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Antistaphylococcal activity of *Thymus vulgaris* and *Origanum vulgare* essential oils: time-lapse kinetics, antibiofilm activity and synergistic potential

Original Article

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Abstract:

Staphylococcus aureus is one of the most frequent human pathogens, whose high virulence and severity of chronic infections are related to their ability to produce biofilms. In the present study, antimicrobial potential of oregano and thyme essential oils on the planktonic growth of *S. aureus* clinical strains (isolated from nose, throat and wound), as well as the effect of the oils on the biofilm production, compared with conventional antibiotic streptomycin were investigated. In addition, time-lapse kinetics and combinations of individual oils with streptomycin were investigated for synergism against the selected staphylococcal strains. The results showed high potential of both oils where minimal inhibitory values ranged between 0.078 and 2.50 mg/ml, while biofilms were reduced up to 96%. In the case of biofilms, the reverse concentration dependency has been observed. Time-lapse kinetics showed recovery of some strains after 4 h of contact with the oils, but these strains demonstrated significantly reduced growth in comparison to the control. All mentioned assays showed slightly higher efficacy of oregano essential oil. Calculated FICs showed either synergistic or additive effect of all tested combinations (thyme-oregano, thyme-streptomycin, oregano-streptomycin), with the thyme-streptomycin as the most efficient one. All mentioned results point to a very high potential of both oils to be used as an adjuvant agent for control of human staphylococcal infections.

Key words:
thyme, oregano, *Staphylococcus aureus*, antibiofilm activity, synergism, clinical isolates

Apstract:

Antistafilokokna aktivnost etarskih ulja *Thymus vulgaris* i *Origanum vulgare*: vremenska kinetika, antibiofilm aktivnost i sinergistički potencijal

Staphylococcus aureus is one of the most frequent human pathogens, whose hStaphylococcus aureus je jedan od najčešćih humanih patogena, čija se visoka virulencija i ozbiljnost hroničnih infekcija povezuju sa sposobnošću produkcije biofilmova. U ovom istraživanju, ispitivani su antimikrobni potencijal etarskih ulja origana i timijana na planktonski rast kliničkih sojeva S. aureus (izolovanih iz nosa, grla i rane), kao i efekat ulja na produkciju biofilma u poređenju sa konvencionalnim antibiotikom streptomicinom. U dodatku, u odnosu na odabrane sojeve stafilokoka istraživani su vremenska kinetika i kombinovan efekat etarskih ulja sa streptomicinom u smislu sinergizma. Rezultati su pokazali visok potencijal oba ulja gde je minimalna inhibitorna aktivnost bila u opsegu između 0,078 i 2,50 mg/ml, dok su biofilmovi bili smanjeni za do 96%. U slučaju biofilmova, uočena je obrnuta koncentraciona zavisnost. Vremenska kinetika je ukazala na oporavak nekih sojeva nakon 4 h kontakta sa uljem, ali su ovi sojevi imali značajno umanjen rast u odnosu na kontrolu. Svi pomenuti testovi pokazali su nešto značajniji efekat ulja origana. Preračunati FIK-ovi pokazali su ili sinergistički ili aditivni efekat svih testiranih kombinacija (timijan-origano, timijan-streptomicin, origano-streptomicin), dok je najefikasnija bila timijan-streptomicin. Svi pomenuti rezultati ukazuju na veoma visok potencijal oba ulja za primenu u vidu dopunskih agenasa za kontrolu humanih infekcija izazvanih stafilokokama.

Ključne reči:
timijan, origano, *Staphylococcus aureus*, antibiofilm aktivnost, sinergizam, klinički izolati

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Introduction

In the past few decades, increased number of bacterial infections, reduced antimicrobial efficacy and newly emerged microbial resistance have become global public concern, leading to improving interest in the examination of the antimicrobial activity of plant-based therapeutics (Xiao et al., 2019; Zhang et al., 2020). Among all alternative natural antimicrobial agents, essential oils display the most significant antimicrobial potential. It is important to note that no tolerance or resistance to essential oils has been discovered yet, which can be explained by the great complexity of their structure and action on several target places at the same time, rather than conventional antibiotics (Man et al., 2019). In the contemporary experimental research, an increasing interest is directed to the modulation of virulence factors of microorganisms, in terms of avoiding resistance development (Zhang et al., 2020). Biofilm is considered to be a major virulence factor that contributed to >80% of all human infections. Biofilms are highly organized multicellular bacterial communities consisting of a complex matrix composed of polysaccharide, proteins, lipids and extracellular DNA. The best way of controlling biofilms is to prevent their development (Abdallah et al., 2020).

Staphylococcus aureus (*S. aureus*) is one of the most dominant human pathogen, responsible for a variety of chronic and severe infections including simple soft skin infections, endocarditis, bacteremia, and severe pneumonia (Xiao et al., 2019). This bacteria is the member of the upper respiratory microbiome in more than 30% of healthy individuals (Prince, 2013). Once hosts defense mechanisms are disrupted, *S. aureus* can proliferate and the host begin to be a nasal carrier (Najem, 2020). There are substantial epidemiological data indicating an association between nasal (or other mucosal site) colonization and subsequent *S. aureus* infection. Surgical wounds contamination with skin staphylococci is not a rare case, and the results of the one study confirmed the molecular similarity of strains isolated from wounds and those found in the nose of the same patients (Prince, 2013; Najem, 2020). Since 1960, with the emergence of MRSA-strains (methicillin-resistant *S. aureus*), *S. aureus* has become a key pathogen associated with many health-care infections, that are usually difficult to treat (Uzair et al., 2017). *Staphylococcus aureus* possesses a variety of virulence factors and their ability to form biofilm plays a critical role in chronic infections. A negative modulation or complete inhibition of biofilm formation may represent an important strategy for infection control and is considered as a major target for the development of novel therapeutic agents (Papa et al., 2020).

Aromatic plants from the *Lamiaceae* family present great sources of biologically active substances with wide use and proven antimicrobial and antioxidant properties (Pecarski et al., 2016; Uzair et al., 2017; Man et al., 2019; Zhang et al., 2020). Anti-virulence activity of these essential oils have been well investigated and some of them such as cinnamon, oregano, clove, and ginger oil (Zhang et al., 2020) are classified as GRAS (**G**enerally **R**ecognized **A**s **S**afe) by the U.S. Food and Drug Administration (FDA). Anti-biofilm activity of these oils and its main compounds (thymol and carvacrol) against food-born (Bilge et al., 2010; Vázquez-sánchez et al., 2014) and ocular infections isolates (Nostro et al., 2007) or MRSA strains (Abdallah et al., 2020) has been investigated in several research papers. In the recently published study, the effectiveness of the essential oils (eucalyptus, cinnamon, clove, and tea tree) on the biofilm-producing ability of *S. aureus* nasal isolates was demonstrated (Najem, 2020).

In the present study we investigated antimicrobial potential of oregano and thyme essential oils on the planktonic growth of *S. aureus* clinical strains (isolated from nose, throat and wound), as well as the effect of the oils on the biofilm production, compared with conventional antibiotic streptomycin. In addition, time-lapse kinetics analysis was performed to determine the time required for exhibiting antimicrobial activity and the time necessary for recovery of the cells at sub/inhibitory concentrations. Finally, to explore the potential of these two oils in enhancing the effect of other antimicrobials, the combined oils and also combinations of individual oils with streptomycin were tested for synergism against the selected staphylococcal strains.

Materials and Methods

Isolation of essential oils

Plant material of *Origanum vulgare* L. and *Thymus vulgaris* L. in pulverized form (aerial parts) was purchased at the local herb market and used for extraction of the essential oils. Isolation of the essential oils was performed by using the original Clevenger-type apparatus and the obtained oils were separated by extraction with diethyl ether, dried over anhydrous $MgSO_4$ and stored at -20 °C before use.

Test microorganisms

The essential oils were tested against a total of 12 strains of *Staphylococcus aureus* clinical isolates of different origin (nose, throat, wound and ATCC – American Type Culture Collection strain 6538).

After isolation on blood agar and identification (selective media-MSA agar and coagulase test), the cultures were maintained on Nutrient agar until used for testing.

Determination of minimal inhibitory concentration (MIC)

Sensitivity of the staphylococcal strains to the action of oregano and thyme essential oils was determined by a broth microdilution method as previously described (Stojanović-Radić et al., 2020). Overnight cultures (18 h, exponential phase) were used for making suspensions of bacteria in sterile physiological saline (0.85% NaCl). The obtained suspensions were adjusted to 0.5 McFarland turbidity (DEN-1, Biosan densitometer) and further used to inoculate the wells of a 96-well microtiter plates. Stock solutions of the essential oils made in 100% DMSO were serially diluted in the microtiter plate in the concentration range from 0.01-5.00 mg/ml and then inoculum was added to each well. The final density of bacterial inoculum was 1-5 x 10^6 CFU (colony forming unit)/ml in each well. The plates included growth control (Mueller Hinton Broth + 10% DMSO + inoculum), sterility control (MHB + 10% DMSO + test oil) as well as positive control (MHB + inoculum + serial double dilutions of streptomycin).

After incubation of the plates at 37 °C for 24 h, the bacterial growth was determined by adding 20 µl of 0.5% (w/w) 2,3,5-triphenyltetrazolium chloride aqueous solution. Minimal inhibitory concentration of the oil was defined as the lowest concentration of the tested samples where visible growth was not detected after the addition of the growth indicator.

Anti-biofilm assay

To investigate the potential of the isolated strains to form biofilm, the tested isolates were first subjected to biofilm forming ability crystal violet (CV) assay as previously described (Pejčić et al., 2020). The wells of the microtiter plates containing 200 µl of tryptone soy broth containing 0.5% (w/v) glucose were inoculated with suspensions prepared as described for microdilution method, to achieve the final concentration of ~10^6 CFU/ml. The plates were incubated 24 h at 37 °C afterwards the well content was aspirated, and the wells were washed twice with phosphate saline buffer (pH 7.4). Then the plates were dried, stained with 0.5% (w/v) solution of CV for 20 min, washed and distained by addition of 250 µl of ethanol (96%, v/v). Following 45 min of distaining procedure, the obtained solutions were transferred into a new microtiter plate and the absorbance of each well content was measured at 595 nm using an ELISA reader (Multiscan Ascent, Labsystems, Finland). According to their biofilm-producing ability, the strains were classified into the following groups: none, weak, moderate, and strong biofilm producers (Stepanović et al., 2007).

The potential of oregano and thyme essential oils to inhibit biofilm production of the investigated *S. aureus* strains was done by using the same procedure. The only difference was that the wells contained serial doubling dilutions of the essential oils in the desired concentration range (0.01-5.00 mg/ml) together with inoculum and cultivation media. The percentage inhibition of biofilm formation caused by oregano and thyme essential oils was calculated according to the following equation: Percentage inhibition= 100- [(A_{595} of the well containing the essential oil / A_{595} of the well without essential oil) * 100]. The results are presented on the graph as absorbances of the control and experimental wells, where absorbance of the control represents 100%.

Time-lapse kinetics of inhibitory action

To investigate whether the oils achieve long term inhibitory action and the amount of obtained inhibition against each strain, the modified time-kill assay has been performed in microtiter plates. The assay was done by inoculation of the media (final cell density was ~10^6 CFU/ml) containing the essential oils in MIC and MIC/2 concentrations and subsequent incubation of the plates for total of 48 h at 37 °C. After inoculation, the absorbance of each well was measured at 600 nm using an ELISA reader (Multiscan Ascent, Labsystems, Finland) at the following incubation periods: 0, 2, 3, 4, 22, 24, 46 and 48 h. The control wells contained only inoculated medium without essential oils.

Checkerboard assay

The type of interaction between the two essential oils and also between the single oil and antibiotic streptomycin was tested using a microdilution checkerboard assay (Stojanović-Radić et al., 2020). The testing was performed against the three strains, two isolates and one ATCC strain. Briefly, the medium containing various combinations of antibacterial agent in question (essential oil or antibiotic), obtained by two-fold serial dilutions, were inoculated to achieve the final concentration of ~10^6 CFU/ml per well. The plates were incubated for 24 h at 37 °C and the combined effects of essential oils with antibiotic or with other essential oil were calculated. The obtained results are expressed as fractional inhibitory concentrations (FICs) and the calculation was done using the following equations:

$$FIC_{EO} = MIC_{EO\text{-}S}/MIC_{EO}$$
$$FIC_{S} = MIC_{S\text{-}EO}/MIC_{S}$$

$$FICI = FIC_{EO} + FIC_S$$
$$FIC_{EO}^1 = MIC_{EO}^1\text{-}_{EO}^2 / MIC_{EO}^1$$
$$FIC_{EO}^2 = MIC_{EO}^2\text{-}_{EO}^1 / MIC_{EO}^2$$
$$FICI = FIC_{EO}^1 + FIC_{EO}^2$$

where FIC_{EO} is the fractional inhibitory concentration of essential oil (oregano or thyme), $MIC_{EO\text{-}S}$ is MIC of the oil in combination with streptomycin, MIC_{EO} is the MIC of the oil alone; FICs is the fractional inhibitory concentration of streptomycin, $MIC_{S\text{-}EO}$ is MIC of the streptomycin in combination with the oil, MIC_S is the MIC of the streptomycin alone; FIC_{EO}^1 is the fractional inhibitory concentration of oregano, $MIC_{EO}^1\text{-}_{EO}^2$ is MIC of the oregano oil in combination with thyme oil, MIC_{EO}^1 and MIC_{EO}^2 is the MIC of the oregano and thyme oil alone, respectively; FIC_{EO}^2 is the fractional inhibitory concentration of thyme, and $MIC_{EO}^2\text{-}_{EO}^1$ is MIC of the thyme oil in combination with oregano oil. FICI was interpreted as synergistic when the value was ≤0.5, as an additive or indifferent when FICI was >0.5 and ≤2, and as antagonistic interaction when it was ≥2.

The antimicrobial activity of *O. vulgare* and *T. vulgaris* essential oils was exhibited against all tested strains and minimal inhibitory values ranged between 0.078 and 2.50 mg/ml (**Tab. 1**). The oregano essential oil showed higher antistaphylococcal potential by inhibiting the growth of the tested panel of staphylococci in the range 0.156-0.625, with the average MIC of 0.45 mg/ml. On the other hand, thyme essential oil exhibited inhibition in the broader MIC range (0.078 - 2.50 mg/ml, MIC_{AVRG}=1.1 mg/ml) and mostly at much higher concentrations in comparison to oregano essential oil. Among the strains of staphylococci, the strain No. 7 showed the highest resistance, while the most sensitive one has been the strain No. 4. These data are not corresponding to those for antibiotic, since the two strains showed the same sensitivity to streptomycin. This is one more proof that the oils possess different mode of action then the antibiotic, used as a control antibacterial agent.

Previous studies on the *T. vulgaris* essential oils antimicrobial efficacy reported variable active concentrations against *S. aureus*, which were highly dependent on the essential oil composition and ranged from <0.2 µl/ml to 4 mg/ml (Rota et al., 2008; Kazemi et al., 2012; Kon & Rai, 2012; de Carvalho et al., 2015; Pesavento et al., 2015; Benabed et al., 2016; Valizadeh et al., 2016; Fani & Kohanteb, 2017; Gedikoğlu et al., 2019; Pourazar et al., 2018). Studies on the antimicrobial activity of oregano essential oil showed very high efficacy against many bacterial species due to high content of a well-known antibacterial compounds, carvacrol and thymol, also present in the thyme essential oil (Sakkas & Papadopoulou, 2017). It has been reported that oregano essential oil inhibited the growth of MRSA (methicillin resistant *S. aureus* strains) at concentrations of 0.16 and 0.32 mg/ml (Boskovic et al., 2015; Lu et al., 2018). In other studies where efficacy of oregano has been investigated against *S. aureus* strains by microdilution method, inhibitory concentrations ranged from 0.15 µg/ml to 0.8 mg/ml (Nostro et al., 2007; Özkalp et al., 2010; Boskovic et al., 2015; Marques et al., 2015; Pesavento et al.,

Table 1. Antistaphylococcal activity of the *Thymus vulgaris* and *Origanum vulgare* essential oils (MIC: Minimum inhibitory concentration; ATCC: American type culture collection)

No.	Strains	*Thymus vulgaris* MIC *mg/ml	*Origanum vulgare* MIC *mg/ml	Streptomycin MIC *mg/ml	Origin
1	*Staphylococcus aureus*	1.250	0.625	0.00625	nose
2	*Staphylococcus aureus*	1.250	0.312	0.00156	nose
3	*Staphylococcus aureus*	1.250	0.625	0.00078	throat
4	*Staphylococcus aureus*	0.078	0.312	0.00078	nose
5	*Staphylococcus aureus*	1.250	0.312	0.0001	wound
6	*Staphylococcus aureus*	0.625	0.312	0.00039	nose
7	*Staphylococcus aureus*	2.500	0.625	0.00078	nose
8	*Staphylococcus aureus*	1.250	0.625	0.00078	ATCC
9	*Staphylococcus aureus*	0.625	0.625	0.0001	nose
10	*Staphylococcus aureus*	1.250	0.625	0.00312	nose
11	*Staphylococcus aureus*	1.250	0.312	0.00078	nose
12	*Staphylococcus aureus*	0.625	0.156	0.00078	nose

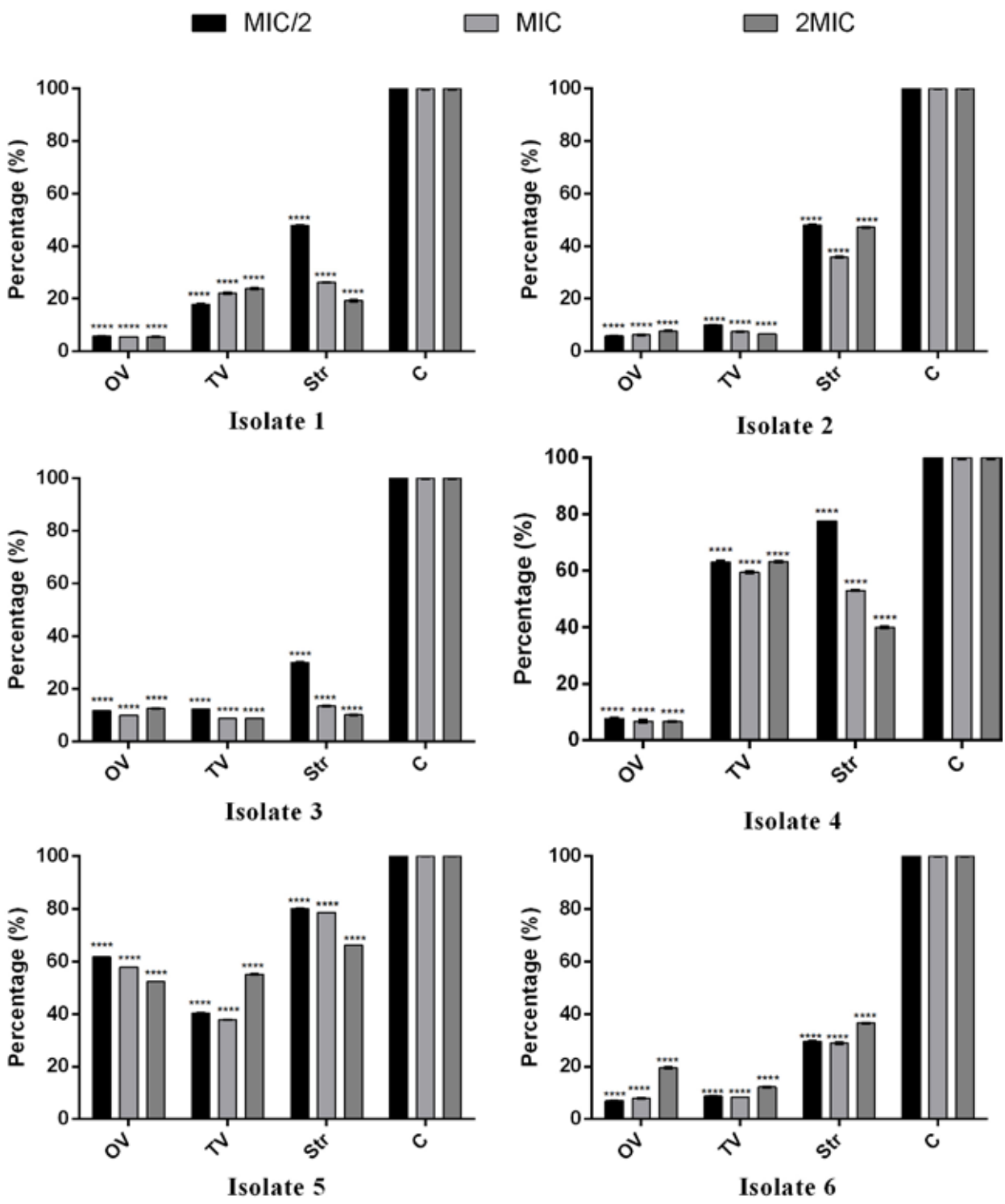

Fig. 1. Antistaphylococcal activity of the *Thymus vulgaris* and *Origanum vulgare* essential oils

*The results are presented as the percent of formed biofilms, where the control biofilm absorbance represents 100%. OV- absorbance of the wells containing *Origanum vulgare* essential oil; TV- absorbance of the wells containing *Thymus vulgaris* essential oil; Str - streptomycin; C - control biofilm.

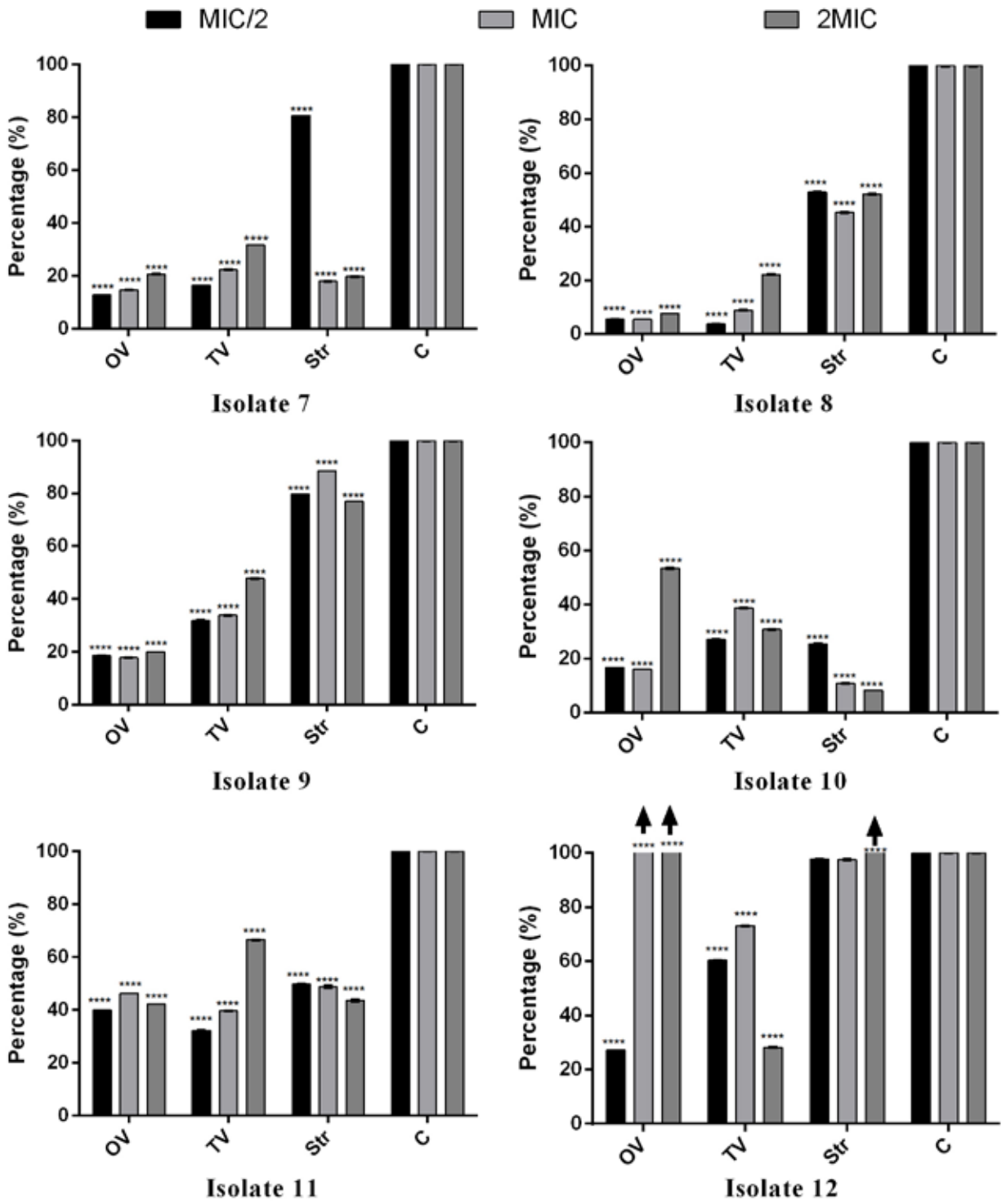

Fig. 2. Antibiofilm activity of oregano and thyme essentail oils against the isolates of *Staphylococcus aureus* No. 7-12

*The results are presented as the percent of formed biofilms, where the control biofilm absorbance represents 100%. OV- absorbance of the wells containing *Origanum vulgare* essential oil; TV- absorbance of the wells containing *Thymus vulgaris* essential oil; Str - streptomycin; C - control biofilm.

2015; Araujo & Longo, 2016; Evangelista-Martínez et al., 2018; Simirgiotis et al., 2020; Xiao et al., 2020). In comparison to these results, the oregano essential oil investigated in this paper fits into the previous range of active concentrations.

Biofilm producing ability and antibiofilm activity

The results on the biofilm producing ability of investigated *S. aureus* strains demonstrated that only one strain was a weak biofilm producer (No. 5), three strains were moderate producers (No. 7, 9, 11), while the remaining strains showed strong biofilm producing ability. The testing of the two essential oils showed that oregano oil had higher antibiofilm activity and that this activity was the most efficient at half-MICs (**Fig. 1** and **Fig. 2**). The double and four-time increment of the oils concentration (MIC, 2 x MIC) did not cause higher reduction. In contrast, reduction was lower with higher oil concentrations. The only exception was the action of this oil against isolate No. 5, a weak biofilm producer. The observed reductions were higher than 90% of the control biofilm for strains No. 1, 2, 4, 6 and 8 and more than 80% in strains No. 3, 7, 9 and 10. In contrast to these strains, the strain No. 5 demonstrated direct concentration-dependency where the reduction ranged from 39% (half MIC) to 48% (2 x MIC). In strain No. 11, the reductions were concentration dependent and ranged from 55 to 60%. Finally, the strain No. 12 demonstrated very unusual effect of oregano essential oil, where half MIC reduced biofilm by 72%, but higher concentrations promoted biofilm formation.

Similar results were obtained when these three concentrations were tested against *P. aeruginosa* in the study of Pejčić et al. (2020) where in many cases, higher concentrations exhibited a lower reduction of biofilm formation. This was explained by an enhanced production of exopolysaccharides (EPS) as a response of the cells to stress caused by high concentrations of antimicrobials. By this production, EPS interfere with diffusion and reduce their toxicity. Also, these high concentrations can cause decreased interaction with cells and/or activation of some resistance mechanisms in the biofilm cells (Pejčić et al., 2020). Therefore, herein obtained results suggest that similar processes occur in *S. aureus* biofilms as well. In the study of Nostro et al. (2007), oregano essential oil antibiofilm efficacy was tested at three sub-inhibitory concentrations (MIC/2, MIC/4 and MIC/8). Their results showed increased reduction with higher concentrations, which is opposite to our results. The explanation might be that in present work, we tested only one subinhibitory concentration and it obtained very high efficiency. Therefore, only subinhibitory concentrations should be tested in the future. Lu et al. (2017) also confirmed high efficacy of oregano essential oil against 24-h-old multidrug-resistant (MRSA) staphylococcal biofilms at concentration of 0.4 mg/ml.

In the case of the thyme essential oil, the mentioned reverse concentration dependence has also been observed. Here, in almost half of the strains (No. 1, 7, 8, 9, 11) the mentioned concentration dependency was observed, while in others the reductions exhibited by all three concentrations were statistically insignificant (No. 2, 3, 4 and 10). Only in strain No. 12, the twice MIC demonstrated the highest efficiency. Up to now, several studies investigated potential of the thyme essential oil against staphylococcal biofilms (Kavanaugh & Ribbeck, 2012; Vázquez-Sánchez et al., 2014; Ben Abdallah et al., 2020). When the thyme essential oil was tested against SC-01, which is a biofilm-forming, oxacillin- and methicillin-resistant clinical isolate, it has been efficient at ~ 0.8% (v/v) concentration against its biofilm (Kavanaugh and Ribbeck, 2012). In the study of Vazquez-Sanchez et al. (2014), the thyme oil showed the highest efficacy with MIC of 0.04% (v/v) among several tested essential oils and the most efficiently eradicated 48-h-old biofilms formed on stainless steel. Finally, the recent research od Abdallah et al. (2020) showed that the essential oil of *Thymus zygis* reduced the biofilms of MRSA isolates up to 91%. In the present study, the reductions exhibited by the thyme essential oil ranged from 31% to 96%.

Time-lapse kinetics of inhibitory action

The present study also dealt with kinetics of inhibitory activity in order to investigate the efficiency of the two oils during a time period of 48 h and the results are presented in **Fig. 3 and 4**. In these experiments, two concentrations were tested, MICs and half-MICs. The strains 7, 8 and 9 were completely inhibited throughout the entire incubation period by both concentrations of both essential oils. In the case of oregano oil, it has been observed that in all strains the inhibition of growth was complete during the first four hours, afterwards the cultures started recovery and exponential growth in strains 1, 2, 3, 5, 6, 10 and 12. However, this growth was significantly reduced in comparison to the control and also the observed growth was noticed only at half MICs (No. 1, 2, 3, 10, 11 and 12) or were concentration dependent (No. 5 and 6). Also, in isolates No. 11 and 12, it is notable that the recovery has been prolonged, and the growth started after 24 h for both oils. The thyme essential oil exhibited similar action, where complete inhibition during the entire period of incubation has been observed in strains No. 1, 7, 8, 9 and 12, while some strains recovered

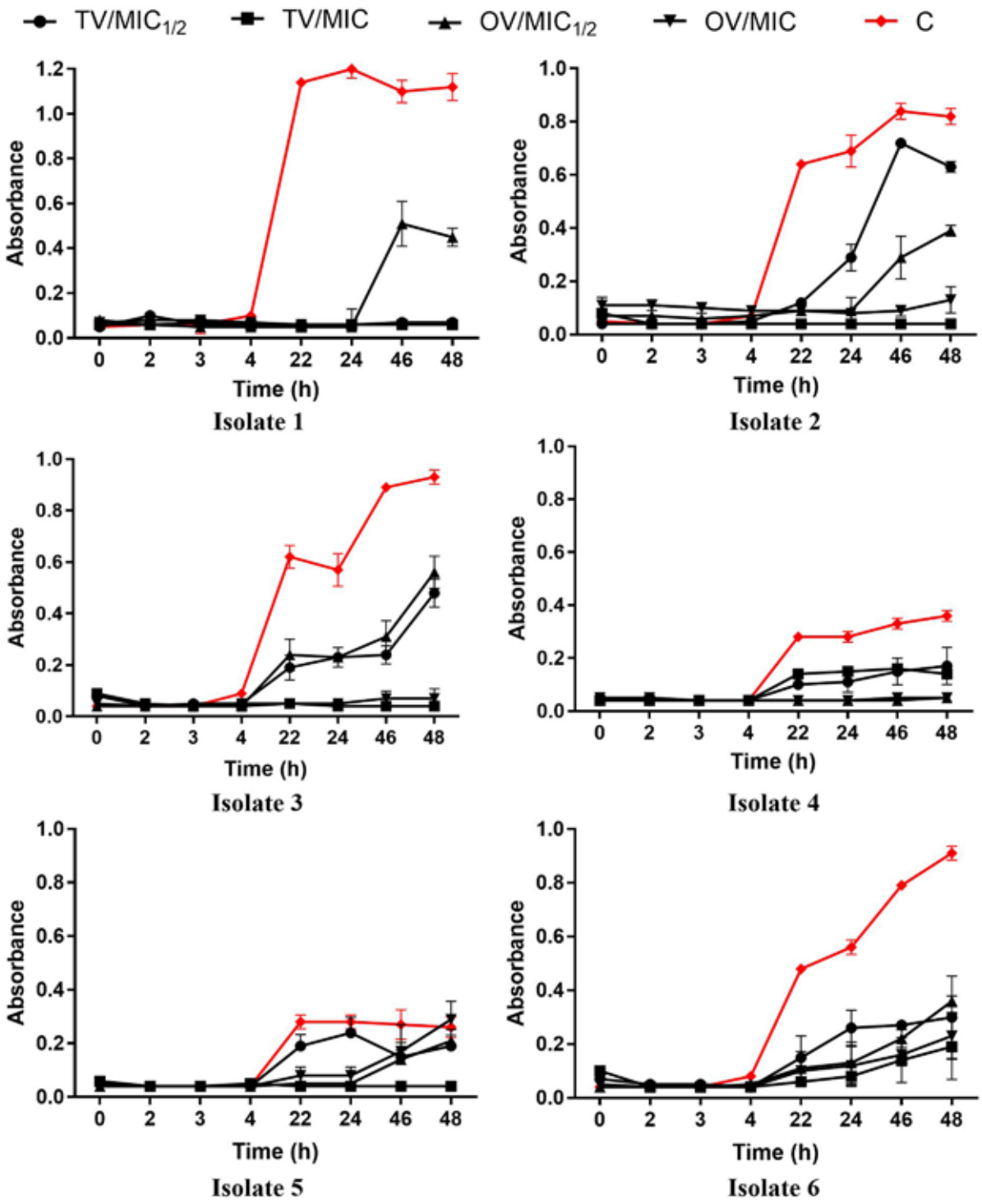

Fig. 3. Time-lapse kinetics of oregano and thyme essential oils inhibitory action against *Staphylococcus aureus* clinical isolates (strains No. 1-6)

but only at the lower tested concentration (No. 2, 3, 10 and 11). Finally, in strains No. 4, 5 and 6, the recovery was observed at both MIC and MIC/2, but the final growth of bacterial populations has been significantly reduced in comparison to the control values. According to this, the efficiency against planktonic cells is the highest during the first day of incubation and this information might be used for application of some synergistic antimicrobials during this period to achieve the complete killing effect at lower oil/antimicrobial agent concentrations.

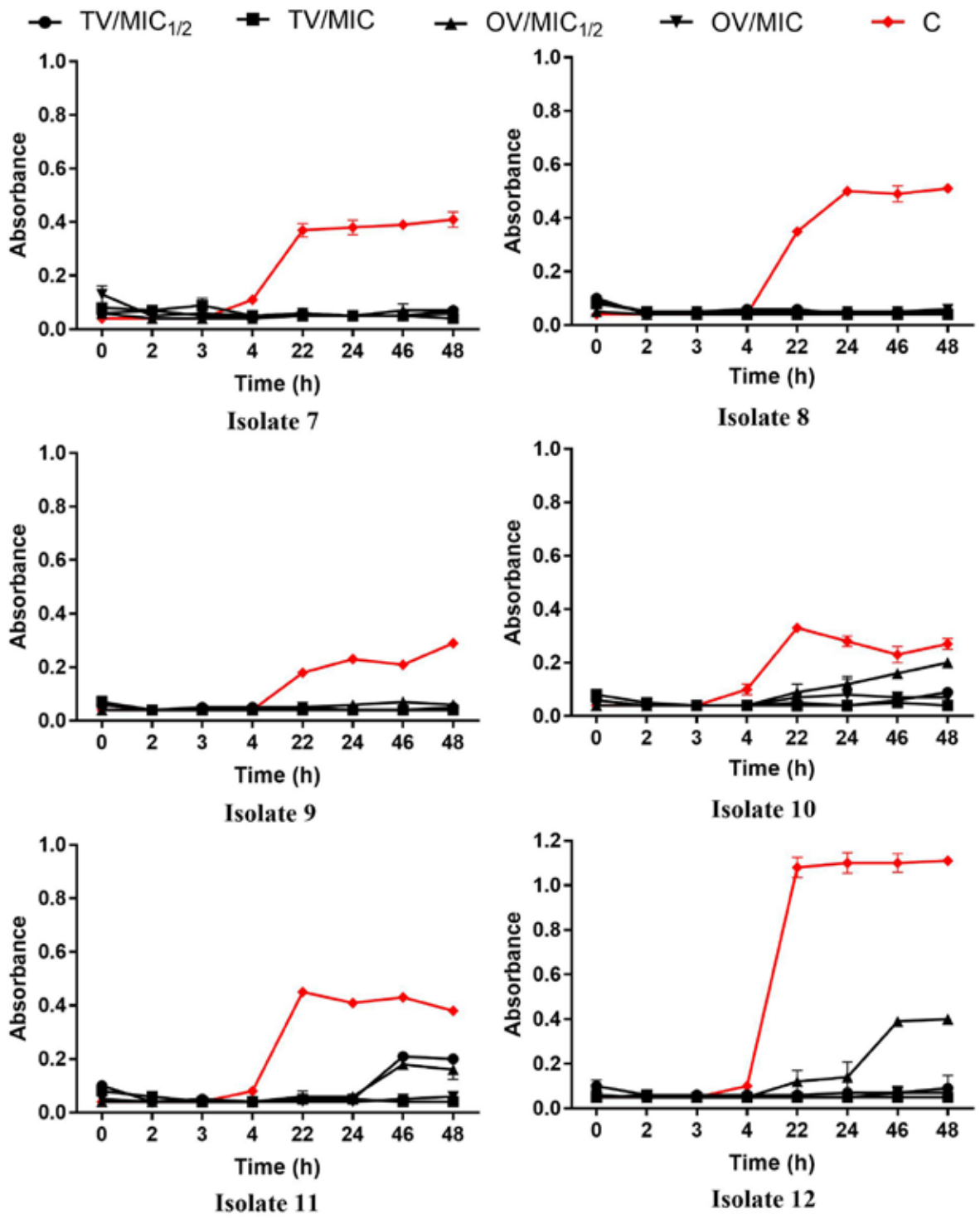

Fig. 4. Time-lapse kinetics of oregano and thyme essential oils inhibitory action against *Staphylococcus aureus* clinical isolates (strains No. 7-12)

Checkerboard assay

To find out which is mutual relationship between the oils and also of the oils alone with the reference antimicrobial drug, the testing of sinergism was done by checkerboard assay. Calculated FICs showed either sinergistic or additive effect of all tested combinations (**Tab. 2**). When the essential oils were combined together, MICs of both oils reduced from two to eight times. In combination with streptomycin, sinergistic and additive effect was observed for both oils, with reductions of MICs up to eight times for both participants in the combination.

Table 2. Minimum inhibitory concentrations (mg/ml) and fractional inhibitory concentration values of the mutual combinations of EOs and with streptomycin

	Combinations	*Staphylococcus aureus* ATCC 6538			*Staphylococcus aureus* * No. 1			*Staphylococcus aureus* * No. 12		
		MIC alone	MIC combined	FIC	MIC alone	MIC combined	FIC	MIC alone	MIC combined	FIC
1	**thyme + oregano**									
	thyme	1.25	0.156	0.125	1.25	0.625	0.5	0.625	0.312	0.5
	oregano	0.625	0.078	0.125	0.625	0.312	0.5	0.156	0.078	0.5
	***FICI**			**0.25**			**1**			**1**
	Interaction			**Syn**			**Add**			**Add**
2	**thyme + streptomycin**									
	thyme	1.25	0.156	0.125	1.25	0.156	0.125	0.625	0.156	0.25
	streptomycin	0.00078	0.00024	0.313	0.00625	0.0009	0.156	0.00078	0.00048	0.626
	***FICI**			**0.438**			**0.281**			**0.876**
	Interaction			**Syn**			**Syn**			**Add**
3	**oregano + streptomycin**									
	oregano	0.625	0.078	0.125	0.625	0.156	0.25	0.156	0.078	0.5
	streptomycin	0.00078	0.00048	0.625	0.00625	0.0009	0.156	0.00078	0.00097	1.25
	***FICI**			**0.75**			**0.406**			**1.65**
	Interaction			**Add**			**Syn**			**Add**

FIC: Fractional inhibitory concentration; FICI: Fractional inhibitory concentration index; MIC: Minimum inhibitory concentration; ATCC: American type culture collection; Syn: synergism; Add: additive

The results pointed to the thyme-streptomycin as the most efficient combination, where sinergism was demonstrated against two out of the three tested strains of staphylococci.

Also, important to mention is the fact that these combinations were sinergistic for the strains with higher MICs of oils. In the third strain, which was more susceptible, additive effect has been observed for all tested combinations. Up to now, it has been reported that the thyme oil exhibits sinergistic potential against staphylococci when combined with garlic (García-Díez et al., 2017), *Myrtus communis* essential oil (Sadiki et al., 2014) and with mustard (Reyes-Jurado et al., 2016). On the other hand, Van Vuuren et al. (2009) demonstrated the predominance of antagonism against staphylococci (and some other microbial species) when the thyme essential oil was combined with cyprofloxacin.

In the case of oregano, it has been reported that it possesses an additive effect in combination with acetic acid (de Souza et al., 2009), basil or bergamot essential oils (Lv et al., 2011), silver nanoparticles (Scandorieiro et al., 2016), as well as with mustard (Reyes-Jurado et al., 2016). The study of Rejes-Jurado et al. (2016) demonstrated that in combination, thyme and mexican oregano exhibited additive effect only. Our study partially confirms these results since additive effect has been observed for one of the three tested strains. Together with this, a recent study of Xiao et al. (2019) revealed that oregano when combined with quinolone drugs (tosufloxacin, levofloxacin, ciprofloxacin) and rifampin completely eradicated all stationary phase *S. aureus* cells. Based on the herein presented results, we can suggest that both oils might be used in combination or for an effective enhancement of the streptomycin efficacy against human infections caused by staphylococci.

Conclusion

The present study tested the antimicrobial potential of the thyme and oregano essential oils against clinical isolates of *S. aureus*. The results showed moderate to high action against planktonic cells and significant antibiofilm potential of both oils. Oregano exhibited higher activity in all tests in comparison to the thyme essential oil. Time-lapse kinetics study revealed that the action of both oils is strain-dependent and that in most cases, the MICs very significantly reduced or even completely eradicated the growth of the cells. Also, inhibitory effect in recovering cultures is the most prominent during the first four up to 24 h of cultivation, so the effect of some compound that possess sinergistic relationship with the essential oils will be the highest if applied during the first 24 h. Finally, the sinergy has been observed when the oils were applied in combination, but also when individually combined with streptomycin. All mentioned results point to a very high potential of both oils to be used as an adjuvant agent for control of human staphylococcal infections.

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