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ANTIBIOFILM ACTIVITY OF *Lamium album* L. EXTRACTS

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ABSTRACT. In this study, the antibiofilm activity of ethanol, acetone, and ethyl acetate extracts of *Lamium album* was evaluated against nine clinical strains from human wound swabs and two reference strains. The extracts of *L. album* exhibited significant antibiofilm activity. The extracts inhibited cell attachment and subsequent biofilm formation. The activity was dose- and strain-dependent, which was confirmed by fluorescence microscopy. The effects on mature biofilms were lower. In addition, the results showed a decrease in bacterial auto-aggregation in the presence of the extracts and no motility reduction. Phytochemical analysis and identification of functional groups by using Fourier Transform Infrared Spectroscopy were also performed. The results obtained in this study provide a basis for further research on this plant species.

Keywords: biofilm, antibiofilm activity, auto-aggregation, phytochemical analysis, clinical bacteria strains, *Lamium album.*

INTRODUCTION

Although bacteria are unicellular organisms, they often show social behavior in the form of biofilm (EL-BASHITI *et al*., 2019). Bacterial biofilm is a three-dimensional community of bacteria, embedded in a self-producing matrix of extracellular polymeric substances (EPS) mainly composed of extracellular DNA, polysaccharides, and proteins (FLEMMING *et al*., 2016). The formation and development of biofilm is a complex process involving four main stages: bacterial attachment to a surface, microcolony formation, biofilm maturation, and dispersal of bacteria which may then colonize new surfaces (AMANKWAH *et al*., 2021). Biofilm-associated cells can adhere irreversible on a wide variety of surfaces, including living tissues and indwelling medical devices. Bacteria within the biofilm exhibit phenotypes that are distinct from free-living cells, so in this form, the bacteria are up to 1,000 times more resistant to antimicrobial agents. Since they are highly resistant to antibiotics and the host immune system, biofilms are regarded as a significant virulence factor that causes persistent, recurrent infections. This makes biofilms a major public health problem as ~80% of all bacterial infections in humans are caused by biofilms (VERDEROSA *et al*., 2019). The growing

biofilm resistance to current treatments increases the need for alternative control strategies. Finding naturally occurring plant compounds that can prevent the production of biofilms is a promising alternative. It is known that some biomolecules from plants can disturb and prevent the attachment of bacterial cells and the formation of biofilms (SAKARIKOU *et al*., 2019; ĐUKANOVIĆ *et al.*, 2020; OSUNGUNNA, 2022).

Lamium album L. is an herbaceous, perennial, flowering plant from the family Lamiaceae. It is a cosmopolitan species, native to Europe and Asia. The common name "white dead-nettle" refers to their resemblance to the unrelated species *Urtica dioica* L. (Urticaceae) known as “stinging nettle”, but unlike *U. dioica*, *L. album* does not have stinging hairs on leaves and stems (YALÇIN and DUYGU, 2006). White dead-nettle expresses a wide range of therapeutic effects, which are linked to various biologically active substances, such as flavonoids, terpenes, phenolic acids, iridoids and essential oil (SHAH *et al*., 2019). In folk medicine, aerial parts (“herba et flos”) of *L. album* are used for lowering high blood cholesterol and glucose concentration, as a remedy in wound healing, menorrhagia and uterine hemorrhage (YALÇIN and DUYGU, 2006).

Considering the above, this study aimed to determine the amounts of total phenolic compounds, phenolic acids, flavonoids, and proanthocyanidins in the ethanol, acetone, and ethyl acetate extracts of *L. album*. The additional aim was to investigate, for the first time, the antibiofilm activity of these extracts on selected bacterial strains as well as their effect on bacterial motility and auto-aggregation.

MATERIALS AND METHODS

Plant material

Fresh and healthy aerial parts of *L. album*, growing wild in Ravna Reka, Central Serbia, were collected in May 2020. Identification and classification of the collected plant were performed at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia. A voucher specimen was deposited in the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia. Plant material was dried in a shadow and then grounded into a powder.

Preparation of plant extracts

Plant material was extracted with ethanol, acetone, and ethyl acetate applying ultrasound-assisted extraction. Ultrasound-assisted extraction was performed using an ultrasonic bath (Vabsonic, Niš, Serbia). In brief, 26 g of the plant material per extract was soaked with 800 mL of each solvent in glass bottles and exposed to an ultrasonic frequency of 40 kHz at 35°C for 20 minutes. Further, for better exhaustion, the mixture of plant material and solvent was stored in a dark place for 24 hours. After extraction and filtration, a rotary evaporator (RV10 basic, IKA, Bensheim, Germany) was used to evaporate the solvent from each filtrate under low pressure and low temperature (40°C). The dry extracts were stored at -20°C until use. For phytochemical analysis, a stock concentration of plant extracts (1 mg/mL) was prepared by dissolving in methanol (>99.8%). For biological assays, a stock concentration of plant extracts was prepared by dissolving in dimethyl sulfoxide (DMSO, >99.8%) and then diluting with a nutrient broth to achieve 10% DMSO (5% DMSO and lower, final).

Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy is used to identify the types of chemical bonds (functional groups) present in the extracts based on the peak values in the infrared radiation region. The wavelength of the absorbed light is characteristic of the attraction,

which can be seen across the entire spectrum. A small amount of dry powders of ethanol, acetone, and ethyl acetate extracts were, separately, encapsulated in a KBr pellet, to prepare translucent sample disks. The samples were examined using an FT-IR spectrometer (PERKIN ELMER SPECTRUM 2, IR, Waltham, USA) with a scanning range from 450 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

Phytochemical analysis

The total phenolic content of the extracts was determined using the Folin-Ciocalteu's method as described by WOOTTON-BEARD *et al*. (2011). Gallic acid (Sigma Aldrich, St. Louis, USA) was used as the standard and the total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract (mg GAE/g of extract).

The total phenolic acid content was determined using Arnow's reagent as described in GAWLIK-DZIKI, 2012. The standard utilized was caffeic acid (Sigma Aldrich, St. Louis, USA), and the total phenolic acid content was expressed as milligram of caffeic acid equivalent (CAE) per gram of extract (mg CAE/g of extract).

The aluminium chloride method, as reported by QUETTIER-DELEU *et al*. (2000), was used to quantify the total flavonoid content in the extracts. Rutin (Sigma Aldrich, St. Louis, USA) was used as the standard and the concentration of flavonoids was expressed as milligrams of rutin equivalents (RUE) per gram of extract (mg of RUE/g of extract).

The total proanthocyanidin content was measured by the butanol-HCl method with ferric ammonium sulfate as a catalyst as described by PORTER *et al*. (1986). The standard utilized was cyanidin chloride (Sigma Aldrich, St. Louis, USA), and the amount of total proanthocyanidins was expressed as milligrams of cyanidin chloride equivalents (CChE) per gram of extract (mg CChE/g of extract).

Bacterial strains

In this study, nine clinical strains from human wound swabs and two reference strains were used. During 2020, four laboratory-confirmed strains of the Gram-positive bacterium *Staphylococcus aureus* (S1, S2, S3, and S4) and five laboratory-confirmed strains of Gram-negative bacteria *Proteus* spp. (Pr1) and *Pseudomonas aeruginosa* (PA1, PA2, PA3, and PA4) were obtained from the Microbiology Laboratory of Hospital in Paraćin, Serbia. Reference strains from American Type Culture Collection (ATCC), *S. aureus* ATCC 25923 (SS) and *P. aeruginosa* ATCC 10145 (PAS), were purchased from Sigma Aldrich, St. Louis, USA. The bacterial strains were kept in 20% glycerol stock at -80°C. Prior to the assay, the bacterial strains were subcultured on Nutrient agar (Torlak, Belgrade, Serbia), twice, at 37°C for 18 hours. Based on our earlier assay, all tested strains are capable to form biofilms *in vitro*.

Determination of minimum inhibitory concentrations

The minimum inhibitory concentration (MIC) of *L. album* extracts was determined using the broth microdilution method (CLSI, 2012). Twofold serial dilutions of plant extracts were prepared in sterile 96-well polystyrene non-treated microtiter plates containing 100 μL of Mueller-Hinton broth (HiMedia Laboratories, Mumbai, India) per well. The tested concentration range was from 0.156 mg/mL to 10 mg/mL. Bacterial suspensions were prepared by a direct colony suspension method and adjusted to 0.5 McFarland turbidity standard using a densitometer (DEN-1, BIOSAN, Riga, Latvia). Ten microliters of adjusted 1:20 diluted suspension of each bacterial strain was added to respective wells to achieve a suspension concentration of approximately 5×10^5 colony-forming units (CFU)/mL per well. The inoculated plates were incubated at 37°C for 20 hours. The growth of the bacteria was monitored by adding resazurin (Alfa Aesar GmbH & Co., Karlsruhe, Germany), the indicator

of microbial growth. Resazurin is a blue, non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by viable cells. MIC was defined as the lowest concentration of tested plant extracts that prevented resazurin color change from blue to pink (SARKER *et al*., 2007). Each experiment included growth control (broth + bacterium), sterility control (broth + extract) and solvent control (5% DMSO and lower). The antibiotic tetracycline (Sigma-Aldrich Co., St. Louis, USA) was used as a positive control.

Determination of antibiofilm activity

Inhibition of biofilm formation

The effect of *L. album* extracts on biofilm formation was evaluated in 96-well flat-bottomed polystyrene tissue culture (TC) - treated microtiter plates following a crystal violet assay as described in STEPANOVIĆ *et al*., 2007. Tryptic soy broth (TSB) (Torlak, Belgrade, Serbia), supplemented with additional glucose to a final concentration of 1%, was used as a nutrient broth in the amount of 100 μL per well. A 100 μL of stock concentration of each plant extract was transferred into wells in the first row of microtiter plates. Then, twofold serial dilutions of plant extracts were prepared resulting in a decreasing concentration range (20 - 0.312 mg/mL). Finally, 10 μL of bacterial suspensions, adjusted to a turbidity of 0.5 McFarland standard was added to the respective wells. The inoculated microtiter plates were incubated at 37°C for 20 hours. After incubation, the content of each well was gently removed by pipetting and wells were washed with 200 μL of sterile distilled water, two to three times, to remove free-floating bacteria. To fix the biofilms, microtiter plates were exposed to hot air at 55°C for 15 minutes, and then the biofilms were stained by adding 100 μL of an aqueous solution of crystal violet (0.1% w/v) (Fisher Scientific, Geel, Belgium). After 15 minutes of incubation at room temperature, the excess stain was rinsed off by pipetting with distilled water, three times, then microtiter plates were dried in an inverted position and 100 μL of 10% acetic acid (v/v) was added to release the dye from the cells. Gentle pipetting was performed to mix the content properly. The optical densities (OD) of samples were measured at 550 nm using an ELISA plate reader (RT-2100C, Rayto, Shenzhen, China). Each experiment included growth control (broth + bacterium), extract control (broth + extract), broth control (broth only) and solvent control (5% DMSO and lower). The percentage of biofilm inhibition was calculated using the following formula presented by ALI *et al*. (2021):

$$\% \text{ Inhibition} = \frac{(OD_{GC} - OD_B) - (OD_S - OD_{EC})}{(OD_{GC} - OD_B)} \text{ x } 100 \quad (1)$$

where OD_{GC} is the OD value of the growth control, OD_B is the OD value of the broth control, OD_S is the OD value of the sample and OD_{EC} is the OD value of the extract control.

Effect on metabolic activity of biofilm

The effect of *L. album* extracts on biofilm metabolic activity was tested by using a resazurin dye test (ALONSO *et al*., 2017). Nutrient broth (TSB with 1% glucose), bacterial suspensions and twofold dilutions of plant extracts (20 – 0.312 mg/mL) were prepared in 96-well flat-bottomed polystyrene TC-treated microtiter plates in the same way as previously described. After incubation at 37°C for 20 hours, the content of each well was gently removed and wells were washed, two to three times, with sterile distilled water. Subsequently, in order to evaluate the metabolic activity, 100 μL of sterile nutrient broth and 10 μL of an aqueous solution of resazurin (0.05% w/v) were added to the wells, and the plates were incubated further for 2 hours at 37°C. After incubation, the lowest concentration of tested plant extracts that prevented resazurin color change from blue to pink was determined as biofilm metabolic

inhibitory concentration (BMIC). Each experiment included growth control (broth + bacterium), sterility control (broth + extract) and solvent control (5% DMSO and lower).

Inhibition of cell attachment

L. album extracts were tested for their potential anti-adsorption properties at concentrations from 0.312 mg/mL to 10 mg/mL. The bacterial suspension (10 µL) adjusted to a turbidity of 0.5 McFarland standard was added into 100 µL of nutrient broth (TSB with 1% glucose) with the presence of different extracts' concentrations (twofold serially diluted). Next, the inoculated microtiter plates (96-well flat-bottomed TC-treated) were incubated for 4 hours at 37°C to allow cell attachment and initial biofilm development. Following incubation, the washing, fixing, and staining procedure was carried out as described previously. The OD values of samples were measured at 550 nm using an ELISA plate reader. Each experiment included growth control (broth + bacterium), extract control (broth + extract), broth control (broth only) and solvent control (5% DMSO and lower). The percentage of inhibition of cell attachment was calculated using the formula (1).

Inhibition of formed biofilm

To form mature biofilms, 20 µL of bacterial suspension (0.5 McFarland turbidity standard) of each strain was inoculated in 180 µL of TSB with 1% of glucose in 96-well flat-bottomed polystyrene TC-treated microtiter plates and incubated at 37°C for 20 hours without adding extracts. After incubation, free-floating bacteria were removed and wells with formed biofilms were rinsed. Then, formed biofilms were treated with 100 µL of plant extracts at varying concentrations (20, 10, 5, 2.5 mg/mL) and incubated further for 24 hours. The washing procedure was repeated, and the formed biofilms were fixed. The biofilms were stained according to the method previously described. The OD values of samples were measured at 550 nm using an ELISA plate reader. Each experiment included growth control (broth + bacterium), extract control (broth + extract), broth control (broth only) and solvent control (5% DMSO and lower). The percentage of reduction of biofilm biomass was calculated using the formula (1).

Effect on auto-aggregation

The auto-aggregation behavior of *S. aureus* (S1, S2), *Proteus* spp. (Pr1) and *P. aeruginosa* (PA2, PA3) in the presence of *L. album* extracts were evaluated (WANG *et al*., 2021). Briefly, 1 mL of bacterial suspension (2.0 Mc Farland standard) in phosphate-buffered saline (PBS) (Fisher Scientific, Geel, Belgium) with 1 mL of tested plant extract (final concentration of 1 mg/mL, in PBS) was incubated at 37°C for 1 hour. After centrifugation at 5000 rpm for 10 minutes, the supernatant was discarded, the pellet was washed and resuspended in 5 mL of PBS. In addition, the auto-aggregation behavior of bacteria, without extracts, was determined as a control. The OD value of each sample was measured at 600 nm, immediately (0 h of incubation) after being vortexed for 30 s (OD_0) and after 4 h of incubation at 37°C (OD_1). Blank was a PBS solution. The percentage of auto-aggregation was calculated according to the following equation:

$$\% \text{ AA} = \frac{(OD_0 - OD_1)}{OD_0} \text{ x } 100 \qquad (2)$$

Effect on bacterial motility

The swimming and swarming motility of *Proteus* spp. (Pr1) and *P. aeruginosa* (PA1, PA2, PA3, PA4) were evaluated with semisolid agar supplemented with or without *L. album* extracts in 6-well microtiter plates. Stock solutions of the plant extracts (10-fold) were dissolved in DMSO and then diluted in PBS to achieve 10% DMSO (1% DMSO final). For swimming motility, 0.3% agar containing 10 g/L of tryptone and 5 g/L of NaCl was used. LB medium supplemented with 5 g/L of glucose and 7 g/L of agar was used for swarming motility. The plant extracts were added to the motility agar to achieve the final concentrations of 1 mg/mL and 5 mg/mL, separately. Media plates were inoculated with 1 µL of bacterial suspension (0.5 McFarlan standard) by stabbing into the center of the agar for swimming motility, and with 1 µL of suspension by adding it on the agar surface for swarming motility (O'MAY and TUFENKJI, 2011). Following incubation at 37°C for 20 hours, the zone diameters (mm) of bacterial migration from the point of inoculation were measured and compared with the control.

Fluorescence microscopy

Antibiofilm activity of *L. album* extracts, in terms of biofilm formation inhibition, was validated by microscopic visualization using a fluorescence microscope (NIKON inverted fluorescence microscope, Ti-Eclipse). The *S. aureus* S2 strain was used as a model strain. The bacterial suspension adjusted to the 0.5 McFarland standard was treated with different extracts' concentrations (0.312 mg/mL to 10 mg/mL). After incubation at 37°C for 20 hours, the wells were washed and attached cells were fixed and stained with 10 µL of an aqueous solution of acridine orange (0.05% w/v) (Fisher Scientific, Geel, Belgium) for 1 min in dark condition and visualized at 400 × magnification.

RESULTS AND DISCUSSION

Fourier Transform Infrared Spectroscopy

The FTIR spectrum is used to recognize the functional groups of the active components present in the extract based on the peak values in the region of IR radiation. The results of FTIR analyses are presented in Figure 1 and Table 1. The peaks were detected in the single bond area (2500-4000 cm^{-1}), the double bond area (1500-2000 cm^{-1}**)**, and the fingerprint region (600-1500 cm^{-1}) while no peaks were detected in the triple bond region (2000-2500 cm^{-1}). The results confirmed the presence of various chemical constituents such as aliphatic alkenes, carboxylic acids, lactones, ketone compounds, amides, aromatic compounds, polysaccharides, P-, S-, Cl-derivatives (HEMMALAKSHMI *et al*., 2017; NANDIYANTO *et al*., 2019).

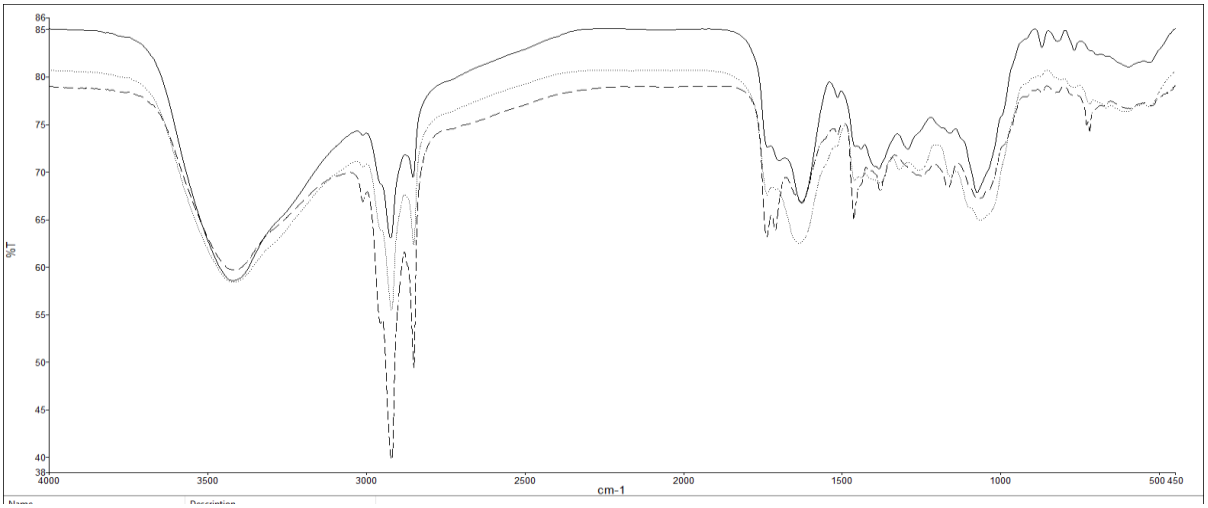

Figure 1. FTIR spectra of *L. album* extracts.
(line - ethanol extract; dash - acetone extract; dot - ethyl acetate extract).

Table 1. Functional groups in *L. album* extracts detected by FTIR spectroscopy.

No	Ethanol extract	Acetone extract	Ethyl acetate extract	Functional group/assignment
	wavenumber (cm^{-1})			
1.	3421	3421	3420	Hydroxy group, H-bonded OH stretch
2.	3011	3011	3011	C–H stretching
3.		2956		Methyl C–H stretching (CH_3)
4.	2924	2920	2920	Methylene C–H stretching (CH_2)
5.	2853	2850	2851	Methylene C–H stretching (CH_2)
6.	1735	1738	1736	Six-membered ring lactone
7.	1698	1711		Carbonyl group, C=O stretching
8.	1629	1627	1637	C=C stretching, Amide
9.	1515	1515		C=C stretching, Aromatic ring
10.	1441	1463	1461	C–H bending
11.	1384	1379	1380	C–H bending
12.	1293		1320	C–H bending
13.		1246	1258	P=O stretching, C–O stretching
14.	1160	1168	1160	C–O stretching, H-bonded stretching
15.	1075	1074	1067	Hydroxy group, C–O stretching
16.	870	872		C–H bending
17.	823	832		Aromatic ring
18.	768		767	Aromatic ring
19.		729		Aromatic ring
20.		720	719	C–S stretching
21.			668	C–S stretching
22.	597	602	600	C–Cl stretching

Phytochemical analysis

The concentration of total phenolic compounds, total phenolic acids, total flavonoids, and total proanthocyanidins in *L. album* extracts were presented in Table 2. Among the studied groups of phenolic compounds, the concentration of flavonoids was highest in ethanol and ethyl acetate extracts, and the concentration of total phenolic acids was highest in acetone extract. Acetone extract had a slightly higher concentration of total phenolics, 39.37 ± 0.19 mgGAE/g, than ethanol 38.95 ± 0.19 mgGAE/g and ethyl acetate extract 29.87 ± 0.07 mgGAE/g. Acetone extract had two times higher concentrations of phenolic acids than ethanol and ethyl acetate extracts. The amount of total phenolic acids in acetone extract was 131.87 ± 3.45 mgCAE/g, while in ethanol and ethyl acetate extracts were 65.05 ± 0.48 mgCAE/g and 59.88 ± 1.33 mgCAE/g, respectively. Ethyl acetate extract has a slightly higher concentration of total flavonoids (87.93 ± 1.27 mgRUE/g) than ethanol (77.79 ± 0.42 mgRUE/g) and acetone extract (57.37 ± 0.42 mgRUE/g). Compared to other tested groups of secondary metabolites, the content of proanthocyanidins was the lowest. The concentration of proanthocyanidins in ethanol and acetone extracts were similar, while in ethyl acetate extract this group of phenolic compounds was not detected.

Table 2. Total phenolic compound (TPC), total phenolic acid (TPA), total flavonoid (TFC) and total proanthocyanidin (TPAC) content in *L. album* extracts.

Type of extracts	**TPC (mg GAE/g)**	**TPA (mg CAE/g)**	**TFC (mg RUE/g)**	**TPAC (mg CChE/g)**
Ethanol	38.95 ± 0.19	65.05 ± 0.48	77.79 ± 0.42	3.19 ± 0.32
Acetone	39.37 ± 0.14	131.87 ± 3.45	57.37 ± 0.42	3.04 ± 0.16
Ethyl acetate	29.87 ± 0.07	59.88 ± 1.33	87.93 ± 1.27	nd

The values are mean ± standard deviations of three replicates; nd-not detected.

According to MATKOWSKI and PIOTROWSKA (2006), the total phenolic compounds in the methanolic extract of *L. album* was 192.5 ± 20 mg GAE/g, while PEREIRA *et al*. (2012) reported 192.5 ± 10.3 mg GAE/g of total phenolic compounds in ethanol extract of *L. album*. The spectrophotometric analysis of methanolic extracts by DANILA *et al*. (2015), showed higher phenolic content in *L. maculatum* samples (105.86 mg GAE/g) when compared with *L. album* samples (72.63 mg GAE/g). The results of total phenolic content obtained in the study by UWINEZA *et al*. (2021), were varied from 234.17 to 650.17 mg GAE/g of extracts for the studied conditions, but it is not directly comparable to other studies of *L. album* extracts because of different extraction methods. According to CZERWIŃSKA *et al*. (2020), high-performance liquid chromatography coupled with diode-array detection (HPLC-DAD) detected iridoids (lamalbid), phenolic acids (chlorogenic acid), phenylpropanoids (verbascoside), and flavonoids (rutin, quercetin malonylhexoside, tiliroside) in aqueous and ethanolic-aqueous extracts from flowers of *L. album*. In *L. album* subsp. *album* flowers, nine phenolic acids (protocatechuic, vanillic, caffeic, syringic, gallic, gentisic, p-coumaric, and chlorogenic acids, and trace amounts of ferulic acid) were detected by the HPLC method (SULBORSKA *et al*., 2020).

Minimum inhibitory concentration of L. album extracts

To obtain the starting biofilm treatment concentrations of *L. album* extracts, MIC values of ethanol, acetone, and ethyl acetate extracts were determined. The MIC values were >10 mg/mL for all three extracts against tested bacterial strains (data not shown). The antibiotic tetracycline was active at concentrations from 0.5 μg/mL to >128 μg/mL. The solvent control did not inhibit the growth of bacteria.

CHIPEVA *et al*. (2013) examined the antimicrobial activities of 18 different extracts of *L. album* against reference bacterial strains. The lowest MIC (0.313 mg/mL) was noticed for chloroform extract from flowers towards *Enterobacter faecalis* NBIMCC 3915, *S. aureus* NBIMCC 3703 and *P. aeruginosa* NBIMCC 3700, and for ethanol extract from leaves towards *E. aerogenes* NBIMCC 3699. In the study by FATHI *et al*. (2018), a much higher inhibitory concentration was observed. Methanol extract of aerial parts of *L. album* inhibited the growth of *E. coli* at 100 mg/mL, and *Klebsiella* spp. at 150 mg/mL.

Antibiofilm activity of L. album extracts

Inhibition of biofilm formation

The antibiofilm activity of *L. album* extracts was evaluated through quantification of biofilm biomass and expressed as a percentage of inhibition of biofilm formation. The solvent control did not inhibit biofilm formation. The obtained results indicated that the effects of *L. album* extracts were dose- and strain-dependent. As shown in Figure 2, all extracts exhibited high inhibition of biofilm formation for strains of Gram-positive bacterium, and lower inhibition for strains of Gram-negative bacteria. Ethyl acetate extract showed the highest inhibition of biofilm formation for *S. aureus* (S2), from 67% to 95% at a concentration range of 0.312 mg/mL to 20 mg/mL. On the other hand, acetone extract exhibited the lowest inhibition of biofilm formation for *P. aeruginosa* (PA1) from 0% to 15%.

To our best knowledge, the antibiofilm activity of *L. album* has not been described in the literature yet, but several studies have described the effect of plants from Lamiaceae family on inhibition of biofilm formation. According to GWIAZDOWSKA *et al*. (2022), the antibiofilm activity of *Glechoma hederacea* var. *longituba* extracts exceeded 90% against *E. coli* and *Enterococcus faecalis*, whereas in the case of *P. aeruginosa* and *Bacillus subtilis* it was 88.6% and 87.9%, respectively. Antibiofilm activity of essential oils from *Origanum majorana*, *Rosmarinus officinalis* and *Thymus zygis* against 29 isolates of methicillin-resistant *S. aureus* was described by BEN ABDALLAH *et al*. (2020). The outcomes of this study indicated that *O. majorana* had the greatest antibiofilm activity (10.29 to 95.91%), succeeded by *R. officinalis* (10.20% to 95.65%) and *T. zygis* (11.67% to 91.48%). MALINOVSKÁ *et al*. (2021) evaluated the antibiofilm activity of essential oils from *Salvia officinalis*, *Thymus vulgaris*, *R. officinalis*, *Origanum vulgare* and *Hyssopus officinalis* plants against *Candida albicans* clinical isolates. The most effective were essential oils of *O. vulgare* and *T. vulgaris*.

Effect on metabolic activity of biofilm

Crystal violet staining was used for biomass quantification lacking information on biofilm viability. To overcome this, the assay with the redox dye resazurin, which was reduced to pink resorufin in the presence of metabolically active cells was used. The effect of ethanol, acetone, and ethyl acetate extracts of *L. album* on the metabolic activity of biofilms is shown in Table 3. At the tested concentration range, from 0.312 mg/mL to 20 mg/mL, ethanol and acetone extracts did not show an effect on the metabolic activity of biofilms, for all tested bacterial strains. Ethyl acetate extract inhibited the viability of *S. aureus* (S1, S2 and S4) biofilms at 10 mg/mL and *S. aureus* ATCC 25923 biofilm, at 2.5 mg/mL. The solvent control did not have a negative impact on bacteria.

Based on our previous study, ethanol extract of *L. album* inhibited the viability of *P. aeruginosa* (PA9) at 20 mg/mL, and at a concentration of 40 mg/mL inhibited the viability of *E. coli* (E16), *Klebsiella* spp. (K6 and K9) and *Proteus* spp. (P17 and P18). Acetone extract showed the most notable activity at concentrations of 5 mg/mL and 10 mg/mL for *Klebsiella* spp. (K9) and *Proteus* spp. (P18), respectively (TERZIĆ *et al*., 2021).

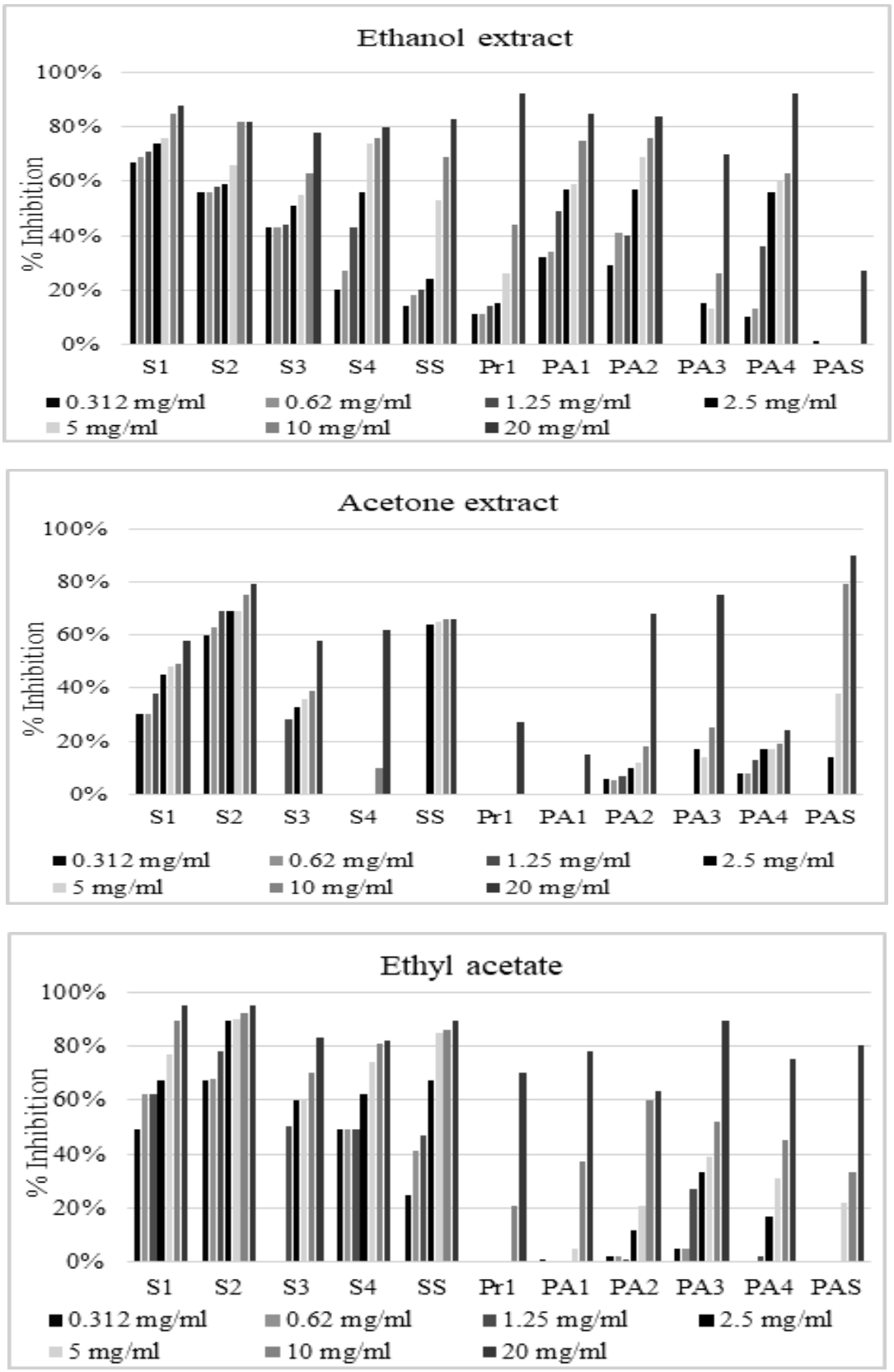

Figure 2. Inhibition of biofilm formation by different concentrations of *L. album* extracts.
(S1, S2, S3, S4, SS – *S. aureus*; Pr1 – *Proteus* spp.; PA1, PA2, PA3, PA4, PAS – *P. aeruginosa*).

Table 3. Effect of *L. album* extracts on the metabolic activity of biofilms expressed as BMIC.

Bacterial strains	Ethanol extract (mg/mL)	Acetone extract (mg/mL)	Ethyl acetate extract (mg/mL)
***S. aureus* S1**	>20	>20	10
***S. aureus* S2**	>20	>20	10
***S. aureus* S3**	>20	>20	20
***S. aureus* S4**	>20	>20	10
***S. aureus* ATCC**	>20	>20	2.5
***Proteus* spp. Pr1**	>20	>20	>20
***P. aeruginosa* PA1**	>20	>20	20
***P. aeruginosa* PA2**	>20	>20	>20
***P. aeruginosa* PA3**	>20	>20	>20
***P. aeruginosa* PA4**	>20	>20	>20
***P. aeruginosa* ATCC**	>20	>20	>20

Inhibition of cell attachment

The first step in biofilm formation is the attachment of bacterial cells to a surface. This phase is irreversible, so the biofilm is unstable, and the effect of extracts is stronger. Ethanol, acetone, and ethyl acetate extracts had great biofilm removal capacity for all *S. aureus* strains, *Proteus* spp. and two *P. aeruginosa* strains (PA1 and PA2). For other *P. aeruginosa* strains (PA3, PA4 and PAS), a lower removal capacity of tested extracts was noticed (Figure 3). The solvent control did not have a negative impact on bacteria.

As far as we know, the inhibition of cell attachment by *L. album* has not been described in literature yet. Prevention of cell attachment by methanol extracts of *Mentha piperita*, *R. officinalis* and *T. vulgaris*, plants from the Lamiaceae family, was described by SANDASI *et al*. (2011). *M. piperita* (57%), *R. officinalis* and *T. vulgaris* (40%) showed good activity against *P. aeruginosa* ATCC 9027, while only *R. officinalis* had an inhibitory effect against *Candida albicans* ATCC 10231 and clinical isolate (> 40%).

Inhibition of formed biofilm

It was observed that the established biofilm was gradually damaged upon treatment with ethanol, acetone, and ethyl acetate extracts of *L. album* (Figure 4). The maximum reduction of formed biofilm was obtained against *S. aureus* (S1), from 85% to 95% with ethyl acetate extract. Ethanol, acetone, and ethyl acetate extracts had no effect on mature biofilms established by *Proteus* spp. (Pr1) and *P. aeruginosa* (PA1, PA2 and PA3). The solvent control did not have a negative impact on bacteria.

According to our best knowledge, the reduction of formed biofilm by *L. album* has not been described in the literature yet, but several studies have described the effect of plants from Lamiaceae family. BEN ABDALAH *et al*. (2020) examined the eradication actions of *O. majorama*, *R. officinalis*, and *T. zygis* essential oils against methicillin-resistant *S. aureus* clinical isolates. The percentage of eradication was ranged from 18.31 to 98.01%, from 12.65 to 94.39%, and from 13.45 to 92.69%, respectively. The effect of ethanol and methanol extracts of *Hyssopus officinalis* on biofilm eradication was described by HASSANSHAHIYAN *et al*. (2018). Among six tested pathogenic bacteria, *P. aeruginosa* was susceptible (95.4%), while *K. pneumoniae* showed the minimum sensitivity. Disruption of established biofilm formed by six human pathogenic bacteria applying ethanol and methanol extracts of *T. vulgaris* was examined by MOHSENIPOUR and HASSANSHAHIAN (2015). Tested Gram-negative

bacteria showed low sensitivity, while *Streptococcus pneumonia* was the most sensitive (65.82%).

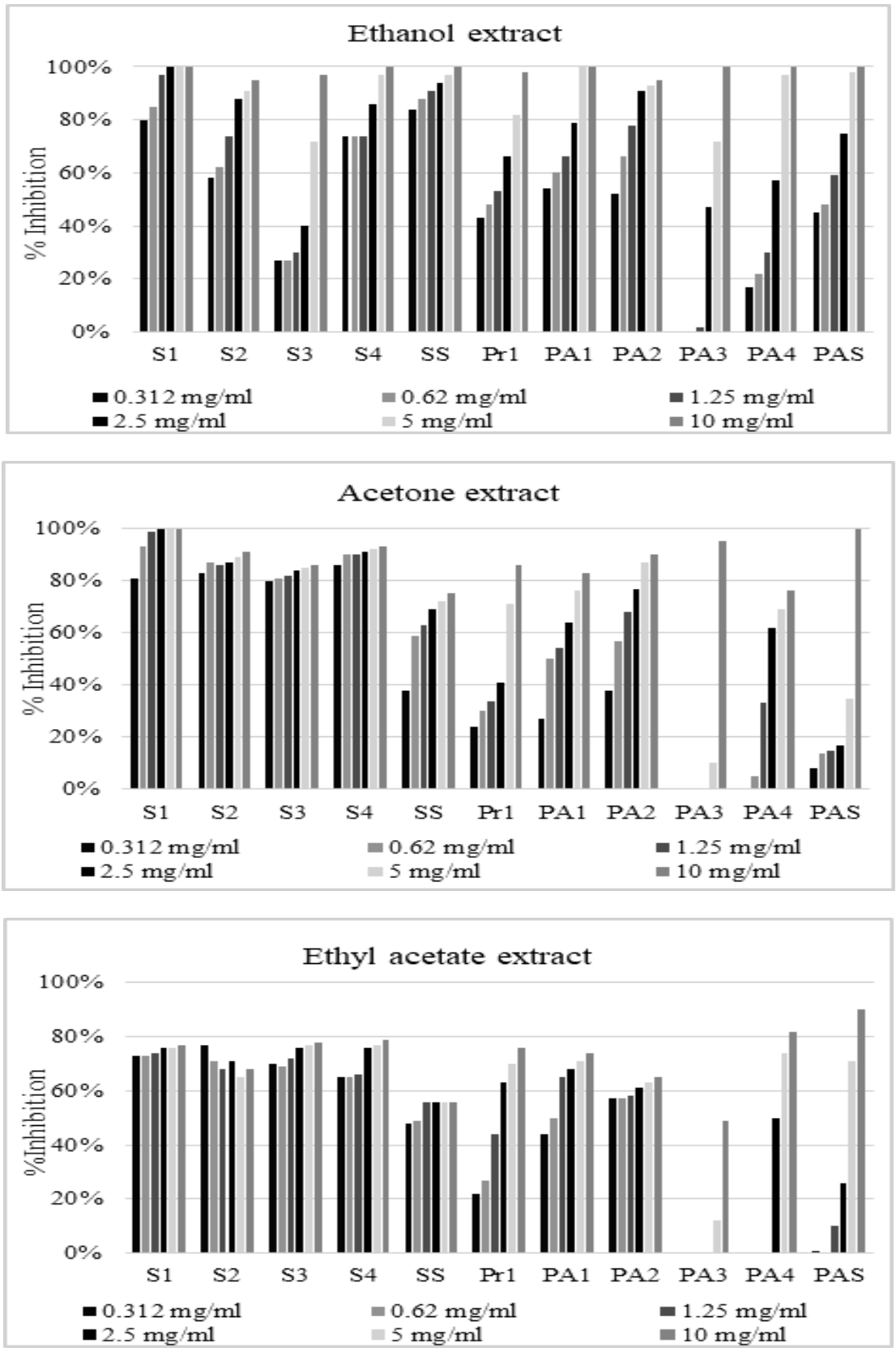

Figure 3. Inhibition of cell attachment by different concentrations of *L. album* extracts.
(S1, S2, S3, S4, SS – *S. aureus*; Pr1 – *Proteus* spp.; PA1, PA2, PA3, PA4, PAS – *P. aeruginosa*).

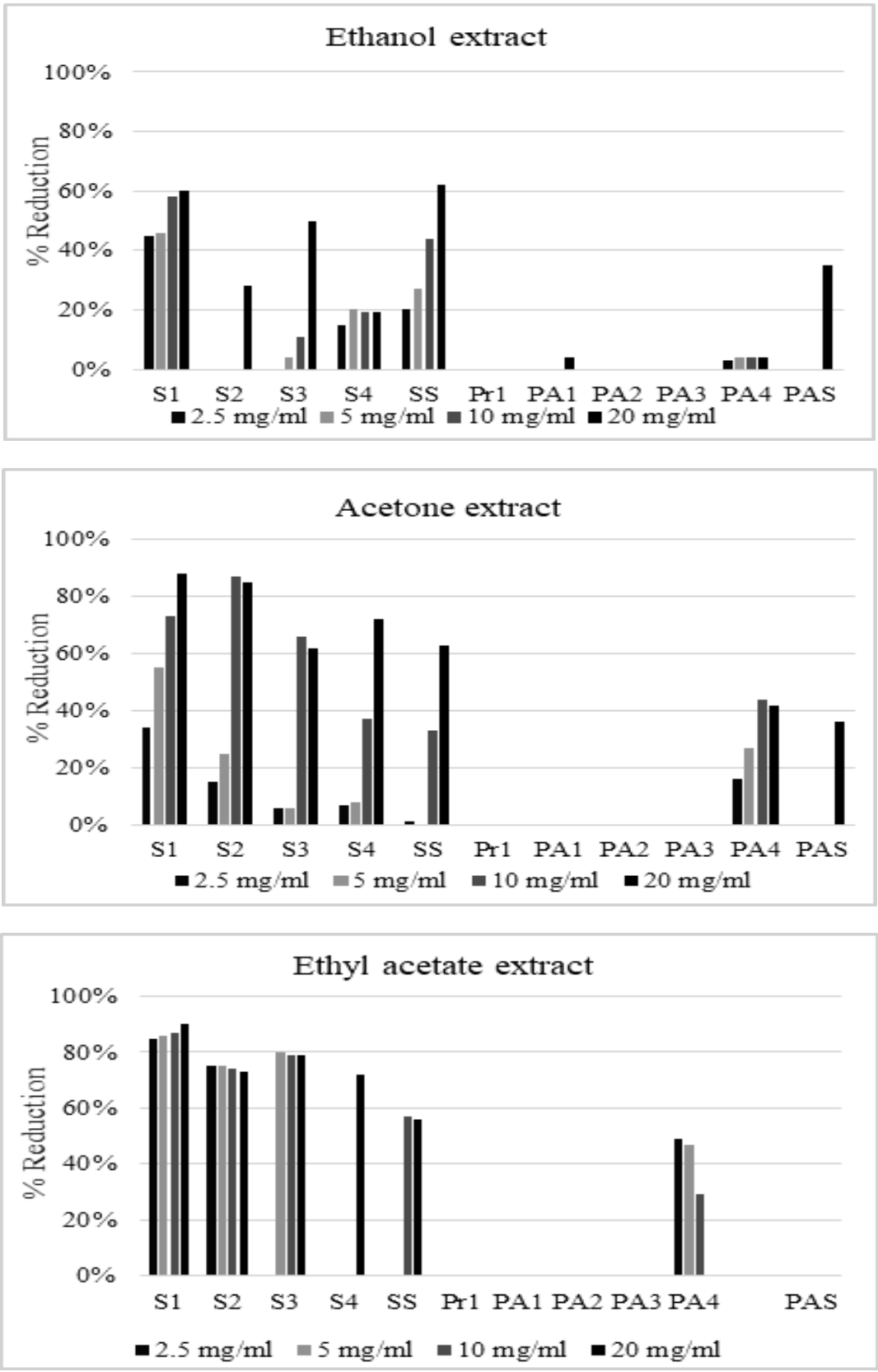

Figure 4. Reduction of formed biofilm by different concentrations of *L. album* extracts.
(S1, S2, S3, S4, SS – *S. aureus*; Pr1 – *Proteus* spp.; PA1, PA2, PA3, PA4, PAS – *P. aeruginosa*).

Inhibition of auto-aggregation

In biofilms, microcolonies are formed when aggregation and proliferation of cells occur simultaneously. Therefore, any treatment that decreases the auto-aggregation behavior may be useful in reducing biofilm development (TAO *et al*., 2022). Auto-aggregation of tested bacterial strains ranged from 8.20 ± 2.99 % to 36.48 ± 1.61 % (Table 4). Ethanol and acetone extracts reduced the auto-aggregation ability for all tested bacterial strains, except *Proteus* spp. (Pr1) where in the presence of acetone extract aggregation of cells has increased. Ethyl acetate extract decreased auto-aggregation in *S. aureus* (S1) and *P. aeruginosa* (PA2) while that effect was not noticed in *S. aureus* (S2), *Proteus* sp. (Pr1) and *P. aeruginosa* (PA3).

Table 4. Effect of *L. album* extracts on auto-aggregation.

Bacterial strains	Control (%)	Ethanol extract (%)	Acetone extract (%)	Ethyl acetate extract (%)
***S. aureus* S1**	17.58 ± 0.75	7.67 ± 1.11	6.69 ± 1.42	9.03 ± 2.58
***S. aureus* S2**	12.66 ± 1.18	0	3.19 ± 2.20	21.33 ± 2.34
***Proteus* spp. Pr1**	8.20 ± 2.99	6.64 ± 0.37	29.27 ± 0.32	47.46 ± 1.17
***P. aeruginosa* PA2**	36.48 ± 1.61	0.52 ± 0.26	0	16.29 ± 1.68
***P. aeruginosa* PA3**	13.67 ± 1.55	10.79 ± 2.30	1.53 ± 0.83	24.79 ± 1.61

The values are mean ± standard deviations of three replicates.

According to our intensive literature search, the effects of *L. album* extracts on auto-aggregation were not evaluated until now, but for some other plants from family Lamiaceae studies with similar aims as ours, were performed. For example, *M. viridis* extracts reduced the capacity of aggregation of *Escherichia coli*, *S. aureus*, *Klebsiella oxytoca* and *Clostridium difficle* (CHELLI-CHENTOUF *et al*., 2015). However, *Thymbra spicata* methanolic extract, depending on the dose, significantly increased the auto-aggregation of *Lactobacillus rhamnosus*, in comparison to the control group (KÖROĞLU and CELEBIOGLU, 2020).

Effect on bacterial motility

Motility plays an important role in the adhesion and biofilm formation of *Proteus* spp. and *P. aeruginosa*, so the swimming and swarming motilities were investigated. All tested strains showed a swimming type of motility (motility zones were 21–37 mm), but swarming motility was noticed by two strains, *Proteus* spp. Pr1 and *P. aeruginosa* PA1 (motility zones were 30 and 35 mm, respectively). The results showed that ethanol, acetone, and ethyl acetate extracts of *L. album* did not have an impact on swimming (Table 5) and swarming motility (Table 6) of tested bacterial strains.

To our best knowledge, the effect of *L. album* on bacterial motility has not been described in the literature yet, but several studies have described the effect of plants from the Lamiaceae family on bacterial motility. PANAYI *et al*. (2022) investigated the effect of six plant extracts from the Lamiaceae family on the motility of *E. coli* MG1655. Swarming motility was inhibited by ethanol extracts of *O. vulgare* and *R. officinalis* (1 mg/mL), and *S. officinalis* (2 mg/mL) significantly by 54.7%, 58.3% and 48.4%, respectively. The highest inhibition (45.7%) of swimming motility was recorded in the presence of the ethanol extract of *R. officinalis*. *O. vulgare* and *S. officinalis* inhibited the swimming motility of *E. coli* MG1655 by 42.3% and 17.2%, respectively. Ethanol extracts of other tested plants had either slight or no effects on both types of motilities. Decreasing in swimming and swarming motility of all tested uropathogens by hexane extract of *Hyptis suaveolens* was described by SALINI *et al*. (2015). The highest inhibition of swimming and swarming motility was recorded by *Serratia marcescens*, from 43.5 ± 1.0 to 4.5 ± 0.6 mm, and from 43.7 ± 0.7 to 5.1 ± 0.2 mm, respectively.

Table 5. Effect of *L. album* extracts on swimming motility (mm).

Bacterial strains	Swimming motility						
	Control	Ethanol extract		Acetone extract		Ethyl acetate extract	
		1	5	1	5	1	5
		mg/mL					
***Proteus* spp. Pr1**	37	37	37	37	37	37	37
***P. aeruginosa* PA1**	37	37	37	37	37	37	37
***P. aeruginosa* PA2**	37	37	37	37	37	37	37
***P. aeruginosa* PA3**	21	37	37	37	37	37	37
***P. aeruginosa* PA4**	27	37	37	37	37	37	37
***P. aeruginosa* ATCC**	26	37	37	37	37	37	37

Table 6. Effect of *L. album* extracts on swarming motility (mm).

Bacterial strains	Swarming motility						
	Control	Ethanol extract		Acetone extract		Ethyl acetate extract	
		1	5	1	5	1	5
		mg/mL					
***Proteus* spp. Pr1**	30	35	35	37	37	37	37
***P. aeruginosa* PA1**	35	37	37	37	37	37	37

Fluorescence microscopy

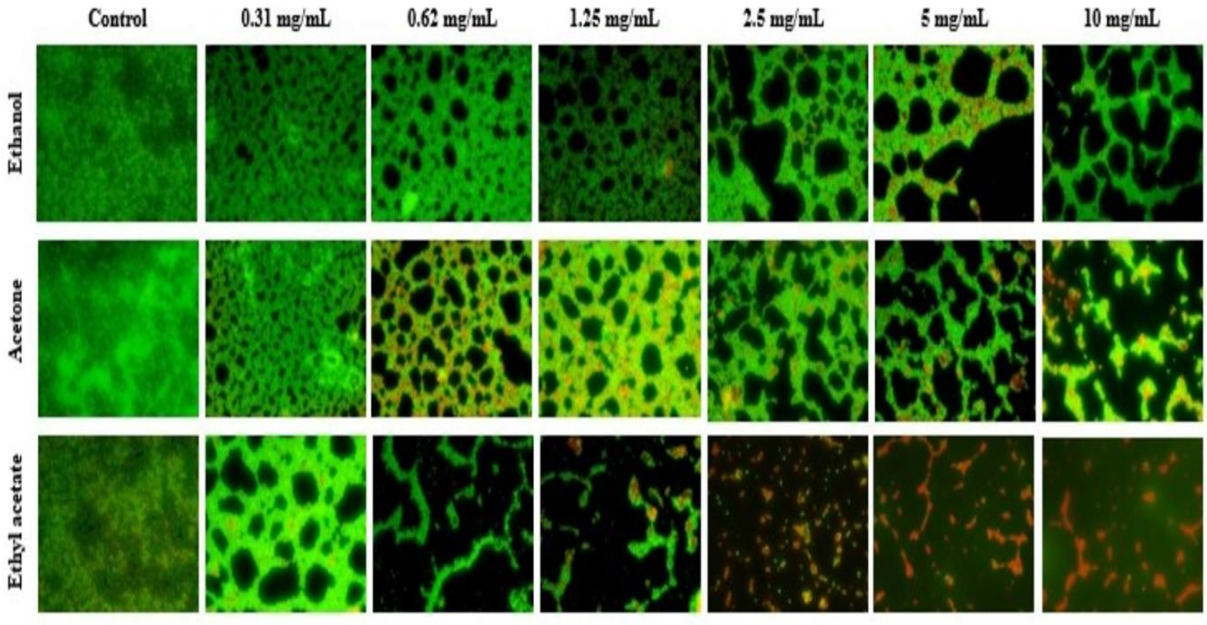

Figure 5. Fluorescence microscope images of *S. aureus* biofilm after treatment with *L. album* extracts.

The effect of *L. album* extracts on biofilm formation was confirmed by microscopic visualization using a fluorescence microscope. Acridine orange stains cells in biofilm, live or dead, and may also bind to nucleic acids that are present in the extracellular matrix (HARRISON *et al*., 2006). *S. aureus* (S2) showed dense and strong biofilm as can be visualized in control samples (Figure 5). As expected, the biofilm-forming ability gradually decreased with increasing extract concentration (Figure 5). The density of adherent bacterial cells was reduced which is in agreement with OD measurements.

CONCLUSION

A biofilm is a widespread form of life of bacteria and a common cause of chronic infections in humans. As compared to conventional therapy, plant-based products have been observed to be useful to cure various ailments. The results of the phytochemical analysis obtained in this work indicate that the concentration of flavonoids in *L. album* was highest in ethanol and ethyl acetate extracts, and the concentration of total phenolic acids was highest in acetone extract. Further, the investigation of *L. album* extracts showed their antibiofilm potential. *L. album* extracts exhibited significant antibiofilm activity by inhibiting cell attachment and biofilm formation, but the reduction of established biofilm has been more difficult to achieve. Generally, the effects of *L. album* extracts were dose- and strain-dependent. With an increase in extract concentration, the antibiofilm rate also increases. In addition, Gram-positive bacterial strains were more sensitive than Gram-negative strains. Moreover, *L. album* extracts reduced bacterial auto-aggregation as an additional factor in biofilm inhibition but did not disturb the motility of bacteria. Our study revealed the potential of *L. album* extracts as a new antibiofilm agent against human pathogenic bacteria. Therefore, isolation and identification of active natural constituents might be potential agents in the control of biofilms.

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