

ANTIFUNGAL ACTIVITY OF THE ESSENTIAL OILS AND COMPONENTS *IN VITRO* AND *IN VIVO* ON EXPERIMENTALLY INDUCED DERMATOMYCOSES AT RATS

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Antifungal activity of the six essential oils (*Mentha spicata*, *Ocimum basilicum*, *Lavandula angustifolia*, *Salvia officinalis*, *Citrus limon* and *C. aurantium*) and five main components (camphor, menthol, linalool, limonene and 1,8-cineole) were tested *in vitro* against three dermatomycetes, *Trichophyton mentagrophytes*, *T. rubrum*, and *T. tonsurans*. The *in vivo* evaluation of the toxicological and antifungal activity of the six essential oils and their components were made on 2-month-old male Wistar rats. We examined the therapeutic potency against experimentally induced dermatomycoses in rats, using the most frequent dermatomycete, *T. mentagrophytes*. The therapeutic efficacy of a 1% solution of the essential oils and their components as well as the commercial preparation bifonazole were evaluated. Antifungal activity of essential oils and components showed potential against dermatomycetes *in vitro* and *in vivo*. The most promise oils and components could be *S. officinalis* and *L. angustifolia* and menthol which showed very good therapeutic and antifungal effect *in vivo*.

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1. Introduction

Human infections diseases have markedly increased during the past ten years, especially in immunocompromised patients. 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 [1]. Commercial antifungal agents can have adverse effects such as gastrointestinal disturbances, hepatotoxicity and leucopenia and these primarily occur with systemic administration. Therefore, the development of more effective and less toxic antifungal agents is required for the treatment of dermatomycosis [2]. Recent research showed that higher plants may serve as promising sources of novel antimycotics with no side effects on human and animals. Essential oils play a great role in these investigations. 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 [3].

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Previous *in vitro* and *in vivo* investigations of the antifungal activity of the essential oils suggested that they could be used as effective antifungal agents [4]. The selection of plants for evaluation was based on traditional usage for treatment of infection diseases [5, 6, 7]. However, there are only limited data in the literature on the antifungal activity of essential oils toward human fungal pathogens *in vivo*. The purpose of this *in vitro* and *in vivo* studies is to examine the antifungal potential of a selection of essential oils and their components against dermatomycetes.

2. Experimental

2.1. Plant material

Materials of *Mentha spicata* (L.), *Ocimum basilicum* (L.), *Salvia officinalis* (L.) and *Lavandula angustifolia* (L.) (Lamiaceae) were collected in August, 2010 at the experimental field, of the Institute for Medicinal Plant Research "Josif Pančić", in Pančevo (Serbia). *Citrus limon* (L.) Burm. (Rutaceae) (08600053-9944) and *C. aurantium* (L.) (Rutaceae) (08600030-8790) oils are commercial samples (AKRAS International Austria). Essential oils components used (camphor, menthol, linalool, limonene and 1,8-cineole) were commercial samples (Aldrich Chemical Co., Milwaukee, WI).

2.2. Antifungal activity *in vitro*

For the antifungal bioassay, three dermatomycetes were used *Trichophyton mentagrophytes*, *T. rubrum* and *T. tonsurans*. The organisms were isolated from patients at the Center for Preventive Medicine, Medical Military Academy, Belgrade, Serbia. The culture was maintained on Sabouroud dextrose agar (SDA) and stored at 4°C [8]. In order to investigate the antifungal activity of the oils and components, a modified microdilution technique was used [9]. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v) and adjusted with sterile saline to a concentration of 1.0×10^5 CFU in a final volume of 100 µl per well. The microplates were incubated for 72 h at 37 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 µl of tested compounds dissolved in medium and inoculated for 72 h, into microtiter plates containing 100 µl of broth per well and further incubation 72 h at 37 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Commercial fungicides, bifonazole (Srbolek, Belgrade, Serbia) was used as positive controls. All experiments were performed in duplicate and repeated three times.

2.3. Antifungal activity *in vivo*

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.

2.4. Analysis of nonharmful effects

To determine the no harmful 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 oils and components were diluted in ethanol (0.01–1%, vol/vol) and injected

intraperitoneally. The ointment is considered as no harmful if all the five animals in a group survive 48 h after application [14]. Concentrations that are no harmful (0.1%) to the feet of animals were used for further investigation.

2.5. *In vivo* fungitoxicity assay

The *in vivo* investigation of the antifungal activity of essential oils and components was made according to Adam et al., (1998) [4]. *T. mentagrophytes* was isolated from patients at the Center for Preventive Medicine, MMA, in Belgrade. 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 oils (every oil separately), treated animals with components (every components separately), and bifonazole. On the back of each animal, 4 cm² areas were cleaned and depilated. The fungal inoculum was prepared from 7-day-old cultures of *T. mentagrophytes*, 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 10⁷ 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) [11]. 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 post inoculation and continued until complete recovery from infection was achieved. The ointments contained 0.1% (vol/vol) of essential oils and components, 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 [12]. 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.

2.6. Statistical analysis of results

Results were analyzed by statistical and graphics package STATGRAPHICS, version 4.2 (STScI Inc. And Statistical Graphics Corporation, 1985-1989, USA), ANOVA procedures, with multiple range test based on LSD (Less Significant Differences), the level significance p.

3. Results

The results of the chemical analyses of essential oils investigated and previously reported [13]. The main components of *Mentha spicata* oil are menthone (21.92%) and carvone (49.52%). Limonene is the most abundant component in *Citrus limon* (59.68%) and *C. aurantium* (90.01%) oils. Linalool (27.21%) and linalyl acetate (27.54%) are the most abundant components in *Lavandula angustifolia* oil. Linalool is also the main component in *Ocimum basilicum* oil with 59.25%. Camphor (16.67%) and α -thujone (31.65%) are the main components in *Salvia officinalis* oil [13].

The results of antifungal activity of essential oils and components *in vitro* are presented in Tables 1. and 2. All the oils tested showed antifungal activity against all the fungi. Essential oil of *M. spicata* showed inhibitory activity at 1.0 μ l/ml and fungicidal at 2.0 μ l/ml as was shown by [14]. *L. angustifolia* showed MIC at 6.0-6.5 μ l/ml and MFC at 7.0-8.0 μ l/ml, while *O. basilicum* possessed inhibitory activity at 2.0 μ l/ml and fungicidal at 3.0 μ l/ml. Oil of *S. officinalis* exhibited

MIC at 5.0 $\mu\text{l/ml}$ and MFC at 9.0 $\mu\text{l/ml}$. *C. aurantium* (MIC 8.0 $\mu\text{l/ml}$ and MFC 10.0 $\mu\text{l/ml}$) showed lower antifungal potential than *C. limon* (MIC at 6.0 $\mu\text{l/ml}$ and MFC 8.0 $\mu\text{l/ml}$). Antifungal potential could be presented as follows: *M. spicata* > *O. basilicum* > *L. angustifolia* > *S. officinalis* > *C. limon* > *C. aurantium*. It can be seen that *M. spicata* oil possessed the best antifungal potential among all the oils tested, while *C. aurantium* exhibited the lowest effect.

Table 1. Antifungal activity (MIC and MFC in $\mu\text{l/ml}$) of essential oils in vitro.

Fungi	<i>M. spicata</i>	<i>L. angustifolia</i>	<i>O. basilicum</i>	<i>S. officinalis</i>	<i>C. aurantium</i>	<i>C. limon</i>	Bifonazole
	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC
<i>T. menthagrophytes</i>	1.0 \pm 0 2.0 \pm 0.6	6.0 \pm 0.4 7.0 \pm 0.3	2.0 \pm 0.6 3.0 \pm 0.3	5.0 \pm 0.5 9.0 \pm 1	8.0 \pm 0.4 10.0 \pm 1	6.0 \pm 0.6 8.0 \pm 0.4	10.0 \pm 1 10.0 \pm 1
	1.0 \pm 0.3 2.0 \pm 0.6	6.0 \pm 0.3 8.0 \pm 0	2.0 \pm 0.3 3.0 \pm 0.3	5.0 \pm 0 9.0 \pm 0.5	8.0 \pm 0 10.0 \pm 0.5	6.0 \pm 0.4 8.0 \pm 0.4	8.0 \pm 0.6 10.0 \pm 1
<i>T. tonsurans</i>	1.0 \pm 0.3 2.0 \pm 0.3	6.5 \pm 0.5 8.0 \pm 1	2.0 \pm 0.3 3.0 \pm 0	5.0 \pm 0.3 9.0 \pm 1	8.0 \pm 0.4 10.0 \pm 0	6.0 \pm 0.3 8.0 \pm 0.4	10.0 \pm 1 12.0 \pm 1.5

Values are means \pm standard deviation of three separate experiments.

Commercial fungicide showed inhibitory activity at 10.0 $\mu\text{l/ml}$ and fungicidal activity at 10.0-12.0 $\mu\text{l/ml}$, lower than essential oils with exception of orange oil (Table 1).

Antifungal activity of essential oils components showed that all the components exhibited great potential. Linalool showed MIC at 2.0-3.0 $\mu\text{l/ml}$ and MFC 3.0-4.0 $\mu\text{l/ml}$. Limonene possessed inhibitory activity at 5.0 $\mu\text{l/ml}$ and fungicidal at 7.0 $\mu\text{l/ml}$ [14]. MIC for 1,8-cineole was 3.0 $\mu\text{l/ml}$ and MFC 3.5 $\mu\text{l/ml}$ [14], while camphor showed inhibition at 4.0-5.0 $\mu\text{l/ml}$ and fungicidal activity at 5.0-6.0 $\mu\text{l/ml}$. Menthol showed great antifungal potential with MIC 0.5 $\mu\text{l/ml}$ and MFC at 1.0 $\mu\text{l/ml}$ [14]. Antifungal potential of components could be ranged as: menthol > 1,8-cineole > linalool > camphor > limonene. The best activity could be seen for menthol, while limonene showed the worst antifungal activity. All the components possessed better antifungal effect than bifonazole (Table 2).

Table 2. Antifungal activity (MIC and MFC in $\mu\text{l/ml}$) of essential oils components in vitro.

Fungi	linalool	limonene	1,8-cineole	camphor	menthol	Bifonazole
	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC
<i>T. menthagrophytes</i>	3.0 \pm 0.6 4.0 \pm 0.3	5.0 \pm 0.5 7.0 \pm 0.6	3.0 \pm 0.6 3.5 \pm 0.5	4.0 \pm 0.4 5.0 \pm 1	0.5 \pm 0.3 1.0 \pm 0.5	10.0 \pm 1 10.0 \pm 1
	2.0 \pm 0.4 3.0 \pm 0.3	5.0 \pm 0 7.0 \pm 0.6	3.0 \pm 0.3 3.5 \pm 0.5	5.0 \pm 0.5 6.0 \pm 1	0.5 \pm 0 1.0 \pm 0.5	8.0 \pm 0.6 10.0 \pm 1
<i>T. tonsurans</i>	2.0 \pm 0 3.0 \pm 0.6	5.0 \pm 0.5 7.0 \pm 0.4	3.0 \pm 0.6 3.5 \pm 0.5	4.0 \pm 0.3 5.0 \pm 1	0.5 \pm 0 1.0 \pm 1	10.0 \pm 1 12.0 \pm 1.5

Values are means \pm standard deviation of three separate experiments.

The antifungal and therapeutic potential of the six essential oils and five of their components was evaluated *in vivo*. There was no harmful activity for the 0.1% solutions of oils and components in toxicological test and for further investigation these solutions were used. The chosen essential oils/their component, and concentration are in accordance with literature data; these compounds are traditionally used in herbal medicine as an antiseptic and/or antimicrobial to help treat wounds and sores [15, 16].

For the experiment *in vivo* and inducing experimental dermatomycoses we used the most frequent dermatomycete *T. mentagrophytes*, which is common in rodents but also in human [17]. The first symptoms on the infected rats inoculated with *T. mentagrophytes* were observed on 3th day of the experiment in the form of well defined clinical parameters for this species (small inflammatory vesicles). After 3 days from erythematous lesions on the skin symptoms goes to the mycotic foci well developed with ulceration, which later (day 20) on resulted in wounds. The treatment started on 5th day of the experiment. Animals treated with *M. spicata* oil were completely cured after 15 days of treatment. Essential oil of *L. angustifolia* exhibited therapeutic activity after 13 days of treatment. The group of rats treated with *O. basilicum* oil were cured after 25 days of treatment. The shortest period of currency was observed at animals treated with *S. officinalis*-12 days (Fig. 1). The longest period of treatment was observed at rats treated with oils of *C. aurantium* and *C. limon*, 45 days. For all the oils tested there no visually observed symptoms at the end of the treatment and the culture reinoculated were negative (Fig. 1).

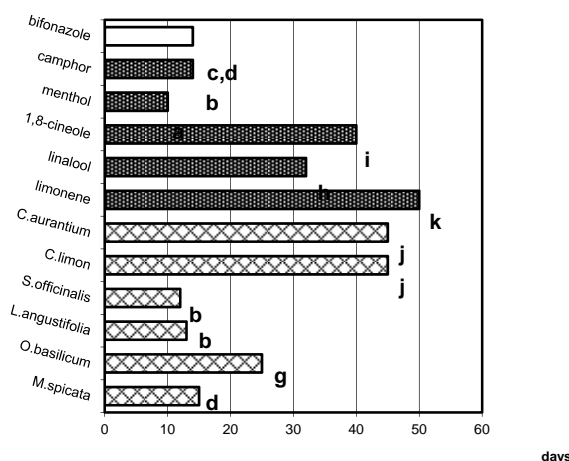


Fig. 1. Time-dependent changes in skin lesion scores in Wistar rats infected with *Trichophyton mentagrophytes* and treatment with essential oils and components, and bifonazole (values marked with the same symbols did not show differences at the level of significance of $p < 0.05$).

All the components used showed therapeutic activity. Linalool showed antifungal activity after 32 days of treatment, while limonene needs 50 days for this activity. Rats treated with 1,8-cineole were cured after 40 days. Camphor exhibited therapeutic and antifungal activity after 14 days of treatment. The best antifungal activity was observed for menthol, which showed therapeutic potential after 10 days of treatment (Fig. 1). 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 (Fig. 1). For untreated rats (control group) symptoms were observed at the same time as in treated animals and were present at the end of the experiment.

4. Discussion

Therapeutic and antifungal activity of selected essential oils and their components *in vivo* could be presented as follows: *S. officinalis* > *L. angustifolia* > *M. spicata* > *O. basilicum* > *C.*

limon = *C. aurantium*. The essential oils of *S. officinalis* and *L. angustifolia* showed the best antifungal activity *in vivo*, while orange and lemon oils possessed the worst antifungal potential in this experiment. Therapeutic and antifungal potential of oils components could be arranged as: menthol > camphor > linalool > 1,8-cineole > limonene. Menthol and camphor showed the best antifungal potential while limonene was the worst one. It is observed that the phenolic and oxygenated components (menthol and camphor) have shown greater therapeutic and antifungal activity, followed by alcoholic components (linalool and 1,8-cineole), while monoterpene hydrocarbons, limonene, proved to be least effective in this case. These results fully confirm our results obtained *in vitro* research, which is in agreement with the literature [4, 5, 18, 19]. In general, studies have shown that oxygenated terpenoids play a bigger role in antifungal activity of essential oils than monoterpene hydrocarbons [20].

After reviewing of the results of the antifungal activity of essential oils and individual components *in vivo* experiment, knowing that the composition of essential oils and the proportion of the tested individual components may be, to some extent, examine and explain the differences between the activities of the tested essential oils. Menthol and camphor showed better antifungal activity than essential oil individually. Since the individual essential oils showed lower antifungal activity than the test individual components, it is evident that the active principles can be explained by individual components. Although it is possible that interactions between the constituents of essential oils block the active principles of individual components when the treatment is the total essential oil. Added to that are antagonistic effect [21], which does not mean that it can be completely neglected the role of individual components of essential oils on the expression of antifungal potential. Menthol and camphor, which showed the best antifungal activity among all tested components, are the dominant components of the essential oils of *M. spicata* and *S. officinalis*, and therefore can be justified by the high antifungal potential of these oils *in vivo*. The essential oil of *O. basilicum*, which is known for the beneficial activity of the skin, healing wounds, etc. is used to treat fungal infections [4] showed good antifungal activity, but only better than lemon and orange. Dominant component of this oils was linalool, which proved to be good, but the tested components as one of the weaker fungicides, in front of limonene. Similarly, lemon and orange oil showed the lowest antifungal potential, as well as among individual components of limonene, which is present in these oils with a high proportion, which is certainly influenced the decrease in the efficiency of oil. Essential oils of *S. officinalis* and *L. angustifolia* have proved to be most effective in the treatment of experimental induced dermatomycoses. If we compare the results obtained during investigation of antifungal activity of essential oils *in vitro*, and this generated *in vivo*, it is obvious that the essential oil of *S. officinalis* and *L. angustifolia* reacted with lower potential *in vitro*. *In vivo* experiments, these oils, in contrast, have proved to be most effective. Obviously, to have better therapeutic activity than other essential oils. In addition, it is known that sage, lavender and above all, always been used to treat various skin diseases and cosmetic products for skin care [22]. Lavender essential oil possessed as the dominant components linalool, linalyl acetate, limonene, cineole and camphor. Good efficacy of essential oil it can be explained by interactions of individual components, but given the importance of some of the components, especially interactions linalyl acetate and linalool [23]. Anti-inflammatory potential and the possibility of easier passage through mucous membrane of the essential oils of lavender and sage, probably contribute to the overall therapeutic effect of these oils in this case [24]. Lavender is also used for healing wounds ethnomedicine [23]. Good efficacy of essential oils of lavender and sage, can be explained by the high content of 1,8-cineole, which is capable of changing the structure and moisture mucous membranes of fungal cells, interfering with the respiratory processes, and therefore comes to the elimination of pathogens [25]. Also, the high content of linalool in the essential oil of lavender contributes to antifungal, therapeutic and antiinflammatory activity of this oil [26]. The presence of limonene and 1,8-cineole which can influence on the changing structure of the lipid layer, the stratum corneum, increased permeability of the epidermis and stratum corneum human, also may be one of the reasons for the good of the essential activities *in vivo* [27]. The therapeutic potential is very important in the healing of inflammatory wounds incurred as a response to infection, and therefore more quickly master the pathogen by an infected organism. It is obvious that some of the activities obtained *in vitro* experiments not be passed or confirmed directly *in vivo* experiments, as confirmed by other studies

with essential oils [23]. Aromatherapy implemented mainly through some of the mucosal sensory cells, which transmit information through the lymphatic system when finally realize the activity. This points to a better activity of essential oils *in vivo*. It can be concluded that in order to obtain an effective antifungal agents with the characteristics (good and antifungal and therapeutic effect), it is best to take into account the results it obtained *in vitro* and *in vivo* experiments. Adam et al., 1998 [4] in their study of antifungal activity of *O. vulgare* oil *in vivo*, showed that this oil is completely cured infected rats for 17 days. Comparing these results with our results, we observed that the essential oils of *L. angustifolia*, *S. officinalis* and *M. spicata* operated effectively, and to a lesser period of time (12-15 days) than oil *O. vulgare*. Soković et al., 2008 [28] showed that the rats treated with the essential oil of *T. vulgaris* were completely cured after 24 days of treatment, whereas thymol showed therapeutic activity for 14 days of treatment. The essential oil of *M. piperita* was completely cured the animals infected with *T. metagrophytes* within 15 days of treatment [29]. By comparing the antifungal activity of commercial mycotic bifonazole, essential oils and individual components, it is noted that the bifonazole showed slightly better therapeutic activity, except menthol. However, as noted earlier, commercial, synthetic products, may have some adverse effects, toxicity, allergic reactions, and possible the emergence of resistance after long period of use. This is especially present in synthetic mycotics who can not completely eliminate the fungus, which are eukaryotic organisms, while they do not damage the infected host cells. The problem of resistance to fungicides that are used long can be overcome by changing the treatment. Fungicide resistance depends on the amount of drug that the patient is exposed, the duration of treatment, and the number of mutations necessary to create resistance.

5. Conclusion

From our previously reported results and results presented here it can be concluded, that essential oils and components used showed very good therapeutic and antifungal effect *in vitro* and *in vivo*. These compounds could represent possible alternatives for the treatment of patients infected by dermatomycetes. Even more, because of the side effects of commercial fungicides and possible resistance of pathogens to the synthetic mycotics, the preparation with natural products have an advantage in treatment of fungal caused diseases. After all, facts, and the obtained results, it can be said that the advantage of products based on medicinal plants that were studied showed no harmful effects on humans and animals, and also proved to be very good antifungal and therapeutic agents.

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