

The effect of fennel essential oil and *trans*-anethole on antibacterial activity of mupirocin against *Staphylococcus aureus* isolated from asymptomatic carriers

Paweł Kwiatkowski¹, Agata Pruss¹, Helena Masiuk¹, Magdalena Mnichowska-Polanowska¹, Maciej Kaczmarek², Stefania Giedrys-Kalemba¹, Barbara Dołęgowska¹, Hanna Zielińska-Bliźniewska³, Jurek Olszewski⁴, Monika Sienkiewicz³

¹Department of Microbiology, Immunology and Laboratory Medicine, Pomeranian Medical University, Szczecin, Poland

²Department of Experimental and Clinical Pharmacology, Pomeranian Medical University, Szczecin, Poland

³Department of Allergology and Respiratory Rehabilitation, 2nd Chair of Otolaryngology, Medical University of Lodz, Lodz, Poland

⁴Department of Otolaryngology and Laryngological Oncology, 2nd Chair of Otolaryngology, Medical University of Lodz, Lodz, Poland

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Abstract

Introduction: *Staphylococcus aureus* constitutes the most pathogenic species within the staphylococcal genus. Humans remain the major reservoirs of this pathogen which colonizes mostly anterior nares of healthy individuals.

Aim: To investigate the effect of fennel essential oil (FEO) and *trans*-anethole (*tA*) on antibacterial activity of mupirocin (MUP) against *S. aureus* strains isolated from asymptomatic carriers.

Material and methods: The content of the FEO was analysed with use of the GC-MS method. The research done on 43 *S. aureus* isolates with different resistance patterns, obtained from nasal vestibule. Antibacterial activity of MUP in combination with FEO or *tA* was examined using the agar dilution method and E-test method. The data analysis was done with the Pearson's χ^2 test.

Results: The chemical composition of FEO was consistent with the European Pharmacopoeia (EP) for the main constituent – *tA* (77.9%) according to the EP recommendations. Macrolide-lincosamide-streptogramin B resistance phenotype was prevalent among 39.5% of *S. aureus* isolates. FEO concentrations of 2.0% and 2.5% revealed antibacterial activity against 76.7% of isolates, whereas *tA* inhibited *S. aureus* growth at concentrations > 4.0%. The MIC values for MUP combined with FEO as well as for MUP combined with *tA* were < 0.064 $\mu\text{g/ml}$ for 79.1% and 86.0% of *S. aureus* isolates, respectively.

Conclusions: Our experiment revealed FEO and *tA* influence on MUP effectiveness. The combination of MUP with FEO as well as MUP with *tA* are worth considering to implement in *S. aureus* eradication procedures. These findings will be useful in designing efficient antistaphylococcal agents which can limit the emergence of antibiotic resistance.

Key words: *Staphylococcus aureus*, mupirocin, fennel essential oil, *trans*-anethole.

Introduction

An extensive transmission of *Staphylococcus aureus* between humans is known to be significantly attributed to asymptomatic carriers. The percentage of persistent carriers of *S. aureus* in anterior nares is reckoned to be about 20%, whereas the transient nasal carriers and non-carriers account for 60% and 20%, respectively. The persistent carrier-state is being more frequently observed among young children than adults; however the incidence of *S. aureus* carriage among listed groups undergoes numerous changes in individuals aged between 10 and 20 [1]. Higher rates of nasal colonization are observed in hospital

staff (around 44%), which doubtlessly poses a great risk of *S. aureus* transmission to hospitalized patients [2, 3].

The carriage of *S. aureus* among medical staff represents an outstanding problem because these can transfer multidrug staphylococcal strains to medical students, other medical staff, and, most importantly, to hospitalized patients. It is estimated that the nasal carriage of *S. aureus* among medical staff ranges between 16.8% and 56.1% [1]. Nasal colonization with *S. aureus* is a risk factor for endogenous staphylococcal infection, especially in patients undergoing surgical procedures with simultaneously reduced immunity. Thus, the eradication is needed in carriers to reduce the risk of infection.

Address for correspondence: Paweł Kwiatkowski MSc Eng, Department of Microbiology, Immunology and Laboratory Medicine, Pomeranian Medical University, 72 Powstancow Wielkopolskich St, 70-111 Szczecin, Poland, phone: +48 91 466 16 52, fax: +48 91 466 16 59, e-mail: pawel.kwiatkowski@pum.edu.pl

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Available guidelines recommend a topical application of mupirocin (MUP) on mucosal membranes of anterior nares to eradicate the *S. aureus* colonization (methicillin-sensitive *S. aureus* – MSSA and methicillin-resistant *S. aureus* – MRSA). Due to a high incidence of re-colonization in individuals treated with MUP, it is recommended to use the ointment for at least several weeks [4]. The worldwide increase in resistance to MUP among *S. aureus* explains the need to evaluate the susceptibility to MUP of each *S. aureus* isolate as well as the necessity to search for new treatment options such as application of essential oils which express good antimicrobial properties [5].

Foeniculum vulgare Mill. (fennel), biennial plant belonging to the *Apiaceae* family has been easily adapted to many geographic regions [6]. Due to its unique taste, aroma and antimicrobial action, fennel is used in food and cosmetic industry [7]. Fennel is also used to treat gastrointestinal and respiratory tracts disorders. It is known to cause intestinal smooth muscles relaxation and to dissolve bronchial secretion, thus to facilitate easier expectoration. Moreover, the fennel seed extract promotes milk supply in breasts of feeding mothers, assuages period pains, increases sex drive and facilitates birth. Available studies reveal that fennel essential oil (FEO) displays antimicrobial, antioxidant, anti-inflammatory, anti-cancer, anti-spasmodic, anti-coagulant, anti-diabetic, cholagogue, diuretic, anti-hypertensive, hepatoprotective and acaricidal properties [8–10].

FEO is composed mostly of *trans*-anethole (*tA*) and fenchone followed by camphene, estragole, limonene, α -pinene, β -pinene, β -myrcene, camfor, 3-carene, α -phellandrene and *cis*-anethole [9].

tA (*p*-methoxypropenylbenzene) is an organic compound, a by-product of terpene synthesis. It constitutes the major ingredient of many essential oils – anise (*Pimpinella anisum* L.), star anise (*Illicium verum* Hook. f.) and fennel (*F. vulgare* Mill.). *tA* is widely used in food industry, but also in cosmetic and health-care industries [11, 12]. Existing evidence proves its antimicrobial, insecticidal, larvicidal and antioxidative properties [7, 13].

Aim

The aim of the study was to evaluate the influence of FEO and *tA* on antibacterial activity of MUP against *S. aureus* strains isolated from asymptomatic carriers.

Material and methods

Strains and their susceptibility to antimicrobial agents

Staphylococcus aureus strains were isolated from medical faculties' students during the 2-years study, in the years 2014–2015 at Department of Microbiology, Immunology and Laboratory Medicine of Pomeranian Medical University in Szczecin, Poland. During the study period,

43 bacterial strains from anterior nares swabs have been collected and routinely all were identified with *S. aureus* screening assay. The specimens were cultivated on Columbia agar with 5% sheep blood (bioMérieux, Poland), identified by colony morphology, tube coagulase test (Biomed, Poland), Staph-Kit test (bioMérieux, Poland) and biochemical test GP Vitek 2 Compact (bioMérieux, Poland). *S. aureus* ATCC® 29213™ strain was used to determine the optimal concentration of FEO and *tA* in combination with increasing (E-test method) concentrations of MUP (bioMérieux, Poland).

The susceptibility to ciprofloxacin, co-trimoxazole and MUP of *S. aureus* isolates was assessed *via* disc diffusion method and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations. MRSA phenotype was determined using cefoxitin. The presence of macrolide-lincosamide-streptogramin B (MLS_B) resistance phenotype was determined using erythromycin and clindamycin in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations [14–17]. Antibiotic discs were provided by Diag-Med, Poland.

FEO and *tA* origin

FEO purchased from Pollena-Aroma (Poland) was used in this study. The essential oil was analysed by gas chromatography-mass spectrometry (GC-MS) at the Faculty of Chemical Technology and Engineering, West Pomeranian University of Technology in Szczecin. The main constituent of tested essential oil was *tA* (77.9%) followed by fenchone (12.8%), α -pinene (3.8%), estragole (2.3%), limonene (2.1%) [14].

tA with a purity of not less than 99.0% was obtained from Sigma-Aldrich, Germany.

Susceptibility of *S. aureus* to FEO and *tA*

The agar dilution method mirrored the one approved by CLSI [18] with the following modification: a final concentration of 1.0% (v/v) Tween® 80 (Sigma-Aldrich, Germany) was incorporated into Mueller-Hinton Agar (MHA) (Sigma-Aldrich, Germany) after autoclaving in order to enhance FEO and *tA* solubility. FEO and *tA* at various concentrations (0.1%, 0.25%, 0.5%, 0.75%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%) (v/v) were independently transferred into MHA with 1.0% (v/v) Tween® 80. Plates were dried at 37°C for 30 min prior to inoculation of each them with suspension containing approximately 10⁴ CFU/ml of particular *S. aureus* isolates, using a multichannel pipette. MHA with 1.0% (v/v) Tween® 80 without FEO or *tA* was used as a positive growth control for *S. aureus*. Minimal inhibitory concentration (MIC) of each tested ingredient was determined after 18 h incubation and recognized as the lowest concentration of oil which completely inhibited the visible growth of *S. aureus* on MHA plate. According

to CLSI recommendations, a single colony or a faint haze caused by the inoculum was disregarded [18].

Determination of the optimal concentration of FEO and *tA* in combination with MUP

Determination of the optimal FEO and *tA* concentration enhancing antibacterial activity of MUP was assessed using ATCC® 29213™ strain of *S. aureus*. The optimization was performed using the agar dilution method and E-test method. For screening of the optimal concentrations of FEO and *tA* enhancing antibacterial activity of MUP, various concentrations (0.1%, 0.25%, 0.5%, 0.75%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%) (v/v) of these chemicals were distributed into MHA with 1.0% (v/v) Tween® 80. Plates were dried for 30 min at 37°C prior to inoculation with sterile cotton swab containing approximately 10⁸ CFU/ml of microorganism. Then, the activity of the antibiotic was studied by applying a plastic test strip (E-test) which consisted a predefined, continuous and exponential gradient of MUP on the surface of MHA plates (with and without chemicals – control). The plates were incubated for 18 h at 37°C in aerobic conditions. After incubation, the MIC of MUP were calculated. Each test was performed with two replicates.

Antibacterial activity of MUP in combination with FEO or *tA*

Antibacterial activity of MUP in combination with FEO or *tA* was examined using the agar dilution method and E-test method in comparison with optimal concentrations revealed by optimization assay. For the screening of antibacterial activity of MUP combined with chemicals, the optimal concentration (v/v) of the chemicals was distributed in MHA with 1.0% (v/v) Tween® 80. Plates were dried for 30 min at 37°C prior to inoculation with 10⁸ CFU/ml of bacterial suspension. The activity of the antibiotic was studied by applying a plastic test strip (E-test) with a continuous and exponential gradient of MUP on the surface of MHA plates (with and without chemicals – control). The plates were incubated for 18 h at 37°C in aerobic conditions. The MIC values were calculated.

Ethical statement

The study was approved (KB-0012/04/01/14) by the Ethics Committee of the Pomeranian Medical University of Szczecin, Poland.

Statistical analysis

Comparison of the study data was done using the Pearson's χ^2 test. Statistical analyses were performed using the Statistica programme version 12.5. A *p*-value < 0.05 was considered statistically significant.

Results

Screening of bacterial susceptibility to antibiotics

All *S. aureus* strains isolated from the asymptomatic carriers, when tested by disc diffusion method, were susceptible to MUP and to ceftiofloxacin (confirmed MSSA phenotype), whereas one of them showed resistance to ciprofloxacin and another one to co-trimoxazole. The resistance to erythromycin and clindamycin was observed in 39.5% of the isolates. The presence of inducible MLS_B and constitutive MLS_B resistance phenotypes was indicated in 34.9% and 4.7% of the strains, respectively. None of the isolates had MS_B resistance phenotype. *S. aureus* ATCC® 29213™ was susceptible to all antibiotics.

Determination of MICs of FEO and *tA*

Our investigation showed that FEO in concentrations of 2.0% and 2.5%, represented an inhibiting activity against 76.7% (*p* < 0.0001) of *S. aureus* isolates. *tA* had much weaker inhibitory activity against staphylococci. An equal number of tested isolates (74.4%) were susceptible to *tA* at a concentration higher than 4.0% (*p* < 0.0001) (Table 1). The minimal concentrations of FEO and *tA* inhibiting growth of *S. aureus* ATCC® 29213™ cells were 2.0% and > 4.0%, respectively. It was also observed that the addition of 1% Tween® 80 has no impact on growth inhibition of reference strain and isolates.

Optimization of FEO and *tA* concentrations against *S. aureus* ATCC® 29213™

The optimal concentrations of FEO or *tA*, enhancing the activity of MUP against reference *S. aureus* strain were 0.75% for FEO and 1.5% for *tA*. It was observed that the addition of FEO and *tA* to MHA with 1% Tween® 80 decreased the MIC of MUP from 0.25 µg/ml to ≤ 0.064 µg/ml (Figure 1). The minimal concentration of MUP combined with 0.75% FEO and 1.5% *tA* (which has been recognized to inhibit the growth of reference strain), was lower than 0.064 µg/ml (Figure 2). It was also noted that the addition of 1% Tween® 80 had no impact on the MIC value of MUP alone.

Influence of FEO or *tA* on antibacterial activity of MUP

A significant increase in antistaphylococcal activity of MUP combined with FEO or *tA* was confirmed by the agar dilution method and E-test method. The influence of FEO or *tA* used in combination with MUP was comparable. MICs of MUP combined with FEO and MUP combined with *tA* were < 0.064 µg/ml for 79.1% (*p* < 0.0001) and 86.0% (*p* < 0.0001) of *S. aureus* isolates, respectively. However, MICs of MUP without FEO or *tA* were 0.19–0.125 µg/ml for 69.8% of *S. aureus* strains. The addition of FEO or *tA* to MHA reduced MIC values from 0.17 ± 0.07 µg/ml to 0.064 µg/ml (*p* < 0.0001), but

Table 1. Minimum inhibitory concentration (MIC) of fennel essential oil (FEO), *trans*-anethole (*tA*) and mupirocin (MUP) for *S. aureus* strains isolated from the asymptomatic carriers

Strain number	Resistance phenotype	MIC of FEO* (%)	MIC of <i>tA</i> * (%)	MIC of MUP [$\mu\text{g/ml}$]
1	–	2.0	3.0	0.25
2	iMLS ^B	2.5	> 4.0	0.19
3	iMLS ^B	2.5	> 4.0	0.38
4	iMLS ^B	1.5	> 4.0	0.19
5	iMLS ^B	2.5	> 4.0	0.38
6	iMLS ^B	2.5	> 4.0	0.125
7	iMLS ^B	2.0	> 4.0	0.125
8	iMLS ^B	2.0	> 4.0	0.19
9	–	2.0	> 4.0	0.125
10	–	2.5	> 4.0	0.25
11	–	2.5	> 4.0	0.125
12	iMLS ^B	1.5	4.0	0.125
13	iMLS ^B	1.5	> 4.0	0.25
14	–	2.5	4.0	0.25
15	–	2.0	> 4.0	0.19
16	–	2.5	> 4.0	0.19
17	–	2.0	> 4.0	0.19
18	–	2.0	4.0	0.19
19	–	2.0	> 4.0	0.25
20	–	2.5	>4.0	0.094
21	–	1.0	> 4.0	0.125
22	–	2.5	> 4.0	0.125
23	–	2.0	> 4.0	0.125
24	iMLS ^B	1.0	> 4.0	0.19
25	–	2.0	> 4.0	0.125
26	–	2.0	> 4.0	0.094
27	–	2.5	> 4.0	0.19
28	–	2.5	> 4.0	0.25
29	–	2.5	> 4.0	0.19
30	–	1.5	> 4.0	0.25
31	–	1.0	> 4.0	0.125
32	–	1.5	> 4.0	0.19
33	iMLS ^B	2.0	> 4.0	0.125
34	cMLS ^B	2.5	3.5	0.125
35	iMLS ^B	2.5	3.5	0.25
36	–	1.5	3.5	0.125
37	iMLS ^B	2.0	3.5	0.19
38	–	2.0	3.5	0.125
39	iMLS ^B	1.5	> 4.0	0.125
40	iMLS ^B	2.0	3.0	0.125
41	iMLS ^B	2.0	3.5	0.19
42	cMLS ^B	2.0	> 4.0	0.094
43	iMLS ^B	2.0	> 4.0	0.125

* $p < 0.0001$. iMLS^B – inducible macrolide-lincosamide-streptogramin B resistance phenotype, cMLS^B – constitutive macrolide-lincosamide-streptogramin B resistance phenotype.

the largest number of isolates (34 and 37 strains of *S. aureus*, respectively for FEO and *tA*) were inhibited by a concentration lower than 0.064 $\mu\text{g/ml}$ ($p < 0.0001$). No correlation was found between resistance phenotypes and susceptibility towards MUP with FEO or *tA* combinations (Table 1).

Discussion

The anethole-dominated FEO represents one of the two reference FEO chemotypes accepted by Pharmacopoeia for medical application [19]. The content of *tA* in FEO ranges from 38.8% (Albania, Yugoslavia) to 84.1% (Turkey, Albania) and is dependent on its geographical origin. The content of *tA* in our tested FEO was close to French and Argentinean fennel oil; those contain 75.5% and 79.9% of *tA*, respectively [20]. The European Pharmacopoeia (EP) requires minimum 60% of *tA* in bitter FEO composition. The remaining needed components are as follows: 15% of fenchone, 1–10% of α -pinene; not more than 6% of estragole and 1–5% of limonene. The chemical composition of the tested FEO was consistent with EP requirements regarding *tA*, α -pinene, estragole and

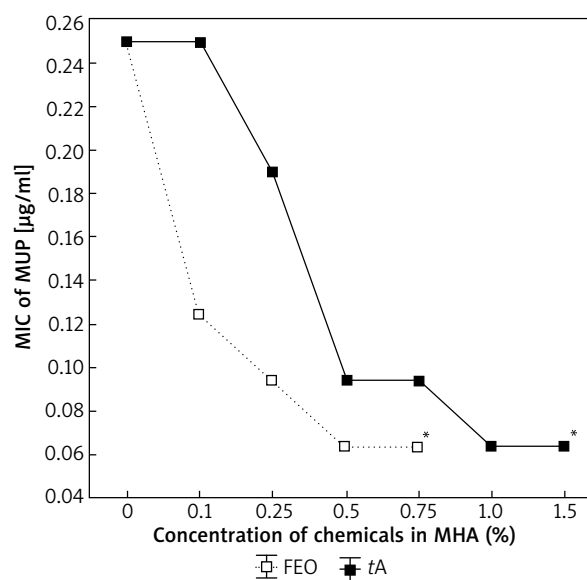


Figure 1. Determination of the optimal concentration of fennel essential oil (FEO) and *trans*-anethole (*tA*) affecting the minimum inhibitory concentration (MIC) of mupirocin (MUP) against *S. aureus* ATCC® 29213™. Concentration lower than 0.064 $\mu\text{g/ml}$ (*), Mueller-Hinton Agar (MHA)

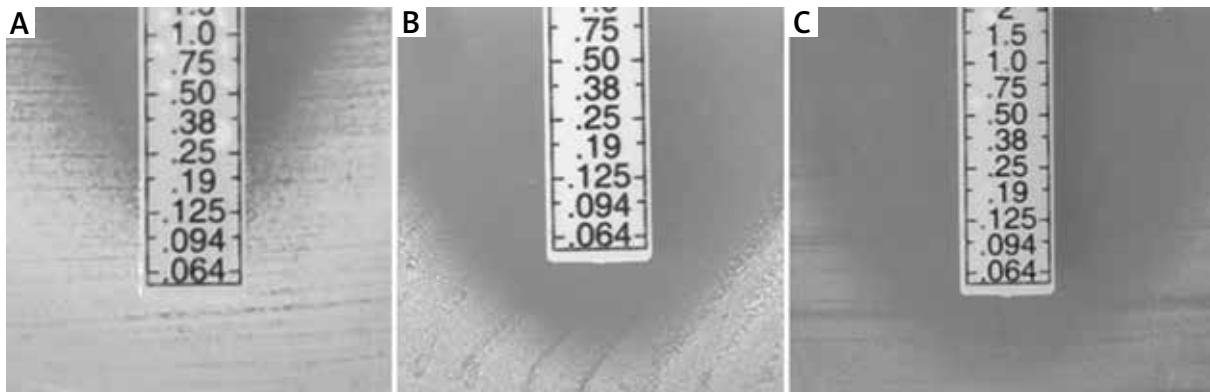


Figure 2. Minimum inhibitory concentration of mupirocin against *S. aureus* ATCC® 29213™: **A** – Mueller-Hinton Agar (MHA) supplemented with 1% (v/v) Tween® 80 without fennel essential oil (FEO) or *trans*-anethole (*tA*), **B** – MHA supplemented with 1% (v/v) Tween® 80 and 0.75% FEO, **C** – MHA supplemented with 1% (v/v) Tween® 80 and 1.5% *tA*

limonene. The content of fenchone was slightly lower when compared with reference data.

According to the available data, the increase in resistance among *S. aureus* to macrolide, lincosamide and streptogramin B antibiotics is being observed [21]. The presence of MLS_B resistance mechanism among staphylococci excludes treatment with the above-mentioned antibiotics, while it is known that erythromycin and clindamycin are commonly used in empirical treatment of Gram-positive bacteria infections. Clindamycin is an alternative therapeutic option for the treatment of staphylococcal skin and soft tissue infections, but also an alternative for patients with penicillin allergies [22]. The degree of resistance to macrolides among MRSA is still increasing, as well in MSSA, and this constitutes a significant challenge for clinicians dealing with skin infections [23, 24]. According to the study of Yilmaz *et al.*, the inducible MLS_B resistance phenotype was detected in 14.8% of MSSA isolates [25]. Similar results were obtained by Prabhu *et al.*, who reported that constitutive and inducible MLS_B resistance phenotype among MSSA occurred in 18% of isolates from different clinical samples [22]. Our results indicate that medical staff is a significant factor in the spread of pathogens resistant to macrolide, lincosamide and streptogramin B antibiotics. Therefore, there is a need for continuous monitoring of this type of bacterial resistance and implementation of new methods to prevent the spread of resistance.

In our previous study we obtained promising results and indicated that FEO was the most effective in combination with MUP [14]. Using the disc diffusion method, the increasing zone of growth inhibition of *S. aureus* was found. We further decided to design an exact research using the agar dilution method and E-test method against higher number of isolates from nasal vestibule with different patterns of resistance. We studied not only the combination of FEO with MUP, but also the combination of FEO main component – *tA* with

MUP which, according to available data, improves cutaneous wound healing. This provides data on its oral LD₅₀ values between 1.8–5.0 g for mice, rats and guinea pigs [26, 27]. It can be concluded that antimicrobial activity of FEO compared with *tA* is determined by more than one particular component, and depends on the synergistic activities of different oil constituents. This is even despite *tA* high content. The MIC values of FEO were much lower than MICs of *tA*, ranging from 1.5 to 2.5% and from 3.0 to > 4.0%, respectively. Summarizing, better results were obtained when combined FEO with MUP. The similar results were presented by Kazemi *et al.* in the study on antistaphylococcal activity of FEO containing mainly *tA*, thymol, α -pinene, 1,8-cineole and fenchone [28]. They found that analysed FEO was more effective against human pathogenic *S. aureus* with MIC – 0.5 μ g/ml than the main constituent – *tA* itself (MIC – 4.0 μ g/ml). Only pure thymol presented comparable activity to phenolic compounds of this oil. Lachowicz *et al.* showed the lesser antimicrobial activity of artificially prepared main constituent mixture (linalool-estragole) compared with anise oil [29]. These results indicated that other less predominant components in anise oil most likely contribute to its antimicrobial activity.

Many scientific centres are investigating effective combinations of chemical agents as well as compounds of natural origin on decrease the resistance of microorganisms. Itaquí *et al.* investigated the interactions between azithromycin and terbinafine in combination with MUP in order to reduce the therapeutic doses of systemic and local treatment against *Pythium insidiosum* responsible for ocular, cutaneous/subcutaneous, vascular and disseminated pythiosis [30]. Sato *et al.* found a synergistic effect of MUP and isoflavanone (Bidwillon B) obtained from *Erythrina variegata* against MRSA [31]. The results showed that Bidwillon B appears to be not only a strong antistaphylococcal agent itself, but also combined with MUP, and thus can be helpful in the elimination of nasal

and skin MRSA carriage. According to the findings of Kifer *et al.*, some combinations of MUP and monoterpenes such as menthol, thymol, 1,8-cineole could be applied in the therapy of infections caused by *S. aureus*, including chronic rhinosinusitis [32]. In this case, the treatment is based on bacterial biofilm growth prevention. Because of the common use of MUP for staphylococci eradication in patients and medical staff, there is a risk of a significant resistance rate increase in the future. The new active compounds acting additively or synergistically with MUP can be effective in prevention of the resistance to this antibiotic.

Conclusions

Our investigation showed that FEO or *tA* could be added to mupirocin-based agents to eradicate the nasal carriage of *S. aureus* and/or to limit the emergence of mupirocin resistance as well. The FEO or *tA* combined with MUP tested in the study was effective against MLSB resistant *S. aureus* isolates and such combination/s may be helpful in nasal eradication of staphylococci with some patterns of resistance. This is not excluded, that in the coming future the FEO or *tA* will be components of agents to mucous membranes antiseptics, but more research in this field are required.

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Conflict of interest

The authors declare no conflict of interest.

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