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Comparative *in vitro* Study of Antimicrobial, Antibiofilm and Quorum Sensing Inhibitory Activities of *Hypericum calycinum L.* and *Parietaria officinalis L.* Extracts

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ABSTRACT

Aim: This study aims to elucidate both the anti-virulence and antimicrobial effects of ethanol extracts from *Hypericum calycinum L.* and *Parietaria officinalis L.*

Material and Methods: Antimicrobial activity was evaluated using the well diffusion method against five bacteria and two yeast isolates involved in human urinary tract infections (UTIs). The potential of the extracts to inhibit quorum sensing (QS), was assessed using the biosensor strain *Chromobacterium violaceum* ATCC 12472. Additionally, the antibiofilm activities were investigated using a microplate biofilm assay on *Escherichia coli* and *Pseudomonas aeruginosa*.

Results: *H. calycinum* exhibited the highest inhibitory effect at a concentration of 100 mg/mL against *Candida albicans* with an inhibition zone of 24.5±0.71 mm, while *P. officinalis* showed its highest effect at the same concentration against *E. coli* with an inhibition zone of 15.5±0.71 mm. Overall, *H. calycinum* demonstrated stronger antimicrobial activity compared to *P. officinalis*. Both plant extracts inhibited QS at similar levels, with inhibition zones ranging between 10-12 mm. The antibiofilm effect varied depending on the bacterial species, but notably, *P. officinalis* extract exhibited over 80% antibiofilm efficacy against *E. coli* at all concentrations.

Conclusion: This study demonstrates that *H. calycinum L.* and *P. officinalis L.* are potent antimicrobial agents against UTI pathogens. While their anti-QS efficacy is not exceptional, the significant inhibition of *E. coli* biofilm formation underscores their potential as formidable agents. Designed as a fundamental study, it highlights the promising antimicrobial properties of these plant extracts and marks the first investigation into their capabilities as QS and biofilm-preventive agents.

Keywords: Antibiofilm activity; antimicrobial activity; *Chromobacterium violaceum* ATCC 12472; urinary tract infection.

Hypericum calycinum L. ve *Parietaria officinalis L.* Ekstraktlarının Antimikrobiyal, Antibiyofilm ve Quorum Sensing İnhibitör Aktivitelerinin Karşılaştırmalı *in vitro* Çalışması

ÖZ

Amaç: Bu çalışma, *Hypericum calycinum L.* ve *Parietaria officinalis L.* bitkilerinden elde edilen etanol ekstraktların hem anti-virülans hem de antimikrobiyal etkilerini açıklamaya çalışmaktadır.

Gereç ve Yöntemler: Antimikrobiyal aktivite, insan idrar yolu enfeksiyonlarında (İYE) rol oynayan beş bakteri ve iki maya izolatına karşı kuyu difüzyon yöntemi ile değerlendirilmiştir. Ardından, ekstraktların Quorum Sensing (QS) mekanizmasını inhibe etme potansiyeli biyosensör suşu *Chromobacterium violaceum* ATCC 12472 kullanılarak değerlendirilmiştir. Ek olarak, antibiyofilm aktiviteleri, *Escherichia coli* ve *Pseudomonas aeruginosa* üzerinde mikroplaka biyofilm deneyi kullanılarak araştırılmıştır.

Bulgular: *H. calycinum*, 100 mg/mL konsantrasyonda *Candida albicans'a* karşı 24.5±0.71 mm inhibisyon zonu ile en yüksek inhibitör etkiye sahipken, *P. officinalis* aynı konsantrasyonda *E. coli*'ye karşı 15.5±0.71 mm inhibisyon zonu ile en yüksek etkisini göstermiştir. Genel olarak, *H.calycinum, P. officinalis*'e kıyasla daha güçlü antimikrobiyal aktivite sergilemiştir. Her iki bitki ekstraktı 10-12 mm arasında değişen, benzer seviyelerde QS'i inhibe etmiştir. Ekstrelerin antibiyofilm etkisi bakteri türüne göre değişmekle beraber *E.coli* üzerinde, tüm konsantrasyonlarda %80'in üzerinde antibiyofilm etkinliği sergileyen *P. officinalis* ekstraktı özellikle dikkate değerdir.

Sonuç: Bu çalışma, *H. calycinum L.* ve *P. officinalis L.*'in İYE patojenlerine karşı güçlü antimikrobiyal ajanlar olduğunu ortaya koymaktadır. Ayrıca, anti-QS mekanizmasına karşı etkinlikleri istisnai olmasa da, *E. coli* biyofilm oluşumunun belirgin şekilde engellenmesi, zorlu ajanlar olarak potansiyellerinin altını çiziyor. Temel bir araştırma olarak planlanan

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bu çalışma, bitki ekstraktlarının umut verici antimikrobiyal özelliklerini vurgularken, bunların QS ve biyofilm önleyici ajanlar olarak yeteneklerinin ortaya koyulduğu ilk çalışma niteliği taşımaktadır.

Anahtar Kelimeler: Antibiyofilm aktivite; antimikrobiyal aktivite; *Chromobacterium violaceum* ATCC 12472; idrar yolu enfeksiyonu.

INTRODUCTION

Quorum sensing (QS), a bacterial communication system, regulates the release mechanisms determining the virulence of bacteria, plasmid transfer, and biofilm formation. Biofilm is microbial communities where cells are encased in their self-produced matrix (EPS), posing medical concerns in bacterial infection pathogenesis (1). Biofilm formation is a dynamic process that plays a role in the development of antibiotic resistance through factors such as the inhibition of antibiotic penetration by EPS, mutation of the target site, increased expression of efflux pump genes, and accumulation of antibiotic-degrading enzymes. The escalating rise in antibiotic resistance has made the prevention of bacterial virulence rather than just controlling bacterial viability a new strategic target (2,3).

Blocking the QS mechanism and biofilm formation may offer an alternative approach to eliminating bacterial infections, as it does not exert the selection pressure typically associated with traditional antibiotic treatment (1).

The use of medicinal plants in traditional medicine has become a vast research field for the discovery of new drugs and bioactive compounds. Secondary metabolites found in medicinal plants, such as flavonoids, terpenoids, and phenolic acids, exhibit antimicrobial effects on microorganisms through various mechanisms, including altering cell morphology, reducing cell membrane permeability, and disrupting cell structure, as well as inhibiting quorum sensing (4).

Turkey is a country rich in a diversity of medicinal plants with proven therapeutic potential and safety in traditional medicine, owing to its geographical location (5).

It has been reported that there are a total of 107 taxa of *Hypericum (Hypericaceae)* in the flora of Turkey 48 of which are endemic (6). The species discussed in this study, *H. calycinum L.*, is a medicinal plant well-known for its antimicrobial, antimalarial, and antioxidant activities, as well as its potent antidepressant and anti-aging properties. This broad range of medical activities is attributed to secondary metabolites such as quercetin, isoquercitrin, quercitrin, rutin, hyperforin, and hyperoside, which have been identified in studies of *H. calycinum L.*'s chemical composition(5-7).

P. officinalis L. (Urticaceae) is traditionally recognized for its depurative, cholagogue, anti-urolithic, and anti-rheumatic properties. It has been reported that the plant extract is prescribed for certain nervous disorders, epilepsy, threats of syncope, and eclampsia. Additionally, it is used for ulcers and externally for hemorrhoids and inflammations (8). In quite old phytochemical studies conducted on *P. officinalis L.* leaves and flowers, it was reported that they are rich in flavonoids and phenolic acids (9,10).

The anti-virulence effects targeting QS and pathogenic bacterial biofilms of many plants, including *H. calycinum L.* and *P. officinalis L.*, have not yet been systematically evaluated. The aim of the study is to determine the antimicrobial and anti-virulence effects of ethanol extracts of these two plants.

MATERIAL AND METHODS

Preparation of the Ethanolic Extracts

Wild-growing *H. calycinum L.* and *P. officinalis L.* plants were collected from their natural populations around Duzce province in northwestern Turkey in May-June 2023. Plant material (20 g) was air dried in the shade, pulverized, and then extracted with ethanol (Merck) (200 mL) using Soxhlet apparatus. The extracts were passed through a membrane filter (Whatman no: 1) to remove particulate matter. Ethanol was evaporated in a rotary evaporator and dissolved with dimethyl sulfoxide (DMSO) (Merck) and kept in sterile opaque bottles in a +4 ºC refrigerator until use.

Preparation of Testing Microorganisms

In vitro antimicrobial studies were conducted with urinary tract pathogens (*Candida albicans, Candida glabrata, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Providencia rettgeri*) obtained from Duzce University, Faculty of Medicine, Medical Biology Research Laboratory, Duzce, Turkey.

Bacteria were incubated in Nutrient Broth (Merck) at 35-37 ºC and yeasts in Malt Extract Broth (Merck) at 25-27 ºC for 24 hours. The turbidity of fresh cultures was regulated to McFarland 0.5 with sterile saline.

Determination of Antimicrobial Activity

The antimicrobial activity of *H.calycinum L.* and *P.officinalis L.* ethanol extracts was determined by well diffusion method. Fresh microorganism cultures were plated on Mueller-Hinton agar (Merck) in three directions with sterile swabs. Wells with a diameter of 8 mm were made in the plates with a sterile pipette. 50 µl of plant extract was added to the wells at 2 different concentrations (100 mg/mL, 50 mg/mL). DMSO was used as a negative control. Bacteria were incubated at 35-37 °C for 24 hours and yeasts at 25-27 °C for 48 hours. The transparent regions around the wells were considered as inhibition zones and their diameters were measured with the help of calipers. In order to compare the antimicrobial activity levels of the plants, the antibiotics Amikacin and Ampicillin (Bioanalyse) were used for bacteria and Nystatin (Bioanalyse) for yeasts. Experiments were performed independently twice. The results were evaluated by taking averages and calculating standard deviations. Results were interpreted according to CLSI limit values (11, 12).

Determination of Anti-quorum sensing Activity

The anti-QS potential of the plants was qualitatively assessed using the well diffusion method. The *Chromobacterium violaceum* ATCC 12472 biosensor strain was swabbed onto nutrient agar in three directions. Wells were then made using a sterile pipette tip. The crude extracts of the plants were added to the wells at concentrations of 100 mg/mL and 50 mg/mL in a volume of 50 µL and incubated at 30°C for 24 hours. 1% DMSO was used as a control. The experiments were performed in duplicate. Measurements were taken by calculating the turbid zone around the wells where violacein production was inhibited (13).

Determination of Antibiofilm Activity

The inhibitory effect of plant extracts at concentrations ranging from 100 to 12.5 mg/mL on biofilm formation was tested using the method described by Stefanović et al. (14) with polystyrene flat-bottom microtiter plates. Fresh cultures of biofilm-forming *E. coli* and *P. aeruginosa pathogens* (100 µl) were added to Mueller-Hinton broth (MHB) (Merck) supplemented with 1% glucose in the presence and absence of extracts (100 µl). The plates were then incubated at 37°C for 48 hours. Wells containing only medium served as negative controls, while wells containing medium and pathogens served as positive controls. After incubation, the wells were washed with Phosphate-buffered saline (PBS) to remove planktonic bacteria. The biofilm mass was fixed with methanol for 5 minutes, stained with 0.3% crystal violet (CV) solution for 15 minutes at room temperature after the wells were dried, and excess dye was removed by washing three times with distilled water. To dissolve the CV dye, 200 µl of 96% ethanol was added to the wells at room temperature, and absorbance was measured at an optical density (OD) of 595 nm. The following formula was used to determine the inhibition rate. Tests were performed in triplicate.

$$\%\ \text{Inhibition} = \frac{\text{ODpositive control} - \text{ODsample}}{\text{ODpositive control}} \times 100$$

The biofilm inhibition was graded on a scale from 0% to 100%. Inhibition ranging from 0% to 50% represents weak biofilm inhibition, while inhibition above 50% indicates strong biofilm inhibition.

Statistical Analysis

Data were entered into Microsoft Excel 365 and presented as mean ± standard deviation (SD). Analyses were performed using IBM SPSS Statistics 27 software. The Shapiro-Wilk test was used to check if the data were normally distributed. The Levene test was used to assess the homogeneity of variances. For variables that were normally distributed and had homogeneous variances; One-Way ANOVA followed by Dunnett's multiple comparison tests was used for comparisons. For variables that were not normally distributed, the Mann-Whitney U test was used to compare the means of two groups. All p-values were obtained by comparing each treatment group to the control group. The significance level was set at 0.05.

RESULTS

Antimicrobial Activity of *Hypericum calycinum L.* and *Parietaria officinalis L.* Extracts

In this study, the antimicrobial effects of ethanol extracts of two different plants against UTI pathogens were evaluated using the well diffusion test. As shown in Table 1, inhibitory effects were observed at two different concentrations of the plants, particularly the *H. calycinum L.* extract exhibited inhibitory effects on both bacterial and yeast growth compared to the reference antibiotics. The most sensitive pathogen to this extract was identified as *C. albicans*, with an inhibition diameter of 24.5±0.71 mm at a concentration of 100 mg/mL. *E. coli* was the most sensitive pathogen to the *P. officinalis L.* extract at a concentration of 100 mg/mL, showing a lower effect compared to amikacin but closer to ampicillin.

Table 1. Summary of antimicrobial activity of *H.calycinum* L. and *P.officinalis* L. compared to some standard antibiotics

Microorganisms	*Hypericum calycinum* L.		*Parietaria officinalis* L.		AK (30µg)	AMP (10µg)	NY (100 µg)
	50 mg/mL	*100 mg/mL*	*50 mg/mL*	*100 mg/mL*			
	Mean±SD	Mean±SD	Mean±SD	Mean±SD			
Candida albicans	20.5±0.71	24.5±0.71	13.0±1.41	15.0±2.82	NT	NT	17.0
Candida glabrata	18.5±0.71	19.0±1.41	12.5±2.12	13.0±1.41	NT	NT	11.0
Escherichia coli	17.0 ± 0	18.5±0.71	14.5±2.12	15.5±0.71	19.0	15.0	NT
Enterococcus faecalis	19.0±1.41	22.0 ± 0	13.0±1.41	13.0 ± 0	NT	16.0	NT
Klebsiella pneumoniae	20.0±1.41	21.0±1.41	11.5±0.5	14.5±3.54	19.0	14.0	NT
Providencia rettgeri	19.5±0.71	20.0±1.41	12.0±1.41	13.5±0.71	17.0	14.0	NT
Pseudomonas aeruginosa	19.5±0.71	22.0±4.24	11.5±2.12	13.0±0.71	27.0	10.0	NT

*Mean zone diameters of tests performed in duplicate for each strain were taken. AMP: Ampicillin (10 µg); AK: Amikacin (30 µg); NY: Nystatin (100 µg); NT: Not Tested.

Anti-quorum sensing Activity of *Hypericum calycinum L.* and *Parietaria officinalis L.* Extracts

The ability of the ethanol extracts of the two plants to inhibit violacein production regulated by the QS mechanism in *C. violaceum* ATCC 12472 biosensor strain is presented in Table 2. The anti-QS effect of the plant extracts was concentration-dependent; however, both plants exhibited violacein pigment inhibition with inhibition zones not exceeding 12 mm.

Antibiofilm Activity of *Hypericum calycinum L.* and *Parietaria officinalis L.* Extracts

In this study, the ethanol extracts of the two plants were evaluated for their antibiofilm capacity at sub-MIC ranges (100, 50, 25, and 12.5 mg/mL). The study was conducted on two of the most common biofilm-forming Gram-negative pathogens, *P. aeruginosa* and *E. coli* (15). The results were calculated according to the equation and presented in Figure 1 and Figure 2.

Table 2. Summary of antiquorum sensing activity of *H.calycinum* L. and *P.officinalis* L.

Plants	Effective concentrations (mg/mL)	QS inhibition zones (mm)
*Hypericum calycinum*L.	50 mg/mL	10.0 ± 0
	100 mg/mL	11±1.41
*Parietaria officinalis*L.	50 mg/mL	10.5±0.71
	100 mg/mL	11.5±0.71

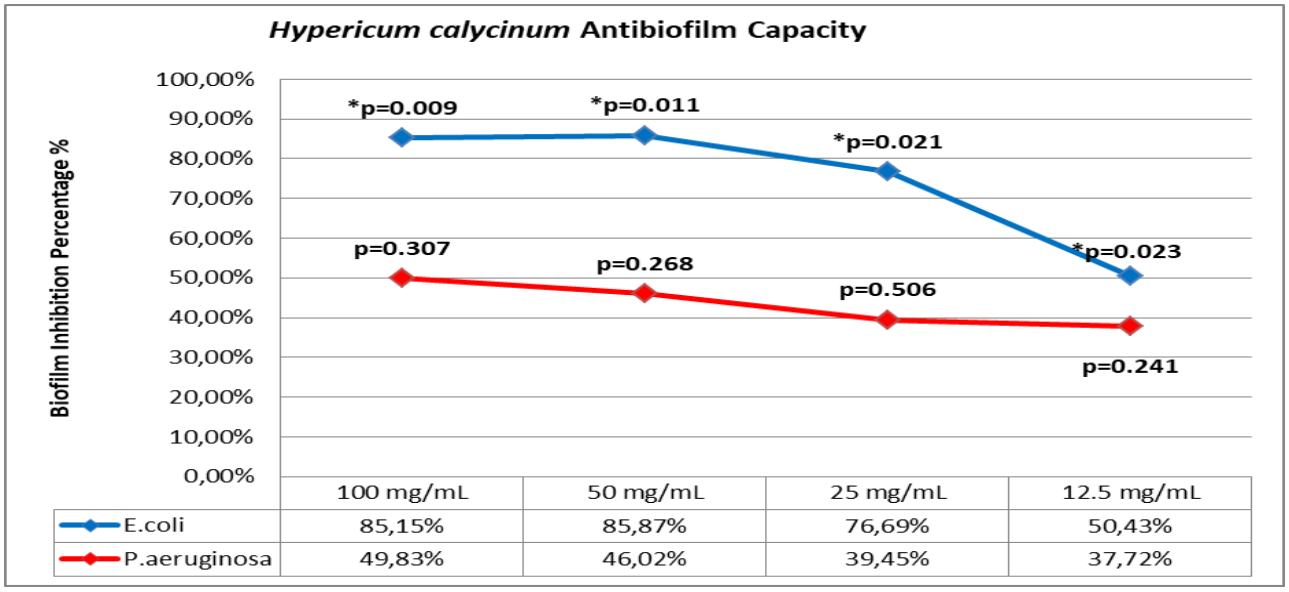

Figure 1. Antibiofilm activity of *H.calycinum* L. extracts against *E. coli* and *P.aeruginosa* biofilms. *Statistically different from the control (p<0.05).

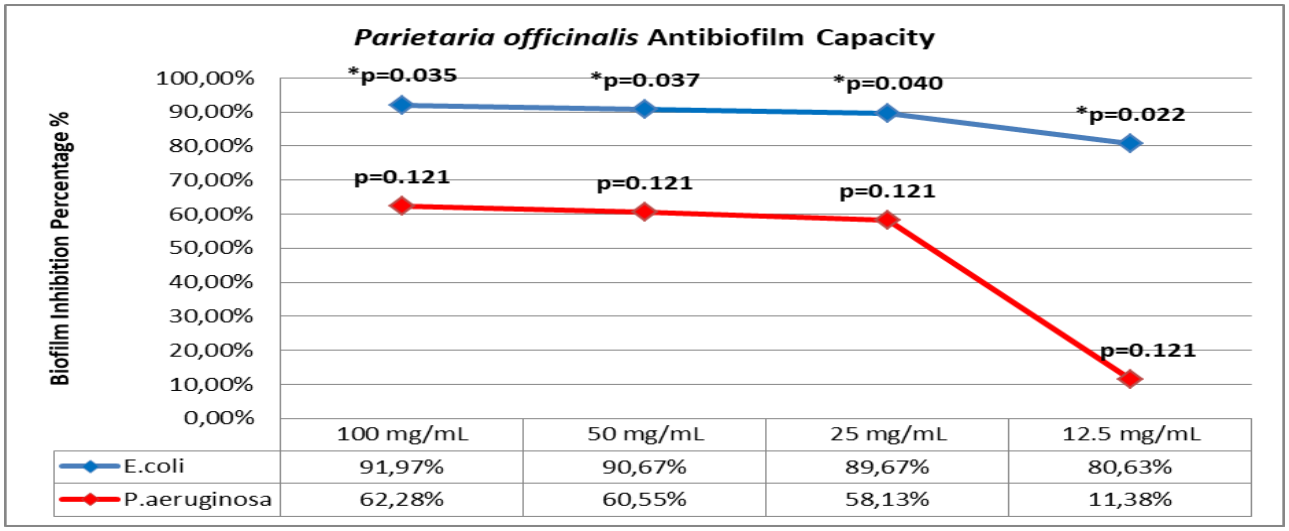

Figure 2. Antibiofilm activity of *P. officinalis* L.extracts against *E. coli* and *P.aeruginosa* biofilms.*Statistically different from the control (p<0.05).

For *H. calycinum*, the biofilm inhibition on *E. coli* at concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL was found to be 85.15% (p=0.009), 85.87% (p=0.011), 76.69% (p=0.021), and 50.43% (p=0.023) respectively. These results also indicate a high level of biofilm inhibition on *E. coli*, with significant results at all concentrations (p<0.05). However, the biofilm inhibition on *P. aeruginosa* at the same concentrations was 49.83% (p=0.307), 46.02% (p=0.268), 39.45% (p=0.506), and 37.72% (p=0.241), with none of these results being statistically significant (p>0.05) (figure 1).

For *P. officinalis*, the biofilm inhibition on *E. coli* at concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL was found to be 91.97% (p=0.035), 90.67% (p=0.037), 89.67% (p=0.040), and 80.63% (p=0.022) respectively. These results indicate a high level of biofilm inhibition on *E. coli*, with significant results at all concentrations (p<0.05). In contrast, the biofilm inhibition on *P. aeruginosa* at the same concentrations was found to be 62.28% (p=0.121), 60.55% (p=0.121), 58.13% (p=0.121), and 11.38% (p=0.121), with none of these results being statistically significant (p>0.05) (figüre 2)

DISCUSSION

Studies on developing new antibiotics from plant extracts continue unabated, but multidrug resistance poses a serious threat. Researchers now agree that developing new strategies to target bacteria's pathogenicity and virulence properties is as important as discovering antimicrobial compounds.

Plant products have the potential to be used as antimicrobial and antivirulent agents safely for extended periods. Plant extracts, rich in phytochemical content, naturally exhibit a synergistic effect, making them potent as bioactive agents. Synergistic effects are stronger than single-molecule effects, and although the mechanism is not fully understood, resistance development against plant extracts is lower (5).

There are not many studies conducted on *P. officinalis*. Studies on the chemical composition analysis of its extracts and its association with pollen allergies date back to before the 2000s (9, 16, 17).

The rich chemical composition revealed in a study conducted with its essential oil in 2023 suggests that this plant deserves further exploration in the field of bioactivity (8). There are no studies determining its anti-QS and antibiofilm effects, and research on different bioactivity aspects of this plant extract is also limited. In the literature, *P. officinalis* has been mentioned as one of the plants with therapeutic potential in the treatment of UTI. It has been reported to be effective in preventing kidney stone formation and reducing infection by increasing urine production (10).

The first antimicrobial study conducted with *P. officinalis* ethanol extract targeted *E. coli*, *P. aeruginosa*, and *S. aureus* bacteria. Among several different methods tested in the study, the best performance was achieved with the well diffusion method. The extract exhibited low-level inhibition against *P. aeruginosa* (6-8 mm) and moderate-level inhibition against *S. aureus* (9-11 mm), but it was not effective against *E. coli*. Phytochemical analysis suggested that compounds responsible for the antimicrobial effect could be steroids, triterpenoids, phenols, and flavonoids (18).

Compared to the study conducted in 2020, the current study results demonstrate that *P. officinalis* extract is more effective against both *E. coli* and *P. aeruginosa* isolates (11-16 mm) (Table 1). The difference in the level of antimicrobial effect may be attributed to variations in phytochemical components of the plant extract. Phytochemical components are affected by many factors such as the climate and seasonal conditions of the region where the plant is collected and extraction methods. Overall, this study has demonstrated the antimicrobial value of *P. officinalis* ethanol extract against UTI microorganisms (6).

The *H. calycinum L.* plant extract has shown higher inhibition compared to the *P. officinalis L.* extract. Its antimicrobial activity, both in yeasts and bacteria, was consistently higher at every concentration compared to nystatin in yeasts and ampicillin in bacteria (Table 1).

Various studies have been conducted on the antimicrobial properties of different extracts of *H. calycinum L.*, revealing its effectiveness against numerous bacteria such as Gram-positive, Gram-negative, and *Mycobacterium tuberculosis* (19-22). Due to its rich phenolic content, *H. Calycinum L.* has demonstrated active results in antioxidant activity analyses (6). These properties may suggest that it may also play a role in its antimicrobial effect.

The two bacteria most commonly associated with UTI, *E. coli* and *P. aeruginosa*, can enhance their competitive abilities with other microorganisms and colonize the bladder through single or multispecies biofilm formation (23).

To summarize, both plants exhibit high antibiofilm activity against *E. coli*, with effectiveness decreasing as the concentration decreases but remaining significant at all concentrations. However, both plants show lower antibiofilm activity against *P. aeruginosa*, with none of the results being statistically significant (Figure 1, Figure 2). This indicates that the antibiofilm capacities of *P. officinalis* and *H. calycinum* vary depending on the bacteria, with a notably higher effectiveness against *E. coli.*

The anti-QS effects of these two plant extracts, ranging from 10 to 12 mm, suggest that they inhibit biofilm formation by disrupting bacterial communication. Through their secondary metabolites, plants have developed a defense system against bacteria. Many studies have demonstrated that these metabolites mimic QS molecules and render receptors in signaling pathways ineffective (24).

Additionally, although the anti-QS effects of the two plant extracts in the study are similar, their antibiofilm effects differ. This indicates that the plant components not only interfere with the QS mechanism but also inhibit biofilm formation through different processes. Furthermore, the variation in antibiofilm properties depending on the bacterial species is indicative of each bacterial species' unique pathways for biofilm formation.

CONCLUSION

The bacterial communication mechanism known as QS signaling and the inhibition of biofilm formation, which is considered a pathogenic property, are viewed as new therapeutic strategies to inhibit the virulence and pathogenicity of pathogens. Present study determined that the plants *H. calycinum L.* and *P. officinalis L.* are potent antimicrobial agents against UTI-causing microorganisms. Additionally, these plant extracts, which have similar anti-QS effects, exhibited different antibiofilm activities depending on the bacterial species. While the use of traditional medicinal herbs has proven to be as effective as synthetic drugs, it is the primary duty of the researchers to adjust the formulation, dosage and duration of treatment to achieve therapeutic effects and to provide adequate information about possible side effects. Present study constitutes one of the fundamental steps in this direction.

Authors's Contributions: Idea/Concept: A.A., G.D.; Design: A.A., G.D.; Data Collection and/or Processing: A.A., G.D.; Analysis and/or Interpretation: A.A., G.D.; Literature Review: A.A., G.D.; Writing the Article: A.A., G.D.; Critical Review: A.A., G.D.

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