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Antimicrobial, Anti-quorum sensing, and Antibiofilm Potentials of Ethanolic Extracts from Lamium galeobdolon (L.) L. and Lamium purpureum L.

Lamium galeobdolon (L.) L. ve Lamium purpureum L. Etanolik Ekstraktlarının Antimikrobiyal, Anti-quorum sensing ve Antibiyofilm Potansiyelleri

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Abstract

Bacteria act through a communication mechanism called quorum sensing (QS) to control pathogenicity. Biofilm formation is a process supported by the QS mechanism and is known to act a part in antibiotic resistance. In this study, antimicrobial, anti-QS and antibiofilm capacities of ethanol extracts obtained from *Lamium galeobdolon* (L.) L. and *Lamium purpureum* L. plants were determined. The antimicrobial activity of plant extracts was evaluated by well diffusion assay against various hospital isolates. The extracts have antimicrobial effects against all bacteria and yeasts. The 27.5 ± 0.71 mm inhibitory effect of *L. purpureum* against *Candida guilliermondii* yeast at a dose of 100 mg/mL is remarkable. The anti-QS potential of the extracts was evaluated by a well diffusion assay based upon violacein pigment inhibition. *L. purpureum* extract showed a higher level of anti-QS effect against *Chromobacterium violaceum* ATCC 12472 biosensor strain. Antibiofilm capacity against biofilm-forming *Escherichia coli* isolate was determined by the crystal violet staining method. *L. purpureum* extract showed an inhibitory effect of 89.66% against *E.coli* biofilm at the highest dose. Both plants have been shown to have anti-pathogenic properties. However, it can be said that the *L.purpureum* plant is a more highly anti-pathogenic plant compared to *L. galeobdolon*.

Keywords: Antibiofilm, Antimicrobial, *Chromobacterium violaceum* ATCC 12472, *Lamium galeobdolon*, *Lamium purpureum*.

Özet

Bakteriler, patojeniteyi kontrol etmek için quorum sensing (QS) adı verilen bir iletişim mekanizması yoluyla hareket eder. Biyofilm oluşumu QS mekanizması tarafından desteklenen bir süreçtir ve antibiyotik direncinde rol oynadığı bilinmektedir. Çalışmada *Lamium galeobdolon* ve *Lamium purpureum* bitkilerinden elde edilen etanol ekstraktlarının antimikrobiyal, anti-QS ve antibiyofilm kapasiteleri belirlendi. Bitki ekstraktlarının antimikrobiyal aktivitesi çeşitli hastane izolatlarına karşı kuyu difüzyon deneyi ile araştırılmıştır. Ekstraktlar, tüm bakteri ve mayalara karşı antimikrobiyal etki göstermiştir. *L. purpureum*'un 100 mg/mL dozunda *Candida guilliermondii* mayasına karşı 27.5 ± 0.71 mm'lik inhibitör etkisi dikkat çekicidir. Ekstraktların anti-QS potansiyeli, viyolasin pigment inhibisyonuna dayanan kuyu difüzyon deneyi ile değerlendirildi. *L. purpureum* ekstraktı, *Chromobacterium violaceum* ATCC 12472 biyosensör suşuna karşı daha yüksek seviyede anti-QS etkisi gösterdi. Biyofilm oluşturan *Escherichia coli* izolatına karşı antibiyofilm kapasitesi kristal viyole boyama yöntemiyle belirlendi. *L. purpureum* ekstraktı en yüksek dozda *E. coli* biyofilmine karşı %89.66 oranında inhibitör etki göstermiştir. Her iki bitkinin de anti-patojenik özelliklere sahip olduğu kanıtlanmıştır. Ancak *L. purpureum* bitkisinin, *L. galeobdolon* bitkisine göre anti-patojenik özelliği daha yüksek bir bitki olduğu söylenebilir.

Anahtar Kelimeler: Antibiyofilm, Antimikrobiyal, *Chromobacterium violaceum* ATCC 12472, *Lamium galeobdolon*, *Lamium purpureum*.

Abbreviations: ATCC, American Type Culture Collection; DMSO, Dimethyl sulfoxide; OD, Optical Density; MHB, Mueller-Hinton Broth; MIC, Minimum Inhibitory Concentration; PBS, Phosphate-buffered saline; QS, Quorum sensing; SD, standard deviation.

1. INTRODUCTION

Today, to combat infectious diseases, the search for new antimicrobial agents that will not cause resistance development continues. However, discovering new antimicrobial agents is not enough because the different strategies developed by bacteria bring microbial challenges. Quorum sensing (QS), a bacterial communication mechanism, causes planktonic cells to aggregate, form biofilms, and develop pathogenic properties. Gene expression regulated through QS causes changes in surface antigens, helping the bacteria to evade host immunity. Bacteria hiding in biofilms are resistant to harsh environmental conditions and nutrient limitations. By remaining dormant within the biofilm, they can hide from the immune system and cause acute infection. In short, bacterial adaptations such as QS and biofilms cause them to be more resistant to antimicrobial therapy by reducing the cellular function requirements that antimicrobials would interfere with or by modulating antimicrobial targets (Tamfu et al., 2020; Vestby et al., 2020; Mirghani et al., 2022; Sharma et al., 2023).

Considering their global use, therapeutic and industrial values, researching plant families with great therapeutic potential becomes important. One of these families that attracts attention with their medicinal plants is the Lamiaceae family. The *Lamium* genus, which is one of the less studied members of the Lamiaceae family compared to other genera, has annual or perennial forms. This genus, which is frequently visited by entomophile pollination representative bumble bee queens and honey bees, is a host for many insect species, making them ecologically valuable. Various species belonging to the *Lamium* genus, comprising nearly 30 species in Turkey, are utilized in public health for treating hypertension, astringency, trauma, paralysis, fractures, constipation, and gynecological diseases. Additionally, they are recognized for their antispasmodic, antiproliferative, antiviral, and anti-inflammatory properties. (Akkol et al., 2008; Bubueanu et al., 2019; Salehi et al., 2019). *Lamium galeobdolon* and *Lamium purpureum* are species belonging to the genus *Lamium*, and their chemical compounds differ qualitatively and quantitatively. These differences can be considered as the reason why the two species have different bioactive effects (Akkoyunlu & Dulger, 2019; Akkoyunlu & Dulger, 2022).

As far as is known, although there are antimicrobial studies on many *Lamium* species, there are almost no studies explaining anti-quorum sensing and antibiofilm effects. The objective of this study is to assess the antimicrobial effects of *Lamium galeobdolon* and *Lamium purpureum* plants against bacteria and yeasts, and their anti-quorum sensing and antibiofilm capacities as a precaution against resistance to antimicrobial treatment.

2. MATERIALS and METHODS

2.1. Plant Extraction Preparation

The above-ground parts of the plants were collected from Duzce province between March and May in 2023, according to the flowering periods of the plants. The collected plants were dried under suitable conditions and ground into powder in the shredder. 20 grams of the powdered plants were placed in the Soxhlet apparatus and extraction was performed with 200 mL of 96% ethanol for 8 hours. The extracts were passed through Whatman filter No.1 and ethanol was removed using a rotary evaporator at 55 °C. The resulting dried extracts were dissolved in dimethyl sulfoxide (DMSO) (Merck, Germany) to a final concentration of 100 mg/mL. The extracts were stored in a sterile dark glass bottle at + 4 °C (Dulger & Dulger, 2018).

2.2. Test Microorganisms Preparation

In vitro, antimicrobial research was conducted with five bacterial isolates (*Bacillus cereus*, *Escherichia coli*, *Listeria innocua*, *Staphylococcus aureus*, and *Streptococcus pyogenes*) and five yeast isolates (*Candida albicans*, *C. glabrata*, *C. guilliermondii*, *C. lypolitica*, *and C. tropicalis*) obtained from Duzce University Research and Application Hospital Medical Microbiology Laboratory. Bacteria were incubated in Nutrient Broth (Merck, Germany) at 35-37 ºC and yeasts in Malt Extract Broth (Merck, Germany) at 25 - 27 ºC for 24 hours. The turbidity of fresh cultures was regulated to McFarland 0.5 with sterile saline.

2.3. Antimicrobial Test

Well diffusion method was used to identify the antibacterial and antifungal activity of *L. galeobdolon* and *L. purpureum*. Fresh microorganism inoculum was coated on Mueller-Hinton agar (Merck, Germany) in three directions with sterile swabs. Wells (8 mm diameter) were made in the plates using a sterile tip. Based on previous studies, plant concentrations of 100 mg/mL and 50 mg/mL were chosen due to their effectiveness and being within safe limits and 50 µL were added to the wells. DMSO was used as a control. Bacteria were incubated at 35 - 37 ºC and yeasts were incubated at 25 - 27 ºC for 24 - 48 hours and the diameters of the transparent zones formed were measured using a caliper. Amikacin (Bioanalyse, Turkey) and Penicillin (Bioanalyse, Turkey) antibiotics were used for bacteria and Ketoconazole (Bioanalyse, Turkey) for yeasts to compare the antimicrobial activity levels of the plants. The experiments were conducted three times independently. The results were assessed using means and standard deviations (Dulger, 2022).

2.4. Anti-quorum Sensing Test

Well diffusion method was used to qualitatively detect the anti-QS potential of the plants. *Chromobacterium violaceum* ATCC 12472 biosensor strain was streaked continuously onto nutrient agar in three different directions. Wells were opened by puncturing with a sterile pipette tip. Crude extracts of plants were added to the wells in a volume of 50 µL, at a concentration of 100 mg/mL and 50 mg/mL, and incubated at 30 ºC for 24 hours. 1% DMSO was used as a control. Experiments were performed in two replicates. Measurements were made by calculating the turbidity zone around the wells where violacein production was prevented (Mulya & Waturangi, 2021).

2.5. Antibiofilm Test

100 µL of sterile Mueller-Hinton broth (MHB) (Merck, Germany) medium with 1% glucose was added to the wells to be studied on a flat-bottomed 96-well microplate. The sub-MICs of the extracts (100, 50, 25, 12.5, and 6.25 mg/mL) were added. A strong biofilm-forming *E. coli* isolate was used in this assay. 100 µL of fresh culture adjusted to 0.5 McFarland turbidity was pipetted into the wells, and the microplate was incubated at 37 °C for 48 hours. The positive control contained 100 µL of bacterial culture and 100 µL of medium. Only 200 µL of medium was used in the negative control. The experiment was performed in duplicate.

After incubation, the wells were washed three times with 200 µL of PBS and dried. Biofilms were fixed with 200 µL of 99% methanol for 5 min. The wells were inverted, their contents drained and dried. The wells were stained with 200 µL of 0.3% crystal violet dye for 15 minutes. Microplates were washed three times with distilled water. The dye bound to the biofilm mass was dissolved with 96% ethanol solution and the absorbance was established at 560 nm in a microplate reader (Robonik, India). Percent inhibition values of plant extracts were calculated with the following formula (Balli et al., 2019; Haney et al., 2021):

$$(\text{OD positive control} - \text{OD sample}) / \text{OD positive control} \times 100) \text{ (Equation 1)}$$

2.6. Statistical Analysis

The data were entered into Microsoft Excel version 365 and presented as mean ± standard deviation (SD) values. Analyses were conducted using IBM SPSS Statistics 27 software. Shapiro-Wilk normality test was applied to determine whether the data showed a normal distribution. The p-value obtained from the Shapiro-Wilk test was found to be greater than 0.05 (For *L. galeobdolon* p = 0.054; for *L. purpureum* p = 0.153). This result indicates that the data follow a normal distribution. To determine whether the inhibitory effect of the concentration groups was significant compared to the control group; a One-Sample t test was applied. The p-value obtained from the One-Sample t test was found to be less than 0.05 (for all p = 0.006). This result indicates that the inhibitory effect of the concentration groups is statistically significant compared to the control group.

3. RESULTS and DISCUSSION

3.1. Antimicrobial Activity

Table 1 shows the antimicrobial effects of *L. galeobdolon* and *L. purpureum* plants on 5 bacteria and 5 yeasts. The results show that plant extracts inhibit microbial growth in a dose-dependent manner (Figure 1). *L. galeobdolon* created the highest inhibition zone (> 15 mm) against *C. glabrata* at both doses. The plant gave better results than the penicillin antibiotic against *B. cereus*, *E. coli*, *S. aureus*, *S. pyogenes* bacteria. However, its effect on bacteria remained low compared to the amikacin antibiotic.

Another plant, *L. purpureum*, has more antimicrobial effects than *L. galeobdolon*. It is highly effective in inhibiting *C. guilliermondii* (≥ 25mm). It is noteworthy that it is as effective as the antibiotic amikacin in inhibiting the growth of *B. cereus*. Compared to the ketoconazole antibiotic used for *Candida* strains, the high dose of the plant showed a more inhibitory effect on the strains.

Table 1. Summary of Antimicrobial Activity of *Lamium galeobdolon* and *Lamium purpureum**

Microorganisms	***Lamium galeobdolon* L.**		***Lamium purpureum* L.**		**P**	**AK**	**KETO**
	50 mg/mL	*100 mg/mL*	*50 mg/mL*	*100 mg/mL*			
	Bacteria						
Bacillus cereus	12.0	13.5 ± 0.71	17.0 ± 1.41	20.0 ± 1.41	10.0	20.0	NT
Escherichia coli	13.5 ± 0.71	14.0 ± 2.82	14.5 ± 2.12	16.5 ± 2.12	7.0	25.0	NT
Listeria innocua	11.0 ± 1.41	12.0	15.5 ± 0.71	19.0 ± 1.41	24.0	30.0	NT
Staphylococcus aureus	12.5 ± 2.12	14.0 ± 5.65	14.5 ± 2.12	16.5 ± 2.12	12.0	26.0	NT
Streptococcus pyogenes	12.0	14.0 ± 1.41	17.0 ± 2.82	19.0 ± 2.82	9.0	29.0	NT
	Yeasts						
Candida albicans	11.5 ± 0.71	14.0 ± 2.82	16.0	18.0	NT	NT	10.0
Candida glabrata	15.5 ± 0.71	15.5 ± 2.12	15.0	16.5 ± 0.71	NT	NT	15.0
Candida guilliermondii	14.0	14.5 ± 4.95	25.0	27.5 ± 0.71	NT	NT	-
Candida lypolitica	13.5 ± 0.71	14.0 ± 1.41	15.5 ± 0.71	18.0	NT	NT	16.0
Candida tropicalis	**12.5 ± 0.71**	**12.0 ± 1.41**	**15.0**	**18.0**	**NT**	**NT**	**15.0**

**Contains zone diameter, which is the average value of three independent experiments (in mm). NT: Not tested; P: Penicillium (10 µg/mL); AK: Amikacin (30 µg/mL); KETO: Ketoconazole (20 µg/mL).*

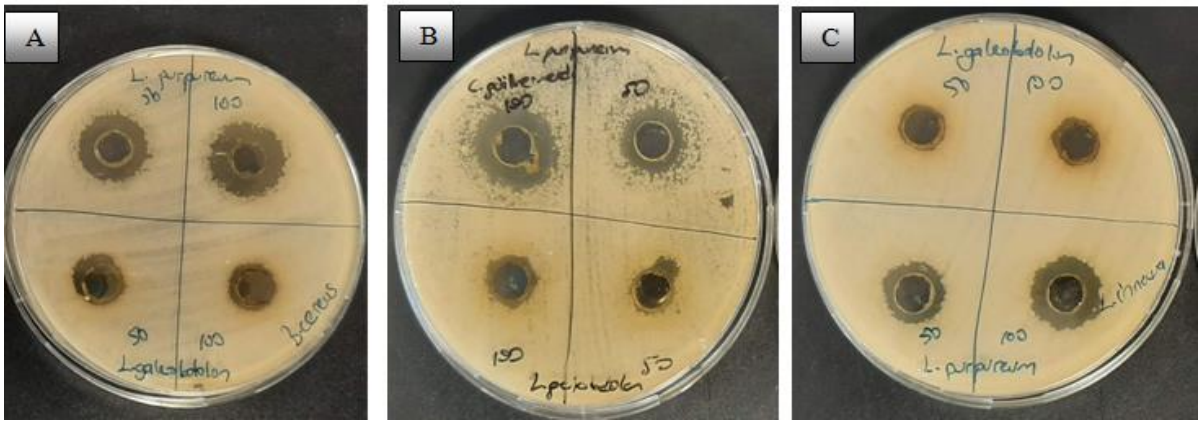

Figure 1. Antimicrobial activity of plant extracts against microorganisms samples (A: *B.cereus* B: *C.guilliermondii*; C: *L.innocua*) measured by well diffusion assay. It was studied in 3 repetitions and the inhibition zones were calculated by measuring with a caliper and presented as the inhibitory effect.

In a study conducted with 4 different *Lamium* species belonging to the genus, including *L. purpureum*, it was determined that all plants had moderate antimicrobial effects and were more effective against yeasts than bacteria. It has been stated that the solvent used also changes the antimicrobial capacity. It has been emphasized that the difference in antimicrobial effects between the species is due to the components they contain (Yalçın et al., 2007).

In a different study, it was stated that *L. galeobdolon* essential oil did not have an antibacterial effect against certain bacteria such as *B. cereus* and *S. aureus*, and had an antifungal effect against *C. glabrata* and *C. tropicalis* yeasts, but this was also weak (Akkoyunlu & Dulger, 2022). It has been stated in previous studies that solvent selection and extraction processes can influence the final results (Rios & Recio, 2005; Yalçın et al., 2007). Our study revealed that the antimicrobial effects of ethanol extracts from these plants are higher than those of the essential oils.

3.2. Anti-quorum Sensing Activity

C. violaceum ATCC 12472 bacteria, a biosensor strain, was used to identify the ability of plant extracts to inhibit the QS mechanism. This bacterial strain is the most popular microorganism used in elucidating QS mechanisms in recent years. Biosynthesis of violacein pigment governed by QS is considered an indicator in defining cell-to-cell signaling pathways (Dimitrova et al., 2023).

The opaque zones that appear around the plant extracts as a result of well diffusion experiments are an indication that only the QS mechanism is stopped without preventing

bacterial growth (Figure 2). The anti-QS effect was presented by calculating zone diameters in two replicate experiments. *L. purpureum* plant had a much greater inhibitory effect than *L. galeobdolon* (Table 2). It is noticed that the anti-QS effect increases depending on the concentration of plant extracts. While *L. galeobdolon* showed an inhibitory effect of 13.33 ± 1.53 at a high dose, *L. purpureum* showed a higher effect than this result even at a low dose (15.67 ± 1.53).

Table 2. Summary of Anti-quorum sensing Activity of *L. galeobdolon* and *L. purpureum*

Plants	**Effective concentrations (mg/mL)**	**QS inhibition zones (mm)**
Lamium galeobdolon	50	12.50 ± 0.58
	100	13.33 ± 1.53
Lamium purpureum	50	15.67 ± 1.53
	100	17.33 ± 0.58

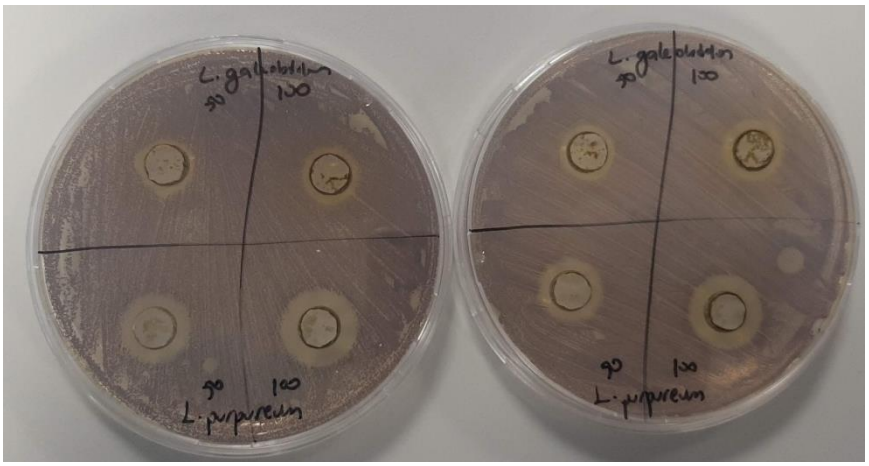

Figure 2. Demonstration of quorum sensing (QS) inhibitory activity of the plant extracts against *C.violaceum* ATCC 12472 biosensor strain

No anti-QS studies on *Lamium* species have been found in the literature. In a study conducted with 10 different plants, it was shown that plant essential oils belonging to the Lamiaceae family, such as *Salvia officinalis*, *Origanum vulgare*, *Clinopodium nepeta*, significantly inhibited the violacein pigment (D'Aquilla et al., 2023). In a different anti-QS study conducted on the pyocyanin pigment of *P. aeruginosa* bacteria, ethanol extracts of 23 plant species were studied. Among these plants, *S. officinalis*, two species belonging to the Lamiaceae family, showed high anti-QS effects, while *Solenostemon scutellarioides* species was ineffective (Elmanama & Al-Refii, 2017).

Attacking the QS mechanism, the key regulatory network of bacterial virulence has become a promising strategy against emerging antibiotic resistance. It was determined that the plant extracts we used in the current study inhibit the QS network of *C. violaceum* ATCC 12472 bacteria.

3.3. Antibiofilm Activity

The antibiofilm capacity *of L. galeobdolon* and *L. purpureum* extracts and the amount of biofilm biomass formed were determined by staining with crystal violet dye (Figure 3). Results calculated as the percentage of biofilm inhibition showed that the effect of plant extracts varied depending on species and dose (Figure 4).

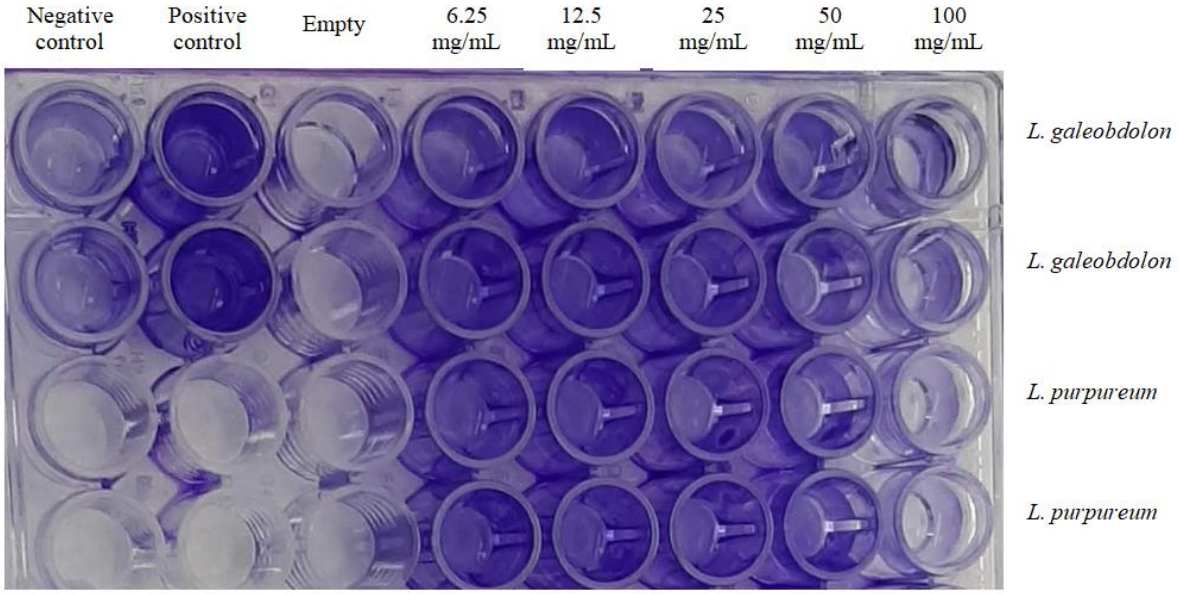

Figure 3. Images show crystal violet stained biofilm corresponding to controls and plant extracts.

L. galeobdolon plant exhibited lower antibiofilm effect than *L. purpureum* plant. *L. purpureum* inhibited biofilm formation by approximately 90% at high doses. Even at its lowest dose, biofilm formation remained below 50%. *L. galeobdolon* prevented biofilm formation by 64.64% at its highest dose, and biofilm formation was below 50% at all doses except the lowest dose. The antibiofilm effects of both plants are remarkable. Antibiofilm studies have not been described for these two species in the literature. However, in a study conducted with *Lamium album*, the most well-known member of this genus, ethanol, acetone, and ethyl acetate extracts were prepared and antibiofilm activity was evaluated against 2 reference strains and 9 clinical isolates obtained from wound swabs. The study found that the ethyl acetate extract was more effective against gram-positive biofilms, destroying *S. aureus* biofilm by up to 95% (Terzić et al., 2023).

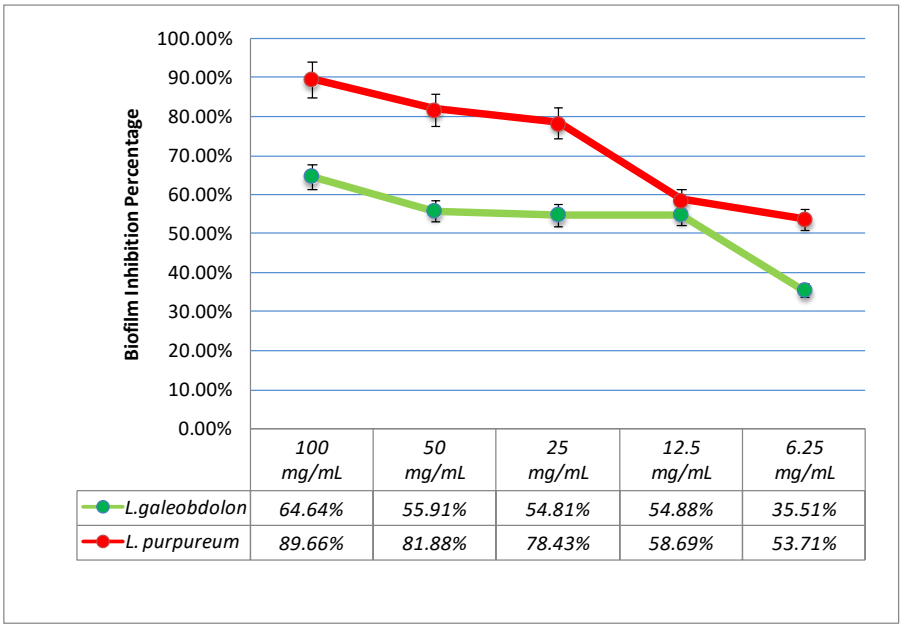

Figure 4. Antibiofilm activity of *L. galeobdolon* and *L. purpureum* extracts against *E. coli* biofilm. *Statistically different from the control ($p < 0.05$).

In treatment with *L. amplexicaule* extract, a different *Lamium* species, it significantly inhibited the Streptococcus mutans bacterial biofilm, which forms strong biofilms in the mouth, at a concentration lower than the MIC. The study indicates that this extract selectively prevents biofilm formation without inhibiting bacterial growth (Lee et al., 2019).

These results encountered in the literature are compatible with our study data. It is evident that *Lamium* species contain active biofilm inhibitory components. It can be said that there is a genus-wide effect against both Gram-positive and Gram-negative bacterial biofilms.

The bioactive properties of plants directly depend on the components they contain. These components depend on many factors such as the physiological development period of the plant, daily and seasonal changes during collection, the composition of the soil, and the type of extraction processes (Azwanida, 2015; Lin et al., 2019).

4. CONCLUSION

Biocontrol research on the use of plant extracts or pure compounds obtained from plants is gaining momentum today. The plants we used in our study are species with significant antibacterial, antifungal, anti-QS, and antibiofilm effects. It is extremely important to distinguish the biologically active natural components of these and many other plants, to determine the stability of crude extracts and to determine their cytotoxic effects before use. Our

study provides a basis for further studies and opens a motivating path for the discovery of potential antimicrobial agents, QS, and biofilm inhibitors.

DECLARATIONS

There is no conflict of interest between the authors.

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