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Antimicrobial efficacy of basil and sage essential oils against *Pseudomonas aeruginosa*: time-lapse kinetics and type of interaction with ciprofloxacin

Original Article

Milica Pejčić
Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, Niš, Serbia
milicapejcic1991@gmail.com (corresponding author)

Zorica Stojanović-Radić
Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, Niš, Serbia
zstojanovic@pmf.ni.ac.rs

Marina Dimitrijević
Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, Niš, Serbia
dimitrijevicmarina92@yahoo.com

Niko Radulović
Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, Niš, Serbia
nikoradulovic@yahoo.com

Abstract:

The increasing resistance of *Pseudomonas aeruginosa* strains to used antibiotics is a big problem of modern medicine. Finding antimicrobial substances which will increase the antibiotics effects is necessary. Therefore, the aim of the present study was to evaluate the time-lapse activity of the basil and sage oils, to evaluate the combined oils effect against *P. aeruginosa* clinical isolates and to determine the oils potential to enhance the activity of ciprofloxacin. The obtained growth curves showed a reduction in the number of bacterial cells in the range of 10.5-94% when basil oil was applied. In the case of the sage oil, higher reduction has been observed (48.5-100%). The basil oil achieved a synergistic interaction with ciprofloxacin in 8 isolates, while the same effect for sage/ciprofloxacin combination was observed in 6 isolates. Indifferent effect was noticed in 64% of the tested isolates for basil/sage combination. These data showed potential benefits of the oils and ciprofloxacin combination therapy in the treatment of *P. aeruginosa* infections.

Key words:
Basil oil, sage oil, time-lapse activity, synergy, ciprofloxacin, *Pseudomonas aeruginosa*

Apstract:

Antimikrobna efikasnost etarskih ulja bosiljka i žalfije na *Pseudomonas aeruginosa*: vremenska kinetika i tip interakcije sa ciprofloksacinom

Povećanje rezistencije sojeva *Pseudomonas aeruginosa* na dosad korišćene antibiotike predstavlja veliki problem u medicini. Neophodno je pronaći antimikrobne supstance koje će povećati efikasnost antibiotika. Zbog toga je cilj ovog istraživanja bio analiza vremenske kinetike ulja bosiljka i žalfije, analiza kombinovanog efekta ispitivanih ulja na kliničke izolate *P. aeruginosa* i određivanje potencijala ulja u povećanju aktivnosti ciprofloksacina. Dobijene krive rasta pokazale su smanjenje broja bakterijskih ćelija u rasponu od 10,5-94% prilikom primene ulja bosiljka. U slučaju ulja žalfije primećeno je smanjenje broja ćelija u rasponu 48,5-100%. Ulje bosiljka pokazalo je sinergističku interakciju sa ciprofloksacinom na 8 testiranih izolata, dok je isti efekat kombinacije žalfija/ciprofloksacin primećen kod 6 izolata. Indiferentni efekat uočen je kod 64% testiranih izolata za kombinaciju bosiljak/žalfija. Ovi rezultati su pokazali potencijalne koristi kombinovane terapije ulja i ciprofloksacina u lečenju infekcija izazvanih vrstom *P. aeruginosa*.

Ključne reči:
Ulje bosiljka, ulje žalfije, vremenska kinetika, sinergizam, ciprofloksacin, *Pseudomonas aeruginosa*

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Introduction

The increase of resistance between the bacterial strains caused by incorrect or over used antimicrobial therapy is becoming a big problem in modern medicine these days. Antimicrobial resistance is a natural evolutional phenomenon where microbes develop resistance against commonly used antibiotics or antimicrobial drugs by different adaptation mechanisms. Owing to increased spreading, the World Health Organization placed antimicrobial resistance among the top 10 urgent threats for the year 2019 (Yu et al., 2020). Among these bacteria, *Pseudomonas aeruginosa* is one of the most frequent multiresistant species and becomes a major concern in public health (El-Hosseiny et al., 2014; Alhazmi, 2015). Therefore, it is necessary to find same antimicrobial substances which will increase the antimicrobial potential of existing drugs, and help in overcoming the bacterial resistance.

The last few decades are marked by a large number of studies on antimicrobial (Akthar et al., 2014; Calo et al., 2015; Tariq et al., 2019), antibiofilm and anti-virulence (Artini et al., 2018; Reichling, 2020) activity of essential oils (EOs) and their components. The use of drug combinations in antibacterial treatment rather than single antimicrobial agents provides better results, because the use of a single agent is highly associated with resistance occurrence. Due to the complexity in chemical composition, no particular bacterial adaptation to essential oils has been described (Mikulášová et al., 2016). Owing to the existing antimicrobial potential, combinations between different antibiotics and other antimicrobial substances, such as EOs, represent one of the most promising advances against drug-resistant microorganisms. The interaction between antimicrobials in combinations can result in three different outcomes: synergistic, additive, or antagonistic effect (Chouhan et al., 2017). In recent years, there have been many studies that investigated the increasing effect of EOs and their components to antimicrobial potential of conventional drugs. These studies have been performed on a number of pathogenic bacteria and *P. aeruginosa* was among them (Hemaiswarya & Doble, 2009; Van Vuuren et al., 2009; Langeveld et al., 2014; Yap et al., 2014; Aelenei et al., 2016; Chouhan et al., 2017).

The antimicrobial potential of *Salvia officinalis* (sage) and *Ocimum basilicum* (basil) essential oils against different pathogenic bacteria is already well known (Delamare Longaray et al., 2007; Bassolé et al., 2010; Fournomiti et al., 2015; Tardugno et al., 2018). Furthermore, different interactions between the sage oil and some conventional antibiotics (El-Hosseiny et al., 2014; Adrar et al., 2016), as well as interactions between the basil oil and antibiotics (Mahmoud et al., 2016; Silva et al., 2016) against *P. aeruginosa* have been reported.

Based on our previous work (Pejčić et al., 2020), we decided to broaden our investigations on the basil (*Ocimum basilicum* L.) and sage (*Salvia officinalis* L.) essential oils, which demonstrated the antibacterial efficacy, as well as potential to reduce the virulence factors (biofilm formation, mature biofilm resistance, motility, and pyocyanin production) of the *P. aeruginosa* clinical isolates. Therefore, the aim of the present study was: a) to evaluate the time-lapse activity of the tested oils; b) to evaluate the combined effects of basil and sage essential oils, used in mixture against *P. aeruginosa* clinical isolates; and c) to determine the potential of the tested oils to enhance the activity of antibiotic ciprofloxacin.

Materials and Methods

Essential oils

The essential oils of sage (*Salvia officinalis* L. (Lamiaceae), Montes, Leskovac) and basil (*Ocimum basilicum* L. (Lamiaceae), MyGreenWay, Niš) were purchased at a local herbal pharmacy (Beyond, Niš). The minimal inhibitory concentrations (MICs) and chemical composition of the oils were studied in detail and compared to the literature data in our previous work (Pejčić et al., 2020).

Used microorganisms

Strains used in this study were isolated from different human material, identified as *P. aeruginosa*, phenotypically characterized and presented in our previous work (Pejčić et al., 2020). Total of 13 isolated *P. aeruginosa* strains were stored at the Microbiology Laboratory, Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš. For the experiments, the investigated strains were grown on Nutrient agar (NA) for 18 h and used to prepare suspensions in physiological saline (0.85% NaCl) that corresponded to the 0.5 McFarland turbidity standard (10^8 cells/ml). The suspension turbidity was adjusted by using a McFarland densitometer (DEN-1 McFarland Densitometer, Biosan).

Time-kill curve

The activity of essential oils against the bacterial strains over time was determined by using the time-kill assay. The initial bacterial suspension was adjusted in Mueller Hinton broth to achieve the final cell concentrations of $5x10^6$ CFU/ml. The effect of oil in MICs concentrations previously determined (Pejčić et al., 2020) on bacterial growth was monitored via measurement of turbidity changes 37 °C during 48 h at 600 nm (Stojanović-Radić et al., 2012).

Checkerboard assay

The antimicrobial agents (basil oil, sage oil and ciprofloxacin) were combined with each other and their interaction against *P. aeruginosa* clinical isolates was investigated using a checkerboard method (Stojanović-Radić et al., 2020). Three combinations were tested: basil oil/ciprofloxacin; sage oil/ciprofloxacin, and basil oil/sage oil. Each antibacterial agent was tested in MICs (previously determined) and several concentrations below the MICs. The test was performed in 96-well microtiter plates, where serial dilutions of each agent were made in horizontal and vertical rows, which resulted in various combinations of both agents. The stocks of tested essential oils were prepared in 100% DMSO and then diluted with MHB. The combination of essential oils and antibiotics was prepared by adding 25 µl of each to the same well of microtiter plate and inoculating with 150 µl of bacterial suspension ($5x10^6$ CFU/ml), afterwards the plates were incubated at 37 °C for 24 h. The interaction between the two antimicrobial agents was estimated by calculating the fractional inhibitory index (FICI). The calculation of FICI was done using the following equations:

$$FIC_A = MIC_{A\text{-}B} / MIC_A$$

$$FIC_B = MIC_{B\text{-}A} / MIC_B$$

$$FICI = FIC_A + FIC_B$$

where FIC_A is the fractional inhibitory concentration of antimicrobial agent A, $MIC_{A\text{-}B}$ is MIC of the agent A in combination with agent B, MIC_A is MIC of agent A alone, FIC_B is the fractional inhibitory concentration of agent B, $MIC_{B\text{-}A}$ is MIC of agent B in combination with the agent A, and MIC_B is MIC of agent B alone. FICI was interpreted as a synergistic effect when its value was ≤ 0.5, as an additive $0.5 < FICI \leq 1$, indifferent effect when FICI was $1 < FICI \leq 2$, and as an antagonistic interaction when it was < 2 (Stojanović-Radić et al., 2020).

Statistical analysis

All experiments were done in triplicate and data are given as average values ± standard deviations. Statistical comparison was done using Two Way ANOVA followed by Tuckey post hoc test (GraphPad Prism version 5.03, San Diego, CA, USA). Probability values (p) less than 0.05 were considered to be statistically significant.

Results and discussion

As a continuation of our previous investigation (Pejčić et al., 2020), where the basil and sage oils antibacterial and anti-virulence potential were determined, the present work reports the time-lapse activity of the tested oils and interactions of combined antimicrobial agents (basil, sage oil and ciprofloxacin) against *P. aeruginosa* clinical isolates.

Growth curve

Our previous investigation (Pejčić et al., 2020) of the herein tested oils antimicrobial activities showed the moderate action of both oils in the range of 5.0–20.0 mg/ml, depending on the isolated strain. The MICs were further tested to investigate the activities of oils during the total incubation period of 48 h, and the results are presented as a growth curve (**Fig. 1**). Inhibition of bacterial growth was observed during the first 21 h of the treatments, after which very slight recovery was detected, but the oils significantly reduced total population number. Following the incubation period of 48 h in total, the basil oil exhibited growth inhibition in the range 10.5-94%. The highest activity has been achieved against isolate No. 7, where reduction of 93.95% was observed after 48 h. On the other hand, the isolate No. 4 showed somewhat higher resistance in comparison to the others, where inhibition of only 10.5% was measured. In the case of the sage oil, higher reduction has been observed (48.5-100%). The most prominent action of the sage oil was detected against several isolates, namely No. 4, No. 7, No. 11 and No. 13, where 100% of growth reduction was measured. The sage oil effectively inhibited regrowth of isolates No. 3, No. 4, No. 6, No. 7 and No. 11 (bacteriostatic effect). After total incubation period, LD_{50} (reduction over 50% of the total population size) was achieved in 92% (sage oil) and 53% (basil oil) of the tested isolates, which demonstrated higher time-lapse efficacy of the sage oil. According to our previous results, the oils showed similar potential considering antimicrobial action, as well as virulence factors-inhibiting potential (Pejčić et al., 2020). However, in the present study, higher effect over time is visible in the case of the sage essential oil.

Studies reported that the antimicrobial activities of the essential oils were due to the presence of some major and minor constituents and some type of interaction between many components (Mitic-Culafic et al., 2005; van Vuuren & Viljoen, 2007; Bassolé et al., 2010). *Salvia officinalis* essential oils containing α-thujone (24%) and camphor (10%) (Delamare Longaray et al., 2007) or 1,8 cineol as a major compounds (El-Hosseiny et al., 2014) failed to show activity against *P. aeruginosa*. In another study, where time-lapse effect of the sage oil was investigated, a very low efficacy against *Pseudomonas aeruginosa* (ATCC 27853) was observed after the total incubation period of 24 h. Each tested concentration (5, 10 and 20 µl) affected only limited number of cells after 10 minutes and 1 h, which resulted in unchanged population size after total incubation period (Khalil & Li, 2011). The mentioned oil was characterized by 1,8- cineole (62%) as a major component while the herein tested oil had different chemical composition with α-thujone (29.7%), camphor (16.6%), and 1, 8-cineole (9.2%) as the dominant compounds (Pejčić et al., 2020). This is probably the reason for better activity of the herein tested oil. On the other hand, *Salvia officinalis* essential oil with very similar content (α-thujone 37%, camphor 14%, and 1,8 cineole 14%) to the oil used in the present study

Table 1. Interaction between antimicrobial agents against clinical isolates of *Pseudomonas aeruginosa*

	basil oil/ciprofloxacin		sage oil/ciprofloxacin		basil oil/sage oil	
Strains	FICI	Outcome	FICI	Outcome	FICI	Outcome
No.1	0.38	S	0.38	S	2.00	I
No.2	0.25	S	2.00	I	2.00	I
No.3	0.09	S	0.16	S	0.56	Ad
No.4	0.13	S	0.17	S	0.63	Ad
No.5	0.12	S	0.15	S	0.53	Ad
No.6	0.53	Ad	0.53	Ad	0.75	Ad
No.7	1.03	I	0.53	Ad	0.53	Ad
No.8	0.38	S	1.25	I	2.00	I
No.9	0.38	S	0.38	S	1.50	I
No.10	0.75	Ad	0.56	Ad	2.00	I
No.11	1.06	I	1.13	I	2.00	I
No.12	0.13	S	0.16	S	2.00	I
No.13	1.03	I	0.53	Ad	2.00	I

FICI -Fractional Inhibitory Index

S-synergistic effect ≤ 0.5; Ad-additive effect 0.5 >1; I-indifferent effect 1 ≥2; A-antagonistic effect >2.

showed promising results on the kinetics of survival of *Staphylococcus aureus* and *Bacillus subtilis*. Sage oil and fractions with high content of α-thujone exhibited a strong bactericidal effect on *S. aureus*, and within 4 hours the bacterial population was completely inactivated. On the other hand, fractions containing other dominant compounds (camphor and 1,8 cineole) exhibited lower effect, demonstrating that α-thujone presents the carrier of antimicrobial activity (Mitic-Culafic et al., 2005).

Previous studies on the basil oil efficacy against *P. aeruginosa* reported MICs from 5-40 mg/ml, (Hammer et al., 1999; Niculae et al., 2009; Pejčić et al., 2020). However, these studies have not performed the time kill assays which would allow us to compare the basil's efficacy against *P. aeruginosa* strains over the time. On the other hand, data for the time-kill kinetic against one *S. aureus* MRSA clinical isolate in the presence of basil oil showed decreased cell number in the first 30 min and this reduction continues until the end of incubation period of 24 h (Opalchenova & Obreshkova, 2003). The same results were obtained for basil oil in MIC concentration against *Candida albicans* ATTC 10231, where after 2 h of incubation the oil reduced the number of living cells (Gucwa et al., 2018). When compared to the literature data, the commercial essential oils tested in the present study showed higher activity during a longer application time. Also, having in mind that the oils that the oils showed efficiency against clinical isolates with higher resistance to antibiotics, it is another confirmation of their higher activity.

Type of interaction between antimicrobial agents Oils combination with ciprofloxacin

Resistance of *P. aeruginosa* to fluoroquinolones, the group of antibiotics that ciprofloxacin belongs to, can be due to mutations in regulation of the efflux systems, or to mutations of the topoisomerase targets (gyrA and also parC). Efflux and target mutations can cooperate to increase the resistance level to other drugs (Rossolini & Mantengoli, 2005). The use of combined therapy has been shown as a better solution in comparison to the use of individual antimicrobial agents in the treatment of bacterial resistance. To investigate the potential of the tested oils for enhancing the activity of ciprofloxacin and to determine the type of interaction between the tested essential oils against *P. aeruginosa* clinical isolates, checkerboard assay was performed. The three combinations were tested: basil/sage oil (BS), basil oil/ciprofloxacin (BC), sage oil/ciprofloxacin (SC) and the results are presented in **Tab. 1**. The synergy was found in 61.5% of the tested isolates and additive effect against the two tested isolates when BC combination was applied. Isolates No. 7, No. 11 and No. 13 expressed somewhat higher resistance to the tested combination in comparison to other isolates, where calculated FICIs showed indifferent

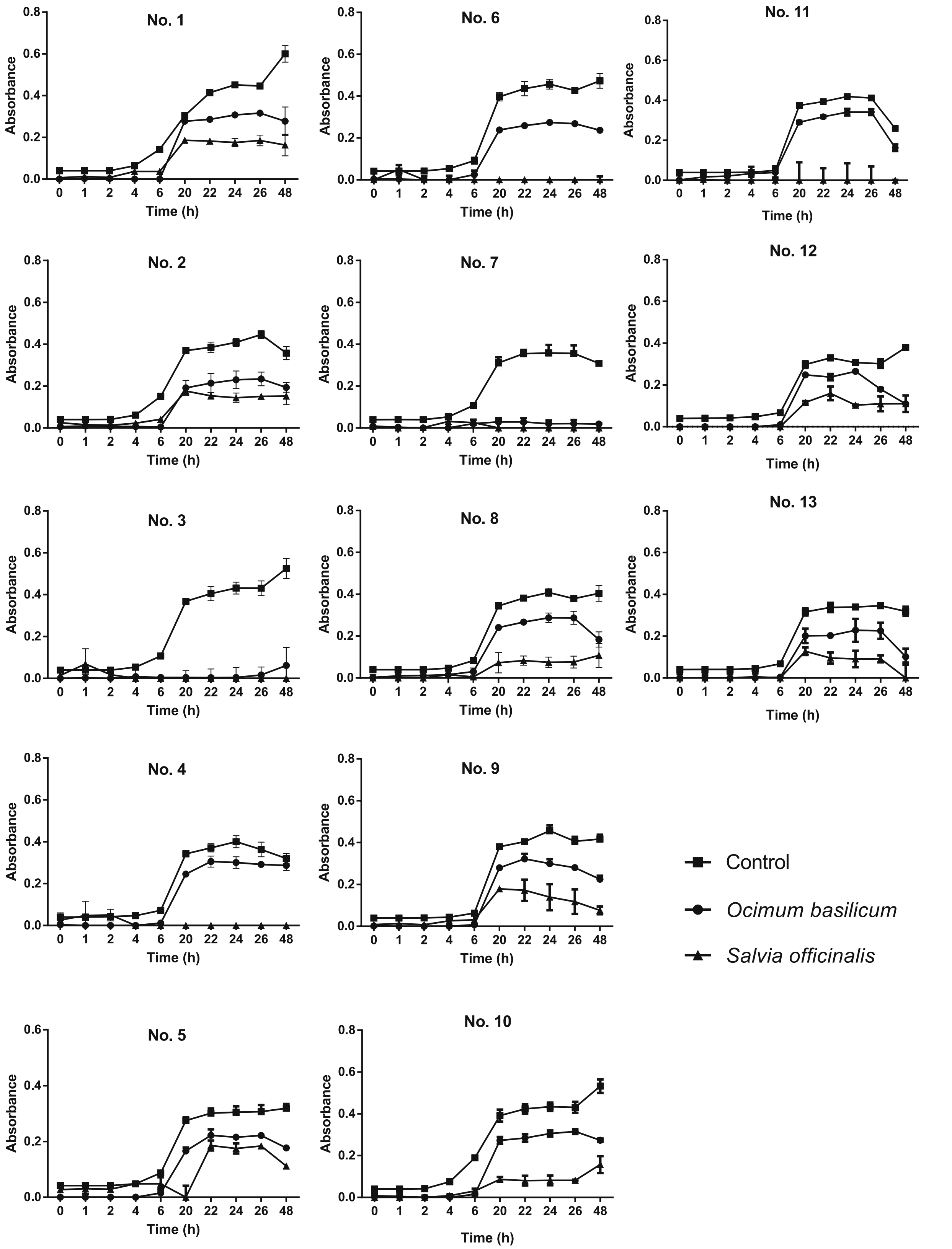

Fig. 1. Growth of *Pseudomonas aeruginosa* clinical isolates in the presence of *Salvia officinalis* and *Ocimum basilicum* commercial essential oils. Data are given as mean ± SD. The analysis was done using Two Way ANOVA followed by Tukey's post hoc test ($p < 0.05$) vs. control.

interaction between the basil oil and ciprofloxacin. On the other hand, SC indicated somewhat weaker activity in comparison to the BC combination. The SC combination expressed synergistic effect in 46.15% and additive in 30.7% of tested isolates. Isolates No. 8 and No. 11 manifested the same sensitivity to SC combination in comparison to the results obtained for these agents alone. These results are very promising and point to the very high potential of basil and sage essential oil as agents that would increase the effectiveness of ciprofloxacin used in antipseudomonal therapy.

The somewhat lower potential of herein tested sage oil in enhancing the ciprofloxacin activity in comparison to the basil may be due to the chemical composition of the oil. The results of chemical characterization of herein tested oils showed α-thujone (29.7%), camphor (16.6%), and eucalyptol (9.2%) as the major constituents of the sage oil and (*E*)-anethole (39.4%), linalool (34.3%), eugenol (5.8%) and longifolene (6.2%) of the basil oil (Pejčić et al., 2020). It has been reported that terpenoid ethers (1,8-cineole) have a lower capacity to sensitize the pathogen to the antibiotic activity as they lack free hydroxyl groups in comparison to both phenolic (eugenol) and alcoholic terpenoids (linalool) which are characterized by the presence of free hydroxyl groups. The hydroxyl group of eugenol is responsible for the inhibition of some enzymes, such as ATP-ase, histidine decarboxylase or proteases (Griffin et al., 1999).

Herein obtained results for basil oil are in agreement with Silva et al. (2016) who reported the synergism for *O. basilicum* oil associations with ciprofloxacin against the clinical isolate *P. aeruginosa* 1662339, while effect of the same combination against *P. aeruginosa* ATTC 25852 was classified as indifferent. Mahmoud et al. (2016) also reported that the basil/ciprofloxacin combination was effective in 88.89% of the total of 9 tested *P. aeruginosa* isolates.

The only study on the sage oil in combination with antibiotics activity reported its potential to significantly increase the efficacy of several conventional antibiotics against *P. aeruginosa* (El-Hosseiny et al., 2014). In the mentioned study (El-Hosseiny et al., 2014) although the sage oil displayed inactivity against the *P. aeruginosa* strain, it potentiated the activity of antibiotics piperacillin, meropenem, and gentamicin, while in combination with antibiotic norfloxacin displayed antagonistic interaction. Another study showed the potential of the sage oil to enhance the activity of ciprofloxacin in control of infections caused by MRSA strains (Milenković et al., 2015).

Sage/basil oil combination

SB combination expressed indifferent effect in 61.54% and additive in 38.46% of tested isolates. There was not synergistic effect noticed between the basil and sage oils. On the other hand, calculated FICIs showed values very close to antagonistic interaction in 7 of the total 13 tested isolates. According to the chemical compositions of investigated oils, these results are expected. The study of Faleiro et al. (2003) reported both antagonistic and synergistic effects of the pure essential oil components in combination, depending on the tested microorganisms. The mixture of linalool plus 1,8-cineole showed antagonistic effect in the case of the *Escherichia coli* strain, while the same mixture showed synergistic interaction when applied to *C. albicans*. Similar results were reported by Adrar et al. (2016), where the sage oil when combined with *Thymus numidicus* essential oil (reach in linalool (8.62–9.26%)), expressed antagonistic effect against *E. coli* 161.

Conclusions

The observed results showed time-lapse efficacy of the basil and sage oils. However, higher effect over time is visible in the case of the sage essential oil. Tested oils have also shown promising effect on increasing activity of antibiotic ciprofloxacin against *P. aeruginosa* clinical isolates. On the other hand, basil and sage oils used in combination require caution due to possible antagonism, and therefore additional research needs to be done on this topic. According to the presented results, basil and sage commercial essential oils can be used as an adjunctive therapy in combination with ciprofloxacin for the treatment of chronical infections caused by *Pseudomonas aeruginosa*.

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