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Microbiological Tests of Natural Limonene and the Compounds Obtained after Isomerization of Limonene in the Presence of Ti-SBA-15 Catalyst—α-Terpinene, γ-Terpinene, Terpinolene, and *p*-Cymene

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Synopsis

The antimicrobial properties of natural limonene and the compounds obtained after isomerization of limonene (α-terpinene, γ-terpinene, terpinolene, and *p*-cymene) were studied. The following microorganisms were selected for the tests: Gram-negative bacteria *Escherichia coli* K12 (ACCT 25922), Gram-positive *Staphylococcus epidermidis* (ACCT 49461), yeast fungi *Candida albicans*, and fungi *Trichophyton rubrum*, *Aspergillus niger*, *Penicillium commune*, *Trichoderma viride*, and *Cladosporium cladosporioides*. During the studies, terpinolene showed the highest activity, and therefore, this compound was chosen for the preparation of therapeutic creams (content of terpinolene: 0.5 and 2 wt%). The obtained creams were active in the microbiological tests even at the lowest content of terpinolene. The mixture of products obtained after the isomerization of limonene also showed antimicrobial activity. Probably, in the future, this mixture of products can be used as a potential and relatively inexpensive ingredient in therapeutic and protective creams that can be applied for the relief of skin lesions and in the treatment of acne or atopic dermatitis.

INTRODUCTION

Essential oils containing terpene compounds can find numerous applications in medicine. Studies conducted with the participation of terpenes isolated from various essential oils showed their bactericidal or bacteriostatic activity. This is connected with the high lipophilicity of terpenes, thanks to which they easily penetrate the cell walls and membranes of various microorganisms, which in turn disrupts the integrity of these structures and

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leads to the disintegration of microbial cells. Toxic action of terpene compounds against microbes consists in coagulation of the cytoplasm and increased cell membrane permeability, resulting in excessive loss of hydrogen and potassium ions, which in turn causes reduction in membrane potential, which disturbs the functioning of proton pumps and, thus, reduces the amount of synthesized adenosine triphosphate (1–4). An example may be tea tree oil, which contains about 100 compounds (monoterpenes, sesquiterpenes, and their alcohol derivatives). The main components of tea tree oil are terpinene-4-ol, α-terpinene, and γ-terpinene (70%). In this oil, the following are also present: *p*-cymene, terpinolene, α-terpineol, and α-pinene (15%). Research on tea tree oil showed (3,5,6) that most of the ingredients in this oil inhibit the development of *Candida albicans* yeast—a commensal organism—that can become pathogenic in immunocompromised people. In addition, these studies showed that the antifungal activity of this oil can be influenced by all of its ingredients, even those that occur in small quantities or are considered as inactive.

Limonene is a terpene compound that is very valuable and of natural origin and can be obtained from waste orange peels (renewable waste from the food industry, biomass). By simple isomerization of this compound (over an appropriate catalyst, e.g., Ti-SBA-15, and without any solvent), it is possible to obtain from limonene other valuable compounds, such as α-terpinene, γ-terpinene, terpinolene, and *p*-cymene (Figure 1). These compounds, the same as limonene, have a lot of applications in medicine and also in cosmetic and food industries. Of particular interest are the cosmetic and medical applications of these compounds (7–9).

α-Terpinene is a fragrant compound of natural origin that is present in various foods, such as oranges, coriander, and oregano. It can also be obtained as the product of isomerization of limonene. Thanks to its characteristic refreshing scent, it is widely used as a fragrance in cosmetic, household, and food products. α-Terpinene is a component of tea tree oil and is considered to be a component responsible for the antioxidant properties of this oil. The properties of tea tree oil allow the use of this oil in the treatment of skin problems, such as acne or mycosis. Studies on α-terpinene showed the efficiency of this compound in the treatment of parasitic infections with auger in horses (10–14).

γ-Terpinene is another product that can be obtained during the isomerization of limonene. In nature, this compound can be synthesized by a plant like rice (γ-terpinene destroys the cell membrane of the bacteria that causes plague among this plant). Generally, this compound exhibits antibacterial properties. Moreover, γ-terpinene in combination with routine

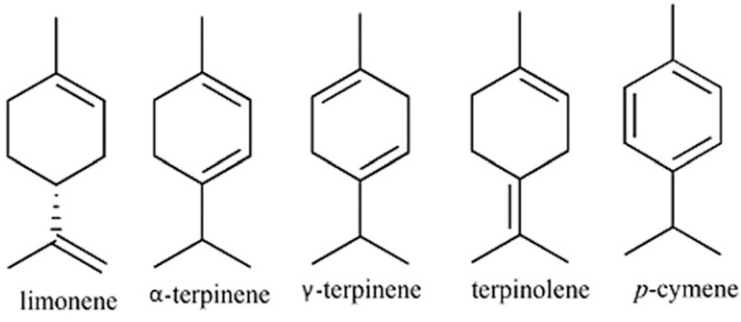

Figure 1. Structure of limonene and the products of limonene isomerization and dehydroaromatization.

antioxidants is used in the treatment of atherosclerosis because of its ability to prevent low-density lipoprotein (LDL) oxidation (14–17).

Terpinolene is used as an additive to fruits, baked products, ice creams, nonalcoholic beverages, and candies. This compound has a calming effect. Terpinolene inhalations affect the functioning of the autonomic nervous system and the human psyche. As a consequence, terpinolene reduces tension and increases the feeling of relaxation. Therefore, this compound can be used in the treatment of mental disorders such as depression. In addition, terpinolene has the potential to treat atherosclerosis. This compound, in combination with β-carotene and α-tocopherol, effectively prevents oxidation of LDL, which plays a key role in the formation of artelectrosclerosis (18–21).

p-Cymene is a product of dehydroaromatization of limonene isomers. This compound is a component of numerous essential oils and is becoming more and more popular among scientists. For example, it is a ligand of many catalysts applied in olefin metathesis—one of the most frequently used reactions in modern organic synthesis. *p*-Cymene can also be used as a solvent because it is less harmful to the environment than commonly used organic solvents (22–25).

Considering the presented antimicrobial properties of limonene, α-terpinene, γ-terpinene, terpinolene, and *p*-cymene, we decided to investigate whether a mixture of the mentioned compounds (the mixture obtained after the isomerization of limonene) exhibited an antimicrobial activity. Such use of the postreaction mixture of terpenes would lower the cost of preparing therapeutic formulations (e.g., creams), as it will not require a step of isolation of individual components from the postreaction mixture, e.g., by distillation methods. Such a mixture could be treated as an "artificial essential oil" with a very narrow composition. The following microorganisms were selected for microbiological tests: *Escherichia coli* K12 (ACCT 25922) and *Staphylococcus epidermidis* (ACCT 49461), yeast fungi *C. albicans*, and fungi *Trichophyton rubrum*, *Aspergillus niger*, *Penicillium commune*, *Trichoderma viride*, and *Cladosporium cladosporioides*. With the compound having the highest antimicrobial activity (terpinolene), therapeutic creams containing 0.5 and 2 wt% of this compound were prepared. The creams used in the research were as simple as possible, so that the compounds included in their composition did not influence the results of the microbiological tests. Therefore, we did not use emulsifiers as ingredients of the investigated creams. These creams can be used as potential therapeutic and protective creams for relief of skin lesions and in the treatment of acne or atopic dermatitis.

MATERIALS AND METHODS

SEPARATION OF NATURAL LIMONENE

Natural orange oil containing up to 98% of limonene can be obtained from waste orange peels by the steam distillation method. This method does not require the application of organic solvents, which makes it environmentally friendly, and the water used for the process can be reused in subsequent processes. The method of obtaining essential orange oil from orange peels by the steam distillation method is based on passing water vapor through fresh orange peels shredded with the use of a food processor. A kilogram of orange peels prepared in this way was placed in a glass reactor and 2.5 L of distilled water was poured into the reactor. The steam passed through the plant material and volatile components

in the steam were condensed in the condenser. The insoluble fraction of the orange oil remained on the surface of the water. The obtained water–oil mixture was then placed in a freezer, and after a few hours, the layer of essential oil was poured from above the ice layer. The essential oil obtained in this way constituted on average 1.06% by mass of the orange peels used (about 10.6 g of oil) and contained 95% of limonene, which was determined on the basis of gas chromatography (GC) analysis.

ISOMERIZATION OF NATURAL LIMONENE OVER TI-SBA-15 CATALYST

Isomerization of the natural limonene was carried over the Ti-SBA-15 catalyst. This catalyst belongs to the group of mesoporous materials. Their unquestionable advantages include high hydrothermal stability and neutrality toward living organisms. In the structure of this catalyst, hexagonally arranged cylindrical pores are present. In addition, the structure of this catalyst is stabilized by micropores, which connect the cylindrical mesopores. The isomerization of limonene was performed in a round-bottom flask with a capacity of 25 cm^3. For isomerization, 5 g of the natural limonene and 0.75 g of the catalyst (content in the reaction mixture 15 wt%) were mixed up. The reaction was carried out at a temperature of 150°C for 24 h. The weight of the postreaction mixture amounted to 4.20 g. Before the microbiological tests, the catalyst was separated from the postreaction mixture by centrifugation. The composition of the postreaction mixture was determined by using the external standard method and GC method. For the GC analyses, a Thermo FOCUS chromatograph (Anchem, Warszawa, Poland) was used, which was equipped with a flame ionization detector and a Quadrex 007-5 capillary column (30 m × 250 μm × 0.25 μm). The chromatograph was also equipped with an autosampler. The parameters of the chromatographic analyses were as follows: helium pressure, 60 kPa; sample chamber temperature, 240°C; detector temperature, 250°C; and thermostat temperature, changed according to the program: isothermally 60°C for 2 min, temperature increase 10°C/min, isothermally 240°C for 4 min, and cooling to 60°C.

As standards for GC analyses, the following were used: limonene (>93%, Sigma-Aldrich, Poznań, Poland), γ-terpinene (97%, Sigma-Aldrich), α-terpinene (85%, Sigma-Aldrich), terpinolene (≥ 85%, Sigma-Aldrich), and *p*-cymene (99%, Sigma-Aldrich). Also, the same compounds were used in the microbial tests described in the following paragraph.

The results of the analyses of the composition of the postreaction mixture (the mixture obtained after the isomerization of limonene) using the GC method showed that the postmixture contained 11.12 wt% (0.43 g) of limonene, 9.02 wt% (0.38 g) of γ-terpinene, 18.17 wt% (0.76 g) of α-terpinene, 13.44 wt% (0.56 g) of terpinolene, and 31.11 wt% (1.31 g) of *p*-cymene. The conversion of limonene (calculated as the amount of moles of limonene that was converted to isomerization products divided by the initial number of moles of limonene) after 24 h of the reaction amounted to 88.80 mol%, and the selectivities of the obtained products were as follows: 10.6 mol% of γ-terpinene, 21.36 mol% of α-terpinene, 15.80 mol% of terpinolene, and 37.12 mol% of *p*-cymene.

MICROBIOLOGICAL TESTS

The antimicrobial properties of natural and synthetic limonene and products of the isomerization of natural limonene were tested against the Gram-negative bacteria *E. coli* K12

(ACCT 25922) and Gram-positive bacteria *S. epidermidis* (ACCT 49461), yeast *C. albicans*, and fungi *T. rubrum*, *A. niger*, *P. commune*, *Alternaria alternata*, *T. viride*, and *C. cladosporioides*. The fungi were obtained from the collection of the Department of Biotechnology, Institute of Inorganic Chemical Technology and Environmental Engineering (IIChTEE), West Pomeranian University of Technology in Szczecin. The microorganisms were isolated from air of habitable buildings by the sedimentation method and identified by morphology and biochemical tests. Twenty-four–hour bacterial cultures on plate control agar (BioMaxima, Lublin, Poland) for *E. coli* and brain heart infusion (BioMaxima) for *S. epidermidis* were used. Solutions of fungal spores were prepared from 7-d cultures carried out at 37°C (yeast) and 25°C (fungi) on agar slants. Sabouraud agar (BioMaxima) for yeast and dermatophytic fungi *T. rubrum* and malt extract agar (Merck, Darmstadt, Germany) for filamentous fungi were used in the tests. The concentration of the microorganism was determined by the spectrophotometric method at wavelength $\lambda = 550$ nm for fungi and $\lambda = 600$ nm for bacteria. A fungal suspension at a concentration of 1.76×10^7 CFU (colony-forming units) $\times$ cm^{-3} and a bacterial suspension at 1.4×10^8 (approximately 0.5 according to McFarland standard) in 0.85% NaCl solution were prepared. Diffusion disk method was used for the assessment of microbial growth inhibition. Sterile paper disks (Whatman No. 1, diameter 5 mm) impregnated with tested compounds (10 μL/disk) were placed at different locations on the surface of agar plates. Incubation of microorganisms was carried out under the following conditions: bacteria and yeasts at the temperature of 37°C for 24 h and fungi at 25°C for 72 h. After incubation, the growth inhibition zones (mm) around the paper disks were measured.

METHOD OF PREPARATION OF THERAPEUTIC CREAMS

To prepare the therapeutic cream, an oil phase consisting of 12.5 g safflower oil and 3 g of beeswax was weighed. Next, the water phase with the following composition was weighed: 3.5 g of urea (pure, Chempur, Pikary Śląskie, Poland), 0.5 g of allantoin (pure, Chempur), and 21.25 g of water. In the next stage to the aqueous phase, terpinolene was added in an amount of 0.2 or 0.83 g [concentration of terpinolene 0.5 wt% (cream named KRT1) and 2 wt% (cream named KRT2), respectively]. The beakers with the water phase and the oil phase were placed in a water bath at the temperature of 80°C. After dissolving the oil phase ingredients, the beakers were taken out of the bath and the oil phase was added to the water, with intense mixing. The whole mixture was stirred until a slightly yellow cream with a homogeneous consistency was obtained (Figure 2).

RESULTS AND DISCUSSION

Natural limonene, the postreaction mixture of compounds obtained after the isomerization of natural limonene (limonene, α-terpinene, γ-terpinene, terpinolene, and *p*-cymene), and each of the products of isomerization of limonene separately were tested for their antimicrobial properties (Table I).

The pure compounds (standards) showed the highest antimicrobial activity. The growth inhibition zones around the paper disks impregnated with limonene and terpinolene were significantly larger for all tested microorganisms (Table I). The mean inhibition zone for

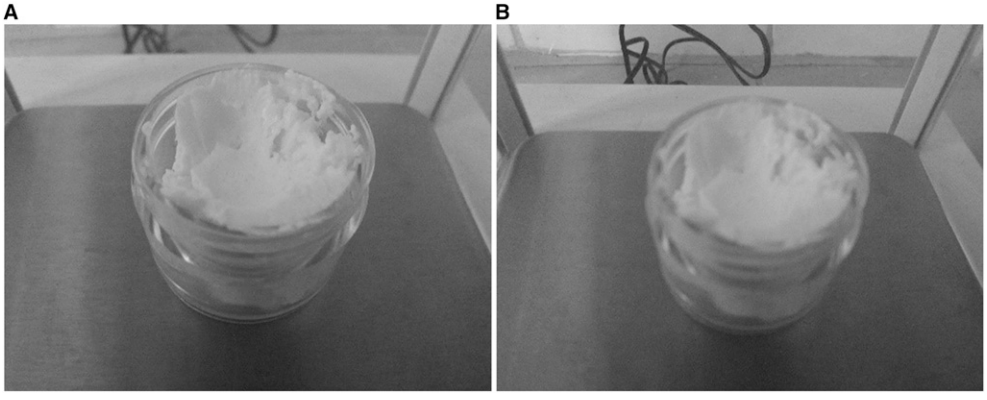

Figure 2. Pictures of prepared creams: KRT1 (A) and KRT2 (B).

the bacteria and dermatophytic organisms (*C. albicans* and *T. rubrum*) ranged from 9.0 to 25.2 mm, indicating a remarkable effect of natural limonene. The terpinolene exerted a particularly strong antifungal effect against the mold fungi (*A. niger*, *P. commune*, *A. alternata*, *T. viride*, and *C. cladosporioides*). The mean inhibition zone ranged from 18.4 to 52.2 mm (Table I). Interestingly, the reaction mixture obtained after limonene isomerization presented the best antimicrobial activity against dermatophytes *C. albicans* and *T. rubrum* and filamentous fungi *T. viride*. *T. rubrum* can be considered as the most sensitive organism because growth inhibition zones were present around disks impregnated with almost all tested substances (the only exception was *p*-cymene). Therefore, the antifungal effects of all tested substances are presented in Figure 3.

By contrast, the most resistant organism was the mold fungi *P. commune*. Growth inhibition zones were observed only around the disk impregnated with terpinolene (Table I).

On the basis of obtained results, terpinolene was assigned as the substance with the best antimicrobial properties against a broad spectrum of microorganisms. For these reasons, terpinolene was chosen as a representative compound for the preparation of therapeutic creams. Two therapeutic creams containing 0.5 and 2 wt% of terpinolene, respectively, were prepared. The performed studies showed that both creams showed activity only against the Gram-negative bacteria *E. coli* and Gram-positive bacteria *S. epidermidis*. The growth inhibition zone around disks impregnated with both creams was not dose dependent and ranged from 8.0 mm for *E. coli* to 10.2 mm for *S. epidermidis* (Table II). It was concluded that 0.5 wt% of terpinolene in a cream is sufficient to inhibit the growth of these microorganisms.

The growing number of multidrug-resistant microorganisms and persons with allergy has become a worldwide public health problem. This concerns in particular to producers of products such as creams, ointments, and toothpastes. Therefore, there is need to find a new effective antimicrobial agent that kills or inhibits the growth of microbes but is safe to use on humans. The best candidates meeting these conditions appear to be natural substances such as monoterpenes or products of their isomerization.

In the presented research, the antimicrobial activity of the postreaction mixture obtained after the isomerization of limonene and its individual components was investigated. Only terpinolene showed the highest activity against all species of tested bacteria and fungi. The considerable inhibition zone obtained for this compound against all dermatophytes

Table I
Growth Inhibition Zone (mm) ± SD for Limonene and Its Isomerization/Dehydroaromatization Products by Disk Diffusion Method

	Substances						
Organisms	Natural limonene	Reaction mixture after isomerization	*p*-Cymene	γ-Terpinene	Limonene (template)	Terpinolene	α-Terpinene
E. coli	9.0 ± 0.1	21.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	21.3 ± 0.1	9.2 ± 0.0
S. epidermidis	9.1 ± 0.1	24.1 ± 0.3	8.3 ± 0.2	0.0 ± 0.0	10.3 ± 0.2	18.4 ± 0.4	18.5 ± 0.1
C. albicans	25.2 ± 0.2	33.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	13.1 ± 0.3	20.1 ± 0.7	20.2 ± 0.4
T. rubrum	9.0 ± 1.0	90.0 ± 2.0	0.0 ± 0.0	42.0 ± 1.5	10.2 ± 0.0	25.2 ± 0.9	27.2 ± 0.7
T. viride	12.0 ± 0.1	21.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	14.2 ± 0.3	42.2 ± 1.0	14.2 ± 0.2
A. niger	0.0 ± 0.0	24.1 ± 0.3	8.1 ± 0.2	0.0 ± 0.0	13.3 ± 0.8	32.3 ± 1.0	0.0 ± 0.0
P. commune	0.0 ± 0.0	33.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	22.3 ± 0.0	0.0 ± 0.0
C. cladosporioides	33.4 ± 0.3	90.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	16.0 ± 0.5	52.2 ± 1.0	14.2 ± 0.0
A. alternata	0.0 ± 0.0	51.5 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	12.0 ± 0.0	23.2 ± 0.0	10.1 ± 0.0

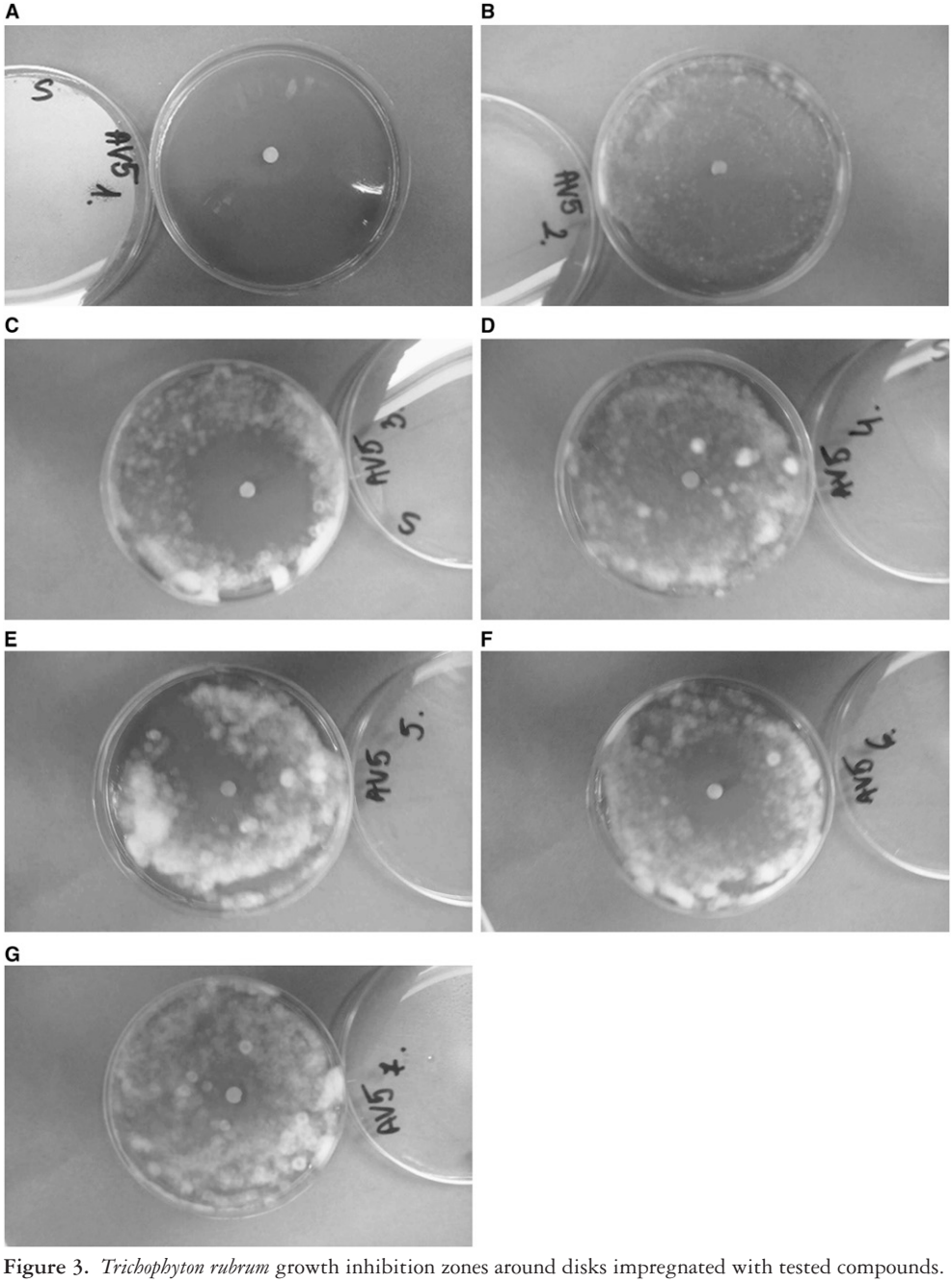

Figure 3. *Trichophyton rubrum* growth inhibition zones around disks impregnated with tested compounds.

tested in the study (including *T. rubrum*) was highly promising. For example, *T. rubrum* is the most common species that causes extremely difficult to cure (due to resistance to most of the commercially available antifungal agents) foot and nail infections (26). Moreover, terpinolene was found to be a very active antifungal agent against *A. niger*. The fungus *Aspergillus* has become a growing concern in the past few years as a causative agent of

Table II
Antimicrobial Properties of Prepared Therapeutic Creams

Cream name	Terpinolene content (wt%)	Mean growth inhibition zone (mm) for *E. coli* and *S. epidermidis*
KRT1	0.5	*E. coli*: 8.0 ± 0.0 mm, *S. epidermidis*: 10.0 ± 0.1 mm
KRT2	2.0	*E. coli*: 8.0 ± 0.0 mm, *S. epidermidis*: 10.2 ± 0.0 mm

bronchopulmonary aspergillosis (ABPA) and "fungal asthma" (SAFS). Treatment for these diseases is performed with steroids by aerosol or mouth, which unfortunately has side effects such as thinning of the bones (osteoporosis) and skin, and weight gain (27).

Our results did not confirm the antimicrobial activity of γ-terpinene against bacteria and the yeast *C. albicans* but proved the highly effective germicidal properties of α-terpinene, which is consistent with the results obtained by other authors (28). It is noteworthy that α-terpineol (as well as linalool) presented antimicrobial activity against periodontopathic and cariogenic bacteria (29).

For other tested compounds, it was found that the postreaction mixture obtained after the isomerization of limonene possesses higher antimicrobial activity than the pure tested compounds against the fungi *T. rubrum*, *C. albicans*, and *T. viride* and bacteria *S. epidermidis* or *E. coli*. This provides evidence that monoterpenes display different mechanisms of action. The mixture of products obtained after the isomerization of limonene also showed antibacterial and antifungal activity. In the future, this mixture of products can also be used as a potential and relatively inexpensive ingredient in therapeutic and protective creams. Interestingly, pure natural limonene, known for its antibacterial and antifungal properties, did not perform favorable properties in comparison with compounds present in the postreaction mixture obtained after its isomerization. The most probable interpretation is the occurrence of synergistic effect.

It was concluded that some products of limonene isomerization showed a potent antimicrobial effect against the tested Gram-negative and Gram-positive bacteria, and fungi.

CONCLUSIONS

The findings of the present study indicated a strong and broad-spectrum antimicrobial activity of terpinolene. It can be recommended as the antibacterial agent in therapeutic and protective creams that can be used in the relief of skin lesions and in the treatment of acne or atopic dermatitis. The mixture of products obtained after the isomerization of limonene also showed antimicrobial activity. In the future, this mixture of products can also be used as a potential and relatively inexpensive ingredient in therapeutic and protective creams. Further investigations are needed for the exclusion of its allergic potential.

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