphene	0.83	340
Sabinene	0.58	379
β-Pinene	0.41	386
β-Myrcene	1.06	408
α-Phelandrene	0.12	435
α-Terpinene	0.53	457
p-Cymene	18.99	471
Limonene	0.46	481
1,8-Cineole	0.76	485
β-Ocimene-(*E*)	1.30	519
γ-Terpinene	4.08	545
Terpinolene	0.14	608
Linalool	0.60	632
Camphor	0.17	734
Borneol	1.72	789
Terpineol-4	1.78	820
Thymol-methyl-ether	0.16	968
Carvacrol-methyl-ether	1.73	990
Thymol	48.92	1113
Carvacrol	3.45	1137
β-caryophyllene-(*E*)	3.45	1442
α-Humulene	0.16	1527
γ-Murolene	0.14	1586
Germacrene D	0.33	1594
σ-Cadinene	2.23	1733
Total identified	96.08	

[a]RI-DB 5 column.

The ointments containing 0.1% of oil and thymol were prepared in petroleum jelly. The therapeutic efficacy of the ointments was evaluated daily by macroscopic examination of lesions and by screening for the presence of the infections by culturing skin scales from the infected area. The lesions were scored as cured only when the infected areas were free of macroscopic lesions and the cultures were negative.

The first symptoms (small vesicles) on the rats inoculated with *T. mentagrophytes* were observed on day 8 of the experiment. For all rats infected, in all groups, symptoms became severe from day 8 to day 20, from erythematous lesions on the skin to the mycotic foci well developed with ulceration, which later (day 20) on resulted in bloody wounds, 20 mm in diameter. We started with the treatment on day 20 of the experiment. The rats treated with the essential oil of *Thymus vulgaris* were completely cured after 24 days of treatment, whereas thymol showed therapeutic activity for 14 days of treatment. The animals treated with the commercial drug, bifonazole, were cured after 14 days of treatment. After this period, cultures taken from the infected region were negative for the animals treated with bifonazole, thymol, and oil. For untreated rats symptoms were observed at the same time as in treated animals, and symptoms became severe from day 8 to day 26. Until the end of the experiments, symptoms were present in these animals (Figures 1 and 2).

On the back of the rats infected with *T. rubrum*, the first symptoms (scaly, erythematous to tawny-brown, bilateral and asymmetric lesions) were observed on day 8 after inoculation. For the rats treated with the essential oil of thyme, symptoms were observed on day 10. Treatment was started on day 20. On day 13 of the treatment, there were no visual symptoms, but cultures were positive, so we prolonged the experiment. After 20 days of treatment with oil, the symptoms became severe (bloody wounds). On day 37 of treatment with oil, the animals were completely cured and cultures were negative. The rats treated with thymol were cured completely after 14 days of

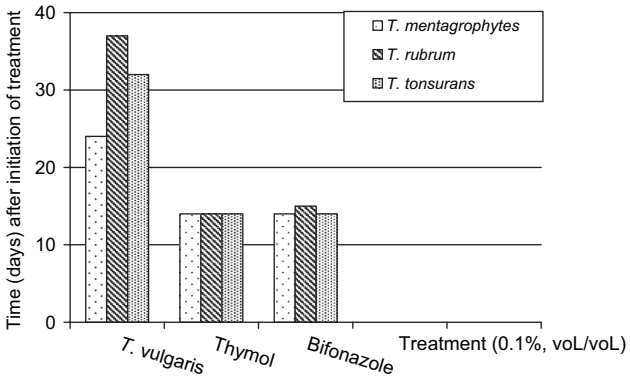

FIGURE 1. Antifungal activity of *Thymus vulgaris* essential oil, thymol, and bifonazole.

treatment and after this period cultures were negative. The animals treated with bifonazole were cured within 15 days of treatment. The control rats, untreated ones, showed few slightly erythematous lesions on the skin after 8 days, symptoms became severe till day 30, while until the end of the experiments symptoms were at the scoring scale 2 (Figures 1 and 3).

First symptoms (small, black-brown lesions) on the rats infected with *T. tonsurans* were observed on day 6 after inoculation. Small lesions had formed behind the ears. Treatment was started on day 20 after inoculation. The rats treated with the oil of *Thymus vulgaris* showed symptoms on day 6. On day 24 after inoculation, the symptoms became severe and the vesicles started to bleed. The rats were completely cured after 32 days of treatment. All the animals treated with thymol had completely recovered after 14 days. The animals treated with bifonazole were cured after 14 days of treatment. Cultures were negative for the treated animals. Control animals possessed the same symptoms as treated animals till day 20 when treatment started. Symptoms became severe for these animals on day 43. The symptoms could be seen until the end of the experiments (Figures 1 and 4).

DISCUSSION

During the 37-day observation period the treated animals were cured completely. For the rats infected with *T. rubrum* and *T. tonsurans*, the symptoms disappeared and appeared again several times, but the fungus was still recovered from the skin. This phenomenon is often observed on either animals or humans infected with dermatomycetes (Ryan, 1994). It should be noted that in many cases macroscopic lesions disappeared before elimination of the infectious agent, indicating that long treatment periods of application and evaluation were necessary (Adam et al., 1998). It is normal because dermatomycete infections typically resolve spontaneously over a variable time period (18 months to 4 years) depending on the immune response. Many of dermatomycetes rarely cause inflammatory reactions, making it very difficult to the immune system to recognize and eliminate the fungus.

It can be seen that clinical parameters were the most characteristic (bloody wounds) development in the animals infected with *T. mentagrophytes*, and that the period of treatment was the shortest, whereas for the rats infected with *T. rubrum* and *T. tonsurans*, symptoms were much temperate, but the duration of treatment was longer than in the previous case. The symptoms caused by *T. rubrum* and *T. tonsurans* disappeared several times during the experiment and reappeared after a few days. The reason for such a phenomenon may be due to the zoophilic characteristics of *T. mentagrophytes* and the antropophilic characteristics of *T. rubrum* and *T. tonsurans*. Some are primarily adapted to the skin of humans, and the others to

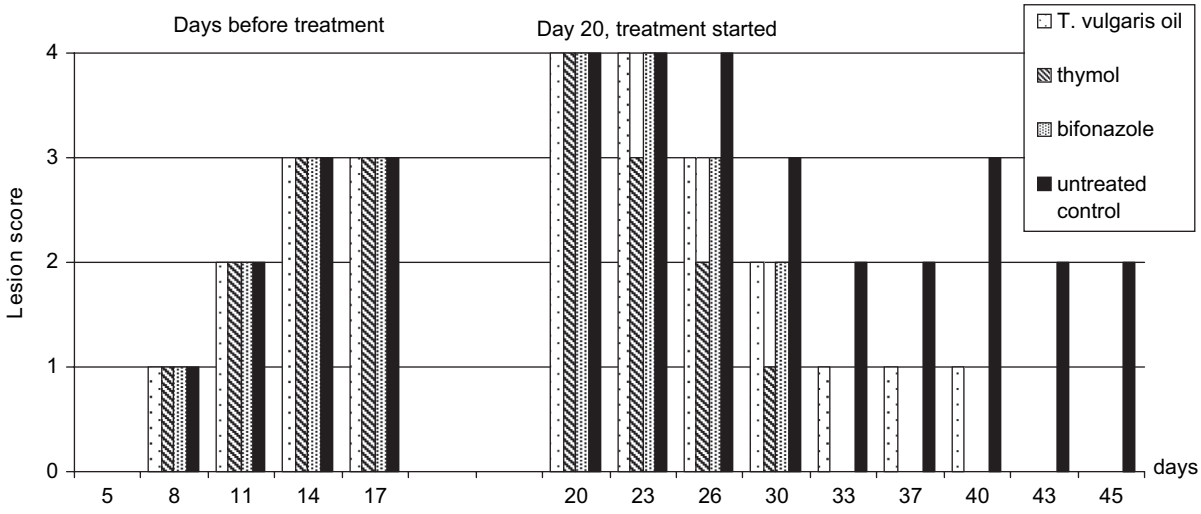

FIGURE 2. Time-dependent changes in skin lesion scores in Wistar rats infected with *Trichophyton mentagrophytes*, and treatment with *Thymus vulgaris* essential oil, thymol, and bifonazole.

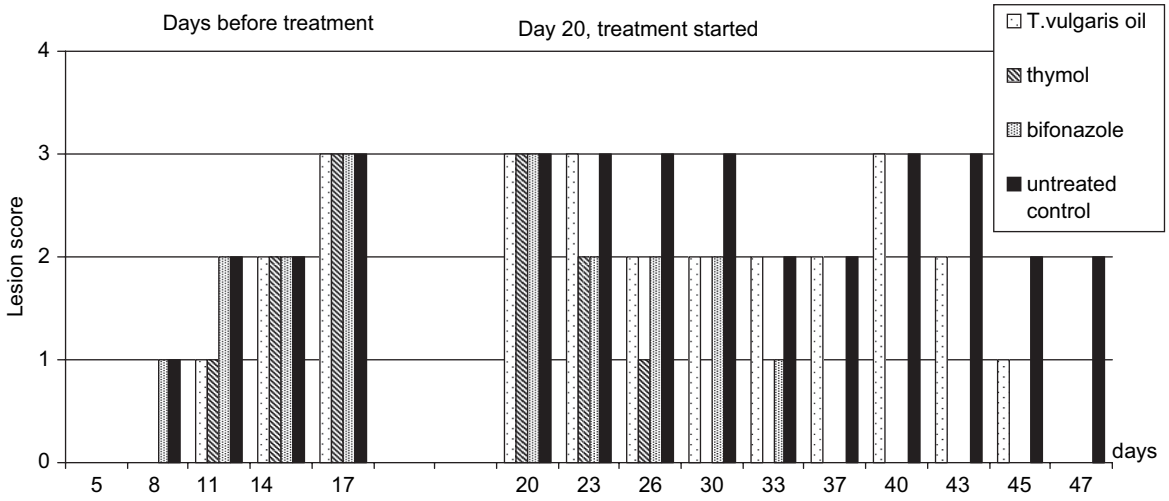

FIGURE 3. Time-dependent changes in skin lesion scores in Wistar rats infected with *Trichophyton rubrum*, and treatment with *Thymus vulgari*s essential oil, thymol, and bifonazole.

animals. The course of the infection is then dependent on the anatomic location, the dynamics of skin growth and desquamation, the spread and extent of the inflammatory response, and the infecting species. If the organisms grow very slowly in the stratum corneum, and turnover by desquamation of this layer is not retarded, the infection will probably be short-lived and cause minimal signs and symptoms (Ryan, 1994).

The essential oil of *Thymus vulgaris* completely cured animals infected with *T. mentagrophytes* within 24 days, *T. rubrum* within 37 days, and *T. tonsurans* within 32 days of treatment.

The animals treated with the commercial drug bifonazole were cured after 14–15 days of treatment. If we compare the activity of this commercial drug and the essential oil investigated, the first one possesses higher therapeutic activity, but, as we commented on earlier, commercial drugs could cause side effects. There were no side effects on animals treated with the secondary metabolites derived from plants.

It can be seen that thymol possessed higher therapeutic and antifungal activities than the essential oil investigated, and the same activity as the commercial drug bifonazole. Because oil showed lower activity than thymol, it is evident that properties of the active principles could be ascribed to this component. It appears that interactions of a chemical nature between oil constituents blocked the actions of the individual active principles as an antagonistic effect (Davidson & Parish, 1989). In addition, the test agents, thyme oil and thymol, assessed had different mechanisms of antifungal action, and the results of these assays may simply reflect these differences.

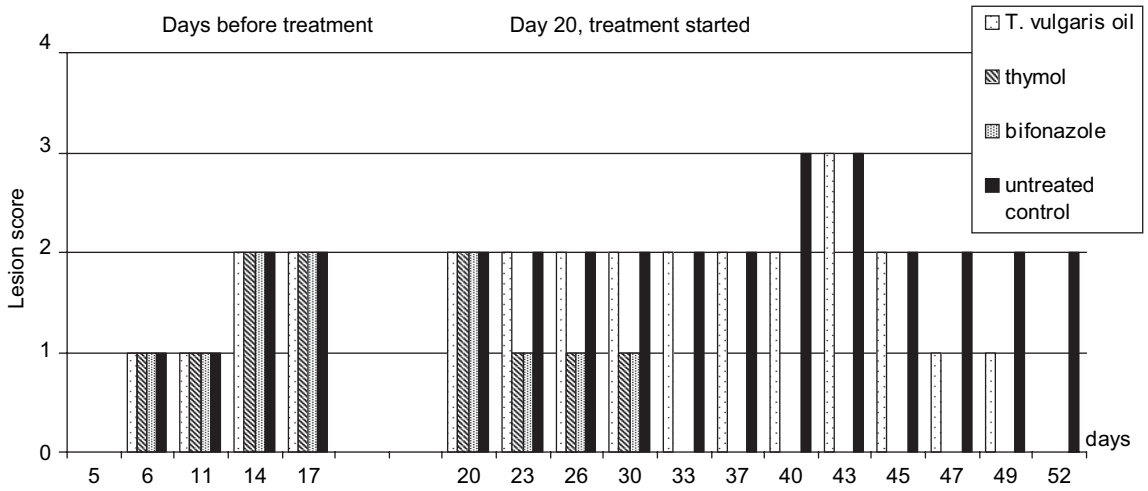

FIGURE 4. Time-dependent changes in skin lesion scores in Wistar rats infected with *Trichophyton tonsurans*, and treatment with *Thymus vulgari*s essential oil, thymol, and bifonazole.

In a recent study, Lee et al. (2007), showed that the experimental therapeutics, eugenol and nerolidol, topically applied after 5 days of infection for 26 days in 10% (vol/vol), possessed antifungal activity in vivo. In our experiment 0.1% of thyme oil solution and thymol showed antifungal activity.

From the above results, it can be concluded that the essential oil of *Thymus vulgaris* and thymol showed very good antifungal and therapeutic activity against the fungal species investigated in this study. These compounds could represent possible alternatives for the treatment of patients infected by dermatomycetes.

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