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Original Research

Potential of *Ocimum basilicum* L. and *Salvia officinalis* L. essential oils against biofilms of *P. aeruginosa* clinical isolates

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Abstract: Biofilms are complex communities of microorganisms, responsible for more than 60% of the chronic human infections and they represent one of the leading concerns in medicine. *Pseudomonas aeruginosa* is human pathogenic bacteria which causes numerous diseases and is known for its ability to produce biofilm. *Ocimum basilicum* L. (basil) and *Salvia officinalis* L. (sage) are widely used plants in traditional medicine for the treatment of different conditions. Therefore, the aim of this study was to investigate the potential of basil and sage essential oils against *P. aeruginosa* biofilm producing strains. The efficacy of two essential oils on *P. aeruginosa* biofilm forming ability was determined using crystal violet method. Out of 15 strains isolated from different clinical biological samples, two were strong, 11 moderate and one weak biofilm producer. Good efficacy of sage essential oil towards strong and weak biofilm producers, but not of basil essential oil, was observed. In the case of moderate biofilm producers, 81.8% showed lower biofilm production after incubation with the sage oil, while 63.6% showed the reduction of biofilm production after basil essential oil treatment. The obtained results showed high potential of both oils for the treatment of persistent infections caused by *Pseudomonas aeruginosa* biofilms.

Key words: *Salvia officinalis, Ocimum basilicum*, anti-biofilm, *Pseudomonas aeruginosa*, multiresistant, clinical isolates.

Introduction

Biofilms are communities of microorganisms (composed of one or several microbial species) irreversibly attached to the surface and incorporated into extracellular polymeric matrix. These complex structures are characterized by different phenotypes of the cells in comparison to the planktonic cells of the same species, considering both growth rate and gene transcription (1, 2). Populations of bacteria in biofilms function as a relatively cooperative communities, capable for higher resistance towards biocides, antibiotics, antibodies and bacteriophages due to the quorum sensing (QS), presence of matrix and reduced metabolism/growth rate of dormant cells inside biofilms (3). Biofilm formation is connected with nosocomial infections, due to its ability to attach and grow on various medical devices (catheters, medical implants, contact lenses, stitching material, etc.) and is considered to be responsible for more than 60% of the chronic infections in humans.

Pseudomonas aeruginosa is an opportunistic human pathogen, typical biofilm producing species that is very often unresponsive to the conventional therapy. When it forms biofilms, bacteria produces three significant polysaccharides (alginate, Pel and Psl) responsible for the biofilm structure stability (4). Also, its high biofilm production ability and resistance are related to highly developed and complex QS systems, *rhl* and *las* systems (5). Although *P. aeruginosa* biofilms often demonstrate (multi) resistance to the conventional antibiotic therapy, there are promising results considering the efficacy of various plant metabolites, especially essential oils, against these bacteria in a form of a biofilm. There are many studies on the topic of *P. aeruginosa* biofilm inhibition by essential oils, where thyme (6), oregano (7), tea tree oil (8), rosemary (9), cinnamon (10,11), mint (12), *Citrus reticulata*, *Syzygium aromaticum* (13), *Cuminum cyminum* (14) showed significant anti-biofilm activity. Some of these oils exhibited QS inhibitory potential, together with the decrease in virulence factors production in the treated biofilms (12,15). According to the results from the studies that investigated anti-biofilm potential of the pure essential oils components, thymol and carvacrol (16), curcumin (17), cinnamaldehide and eugenol (11), they turned out to be very effective and are considered to be the main carriers of anti-biofilm activity. However, these studies were oriented mostly towards standard culture collections strains, while clinical isolates, often characterized by much higher (multi) resistance, were rarely the subject of these investigations.

To the best of our knowledge, there are no studies on the subject of the efficacy of basil (*Ocimum basilicum* L.) and sage (*Salvia officinalis* L.) essential oils, known as potent natural antimicrobial agents, against biofilm production ability of *P. aeruginosa*. Therefore, the aim of the present study was to evaluate the effects of these two essential oils on biofilm producing ability of (multi) resistant clinical isolates of *P. aeruginosa*. Also, susceptibility patterns to antibiotics and tested oils were compared and brought in connection to possible mechanism

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of oils action.

Materials and Methods

Essential oils

Essential oils of sage (*Salvia officinalis*, Montes, Leskovac) and basil (*Ocimum basilicum*, MyGreenWay, Niš) were purchased at a local herbal pharmacy (Beyond, Niš).

Susceptibility of isolates to antibiotics

Susceptibility testing was performed according to the standardized EUCAST protocol v 5.0 (18). Briefly, *Pseudomonas* isolates were precultured on Blood agar at 37 °C for 24 h. Bacterial suspensions of both clinical and reference strains were prepared in 0.85% NaCl (w/w) and adjusted to McFarland 0.5 turbidity standards. Prepared suspensions were used for inoculation of the Mueller Hinton agar plate's surface, afterwards the antibiotic discs (piperacillin-tazobactam (TZP), ampicillin (AMP), imipenem (IMP), meropenem (MER), cefepime (CFPM), ceftazidime (CFT), ciprofloxacin (CPR), amikacin (AMK) and gentamicin (GEN)) were placed on the inoculated agar surface. After the incubation period of 24 h at 37 °C, the inhibition zones around the discs were measured and interpretation of the inhibition zone values (S-sensitive / R - resistant) was based on the EUCAST v 5.0 criteria.

Used microorganisms

Strains used in this study were isolated from different human material (urine sample, skin, throat, eye, ear and wound swab) and identified as *P. aeruginosa* by cultural and biochemical characteristics. Total of 15 isolated *P. aeruginosa* strains of different origin were stored at the Microbiology laboratory (Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš). Cultures were maintained on Nutrient agar (NA) at optimal temperature (37 °C).

For experiments, bacterial strains were grown on NA for 18 h and used for making suspensions in physiological saline (0.85% NaCl), corresponding to 0.5 McFarland turbidity, which were further used for inoculation of the broth in microtiter plates.

Anti-biofilm assay

Biofilm producing ability of the isolated strains under static conditions was tested using crystal violet (CV) method according to Stepanović *et al*. (19) with the slight modifications. All isolates were grown as biofilms in wells of polystyrene flat-bottomed 96-well microtiter plates. Briefly, wells of the each microtiter plate were filled with 200 µl of Tryptone Soy Broth (TSB) containing 0.5% glucose. The initial cell suspension was standardized to 0.5 McFarland turbidity using densitometer (DEN-1, Biosan) and used for the plate inoculation in the adequate volume to achieve the final cell concentrations of ~ 10^6 CFU/ml (colony forming units/ml). The inoculated plates were further incubated for 24 h at 37 °C. After the incubation period, well content was gently aspirated, washed twice with PBS (pH =7.4), dried and stained with 0.5% CV (20 minutes). Stained plates were washed and wells were filled with 250 µl of 96% (v/v) ethanol; 45 min later, 100 µL of the distaining solution was transferred into new microtiter plate and the absorbance of the solutions was measured at 595 nm using an ELISA reader (Multiscan Ascent, Labsystems, Finland). Strains were, according to their biofilm producing ability, differentiated into one of the following groups: non, weak, moderate or strong biofilm producer (19).

The effect of different concentrations of the two tested essential oils on 15 different *P. aeruginosa* strains biofilm production was done using to the same procedure. The essential oils were dissolved in dimethylsulfoxide (DMSO, 10%) and serially diluted in wells (40.0-10.0 mg/ml). The highest concentration of DMSO in wells was 5%. Each well was inoculated with the suspensions, incubated for 24 h and biofilm quantification was done as described previously.

Statistical analysis

All experiments were done in triplicate and repeated three times. Data are given as average values ± standard deviations. Statistical comparison was done using One 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

The results of the isolated strains susceptibility to the wide antibiotic spectrum and their biofilm producing ability are presented in Table 1. Antibiogram assay showed that even five isolated strains (1/3 of the total number of isolates) are resistant to multiple drugs, while three of them demonstrated susceptibility to all tested antibiotics (Table 1). After biofilm production analysis it was determined that two strains are strong biofilm producers, eleven moderate producers, while only one strain did not displayed biofilm producing ability (Table 1). After the treatment with sage and basil essential oils different concentrations, biofilm production of both strong (strains No. 1 and 2) and weak biofilm producers (No.14) was significantly affected only in the case of sage essential oil (Figure 1). On the other hand, these strains showed to be resistant (produced the same amount of biofilm as control or the reduction was not significant) to the effects of basil essential oil. Strains No. 1 and 2 are isolates from the throat swabs, classified as strong biofilm producers. Skin swab isolate No. 14, classified as weak biofilm producer, also showed the biofilm production decrease when the sage essential oil was applied (reduction to 64%), while it was proven to be resistant to the basil essential oil. It is interesting to mention that the same strain was sensitive to all antibiotics (Table 1).

Out of eleven moderate biofilm producers, originated from urine sample, wound, eye and ear swabs, 81.8% showed reduction of biofilm/susceptibility when treated with sage essential oil, while 63.6% demonstrated reduced production of biofilm after the treatment with basil essential oil. Some of the isolated strains showed sensitivity to both oils. Biofilm producing ability of isolated strain No. 3 showed significant ($p<0.001$) reduction of 40-49% when the sage essential oil was present in the medium, while isolate No. 4 showed resistance to the action of this essential oil. On the other hand, *O.*

Table 1. Antibiogram (resistance patterns) of isolated strains to wide spectrum of antibiotics according to EUCAST criteria and their biofilm producing ability.

	Material used for isolation	AMK	GEN	CFPM	CFT	IMP	MER	CPR	TZP	AMP	Biofilm producing ability
1	Throat swab	S	S	S	S	S	S	S	S	S	Strong
2	Throat swab	S	S	S	S	S	S	S	S	S	Strong
3	Urine sample	R	R	R	R	R	R	R	S	R	Moderate
4	Urine sample	S	R	S	S	S	S	R	S	S	Moderate
5	Urine sample	S	S	S	S	S	S	S	S	S	Moderate
6	Urine sample	R	R	R	R	R	R	R	R	R	Moderate
7	Urine sample	R	R	R	R	R	S	R	R	R	Moderate
8	Eye swab	S	S	S	S	S	S	S	S	S	Moderate
9	Eye swab	S	S	R	R	R	R	S	R	R	Moderate
10	Wound swab	S	S	S	S	S	S	S	S	S	Moderate
11	Wound swab	S	S	S	S	S	S	S	S	S	Moderate
12	Wound swab	S	S	S	S	S	S	S	S	S	Moderate
13	Ear swab	S	R	R	R	R	S	S	S	S	Moderate
14	Skin swab	S	S	S	S	S	S	S	S	S	Weak
15	Skin swab	S	S	S	S	S	S	S	S	R	Non producer

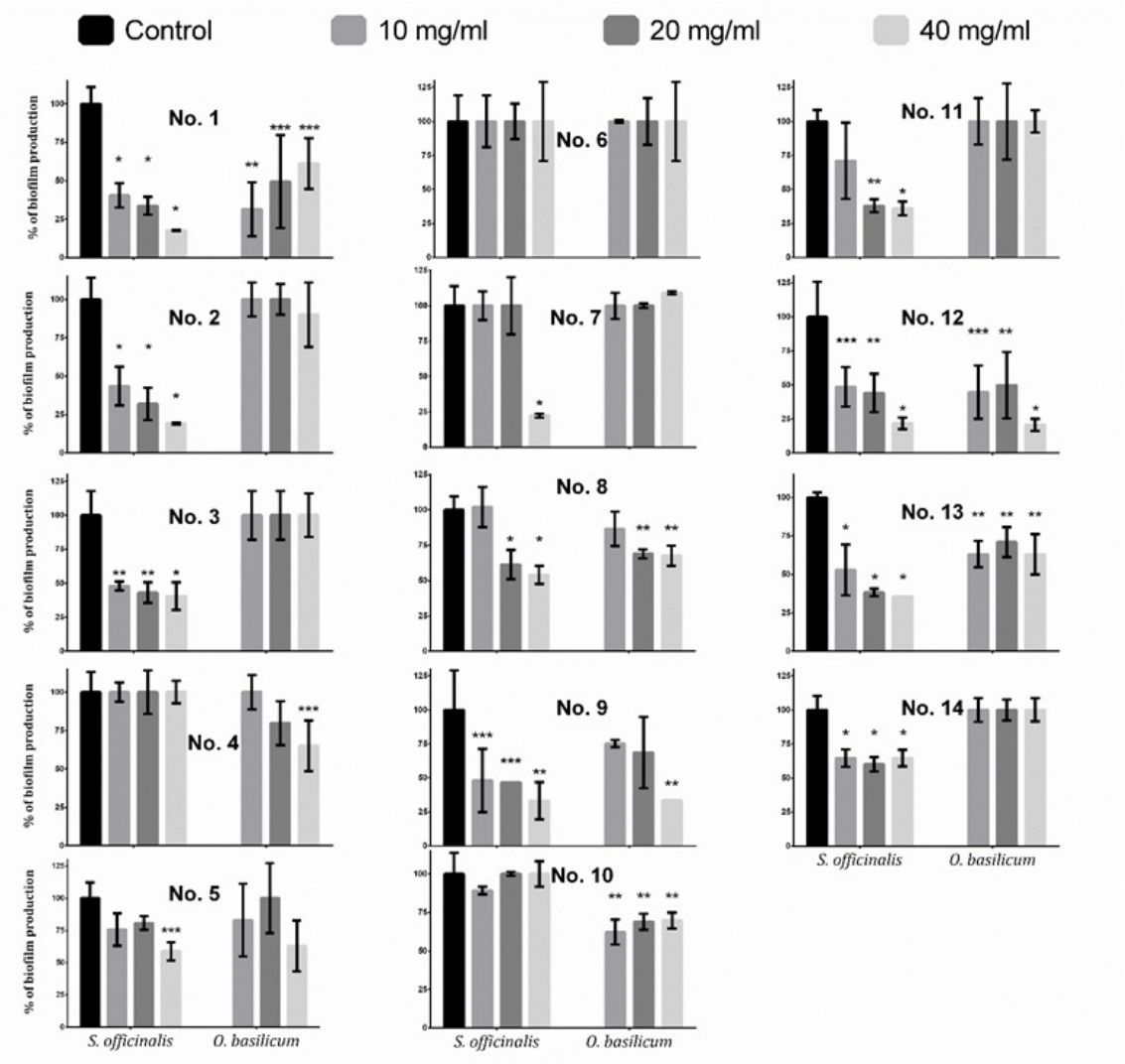

Figure 1. Reduction of *Pseudomonas aeruginosa* biofilm production by *Salvia officinalis* and *Ocimum basilicum* essential oils. Data are given as mean ± SD. Analysis was done using One Way ANOVA followed by Tuckey post hoc test. * $p<0.001$; ** $p<0.01$; *** $p<0.05$ vs. control.

basilicum essential oil reduced biofilm production of the strain No. 4 by 20 and 35%, respectively, at concentrations of 20 and 40 mg/ml. The production of biofilm of isolated strain No. 5 was affected by both oils up to 43% (basil) and 19-42% (sage), but statistical analysis showed significance only in the case of the highest concentration of sage (40 mg/ml, reduction of 42%, $p<0.05$). Two multi drug resistant isolates, No. 6 and 7 demonstrated low sensitivity towards the action of both essential oils. In the case of strain No. 6, both basil and sage oils failed to reduce biofilm production. Biofilm production of the isolated strain No. 7 was only affected by the sage essential oil and significantly reduced it for 78%, however this effect was noted only at the highest tested concentration.

On the other hand, in the case of other isolates (No.

8, 10, 11, 12 and 14) which showed good susceptibility to antibiotics, sensitivity and reduction of biofilm after the treatment with both essential oils was not negligible also (Figure 1). In the case of No.8, high significance ($p<0.001$) was found for the two higher tested concentrations of sage essential oil, but was also significantly sensitive to the same concentrations of basil oil ($p<0.05$). Isolate No. 9, obtained from eye swab, classified as moderate producer but resistant to four antibiotics, showed fair sensitivity to the action of both tested oils (up to 67% reduction for both oils), which was not the case with strain No. 10, which was sensitive to basil and resistant to sage essential oil. Among all tested strains, the most prominent activity of both oils can be seen in the case of isolate No. 12 (moderate biofilm producer from wound, sensitive to all antibiotics), where the reduction of biofilm with sage oil ranged 50 to 80%, while the reduction noted after basil oil treatment ranged from 52 to 79% (Figure 1). However, when statistical significance (and not percentage reduction only) is taken into account, strain No. 13 (multi-drug resistant isolate from ear swab) showed the highest sensitivity, which was observed for both applied essential oils.

Discussion

Pseudomonas aeruginosa is known as very challenging organism in view of its control by both antibiotics and other chemical agents. Its resistance is connected with many factors, among which the most important ones are low cell wall permeability, efflux pumps responsible for the efficient removal of antibiotic molecules and genetic base for many resistance mechanisms, associated with their ability to produce biofilms (20). It is known that biofilms can be up to 100 times more resistant in comparison to the planktonic cells of the same strain. Due to its very high resistance (even in planktonic state), herein tested oils concentrations were very high, in order to reveal potential anti-biofilm action. We found that sage essential oil exhibited higher activity, even against biofilms of multi-drug resistant strains (No. 3, 7, 9 and 13) and strong biofilm producers (strains No. 1 and 2) which was not the case with basil essential oil (except for the strain No.13 whose biofilm was significantly reduced by this oil). The two strong biofilm producers (No. 1 and 2) showed high reduction of formed biofilm after the treatment with sage essential oil, which justifies traditional usage of this plant species for the treatment of throat infections, since we found that all tested concentrations exhibited significant reduction of the biofilm (the concentration of 10 mg/ml reduced biofilm to 50%). Isolated strains No. 3, 6 and 7 were multi-drug resistant and therefore, the fact that they showed some sensitivity highlights the significance of the obtained efficiency of the sage essential oil. Previously, it was determined that the essential oils of the three sage species (*Salvia fruticosa*, *Salvia officinalis* and *Salvia sclarea*) decreased tetracycline efflux and decreased the expression of *tet*(K) gene in tetracycline resistant clinical isolates of *Staphylococcus epidermidis* (21). The sage essential oil composition is known to be relatively constant, since many studies on its chemical composition are performed and three compounds were mostly found to be the dominant ones (α-thujone, camphor and 1,8-cineole) (22-24). These terpenoids are known for their high and wide spectrum antimicrobial potential (25). Monoterpenes are known to exert antimicrobial effect by a perturbation of the lipid fraction of microorganism plasma membrane (owing to their hydrophobicity), resulting in alterations of the membrane permeability and consequential leakage of intracellular materials (26). These effects might be only partial, but they allow penetration of the compounds into the cells and contact with intracellular target places. Previously, essential oil of basil was found to possess linalool, methyl chavicol, eugenol, thymol and *p*-cymene as the dominant compounds (27-29). These compounds were also found to possess antimicrobial efficacy (30-32), and in most cases, mode of action was found to be the same as for the previously mentioned terpenes - cell wall damaging effect and alteration of the permeability (16, 33, 34).

Several studies evaluated the activity of various plant metabolites against *P. aeruginosa* biofilm production. Essential oils containing terpenes such as carvacrol and thymol as the major compounds (*Thymus sipyleus* Boiss. subsp. *sipyleus* Boiss. var. *davisianus* Ronniger, *Origanum vulgare* subsp. *hirtum*, *Melaleuca alternifolia*) were found to significantly reduce *P. aeruginosa* formed biofilms (6-8). This was further confirmed by evaluating these two compounds, where they exhibited very significant anti-biofilm potential (35), affecting both adherence and biofilm forming ability of *P. aeruginosa*. In addition to the structural alterations of the cell membrane, it was found that essential oil's constituents alter QS mechanisms in *P. aeruginosa* biofilms. Clove (*Syzygium aromaticum* L.) essential oil, rich in monoterpenes thymol, carvacrol and eugenol was proven to modify QS and biofilm formation of *P. aeruginosa* PAO1, where the enzyme activity, production of piocyanin, cell mobility and exopolysaccharide concentrations were reduced in a dose dependent manner (36). Similar results were obtained for the effects of eugenol in the study of Zhou *et al.* (37).

When analyzing the results obtained for the tested bacterial isolates antibiotic resistance and their sensitivity to essential oils, one can notice that the strains showing resistance to basil essential oils also showed resistance to gentamicin. Gentamicin belongs to the aminoglycoside group of antibiotics, whose mechanism of action is inhibition of protein synthesis by binding to 30S ribosomal subunit (20). It is known that this antibiotic binds to the A site of the ribosomal subunit (38), and therefore, it can be hypothesized that some active compounds of this oil have, at least partially, this mechanism of action that might act in synergistic manner with the cell wall-targeted mechanism of other compounds. Meropenem and imipenem are members of a broad spectrum antibiotics belonging to carbapenems (class of β lactams), with the mechanism of action that affects cell wall synthesis. In this study, isolated strains showed different sensitivity to these two antibiotics, which can be explained by different sites of these two molecules' penetration into the cell. It is known that cell wall of *P. aeruginosa* represents significant barrier for the various antibiotics penetration, where small molecules including β lactam antibiotics and quinolones can cross only through very small channels, porins. *Pseudomonas*

aeruginosa possess various porins, among which oprD is the site of imipenem passage, which is proven since the loss of this channels cause imipenem resistance (20, 39). On the other hand, resistance to meropenem was not noted in the same experiments (oprD mutant cells of *P. aeruginosa*), which pointed to the fact that meropenem has different site/mechanism to enter the cells.

Previously mentioned results, together with those obtained from this study, highlight the importance of these (mono) terpenes and terpenoid compounds derived from plants, in the control of *P. aeruginosa* biofilm formation. It is known that biofilms exert higher resistance in these states, due to many factors such as reduced mobility of active compounds, inactivation and reduction of penetration into the biofilms dipper layers and persister cells (40). Therefore, the fact that the natural compounds capable to enter, affect metabolism and reduce biofilm development, provides promising and optimistic information in the light of microbial communities' control. Further studies should be done in order to reveal their exact mechanism of action and to use them it in control of such microbial structures. Our data point to the fact that commercial essential oils rich in terpenes and terpenoids altered biofilm production of the majority of the tested *P. aeruginosa* (multi) drug resistant clinical isolates. Therefore, both essential oils, especially sage, which showed significantly higher activity might be considered as a natural and non-harmful agents for the treatment of chronic infections caused by *P. aeruginosa* biofilms.

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