

Original Research Article

Phytochemical profiling, antibacterial and antibiofilm activities of *Delonix regia* flowers and leaves extracts against *Pseudomonas aeruginosa*

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Abstract

Purpose: To evaluate the phytochemical composition and antibacterial potential of *Delonix regia* (Royal Poinciana) flowers and leaves.

Methods: Flowers and leaves extract of *Delonix regia* was prepared using the Soxhlet apparatus. The phytochemical composition of plant extract was evaluated using High-Performance Liquid Chromatography (HPLC)/Mass Spectrometry. The Agar well diffusion method, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) were used to test the antibacterial activity of the plant extract. Microtiter plate method was used to study the effect of extract on *Pseudomonas aeruginosa* biofilm.

Results: Extraction yields were highest in methanol ($\approx 35\%$), suggesting a higher concentration of polar bioactive constituents. The HPLC-MS analysis identified diverse compounds, including phenolic acids, flavonoids, terpenoids, sterols, and carotenoids, with rutin, β -sitosterol, and lupeol as major constituents of the methanolic flower extract. Among all extracts, methanolic flower extract exhibited the broadest antibacterial spectrum. The extract significantly reduced *P. aeruginosa* biofilm formation by over 80 % at concentrations ≥ 25 mg/mL. Scanning electron microscopy confirmed marked biofilm disruption and bacterial morphological alterations.

Conclusion: These results suggest that *D. regia* methanolic flower extract harbors potent bioactive compounds capable of inhibiting both planktonic growth and biofilm formation of pathogenic bacteria. Although the active concentrations appear high for systemic use, they are fully appropriate for topical wound applications and antimicrobial coatings, where higher local doses are acceptable.

Keywords: Antibacterial resistance, Antibiofilm, *Delonix regia*, Phytochemicals, *Pseudomonas aeruginosa*

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INTRODUCTION

Bacterial resistance to antibiotics is a profound global problem and development threat requiring

immediate action. Antimicrobial resistance is one of the leading causes of death around the world. The emergence of resistance is driven by multiple interacting factors and the dynamics of

its spread. To address the growing problem of antimicrobial resistance and the excessive and unregulated use of antibiotics, exploring new therapeutic strategies for managing bacterial infections is critically required. *Pseudomonas aeruginosa* is considered one of the foremost multidrug-resistant pathogens owing to its pronounced ability to form resistant biofilms and acquire multiple resistance genes. This urgent need for new therapeutic approaches to inhibit bacterial growth and biofilm formation highlights natural products and plant extracts as attractive sources of potential antimicrobial agents [1,2].

The flowering plant species *D. regia* (Royal Poinciana or flame tree) is an important tree that is native to Madagascar, but is now distributed in several countries of the tropical region. It belongs to the family, Fabaceae and subfamily, Caesalpinioideae. Various parts of *D. regia* are used in traditional medicine to treat a variety of disorders. Some reported biological activities of the parts of *D. regia* include antioxidant, hepatoprotective, anti-inflammatory, cytotoxic, hypoglycemic, gastroprotective, diuretic, wound healing, antidiarrhoeal, anthelmintic, antimalarial, and antimicrobial activity. Phytochemical screening of *D. regia* revealed the presence of biologically active constituents including flavonoids, phenolic compounds, tannins, alkaloids, glycosides, sterols and terpenoids [3,4]. Given the broad antimicrobial potential of *D. regia* extracts previously reported against Gram-positive and Gram-negative bacteria, it is very important to verify their potential effectiveness against biofilms. This study aimed to determine the phytochemical composition of *D. regia* flowers and leaves extracts and their antibacterial and antibiofilm activity.

EXPERIMENTAL

Collection of plant material

Leaves and flowers of *D. regia* plant were collected from privately owned land in the Dead Sea area, Jordan, in August 2024. Taxonomic identification of the plant was performed by Dr. Feryal Khreissat, Mutah University. The flowers and leaves were shade-dried and powdered in a blender. The powdered plant was kept in an airtight plastic bag for further analysis. No herbarium voucher was deposited in the herbarium.

Extraction of powdered leaf and flower

Thirty grams of dried flower and 60 g of dried leaf were extracted separately using the Soxhlet extraction method with chloroform, methanol and

water (250 mL each). Then the extracts were filtered, concentrated under reduced pressure using rotary evaporator at 45 °C. Finally, the yields were calculated. All extractions were performed in triplicate [5].

Determination of antibacterial activity of extracts

Agar well diffusion method was performed to determine the antibacterial activity of the extracts against *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 9144), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 9027). Bacterial concentrations were adjusted to 0.5 McFarland and 100 µL of each bacterial growth was equally spread on the surface of nutrient agar. Wells (8 mm) were filled with 200 µL of each extract. The inhibition zones were measured after 18 h of incubation at 37 °C. The experiment was conducted in triplicate [6]. Values were interpreted as less active, moderately active and highly active with zone diameters of inhibition of ≤ 1 cm, 1.1 – 1.4 cm, and ≥ 1.5 cm, respectively [7].

Determination of minimum inhibitory concentration (MIC)

The MIC was determined using a 96-well microtiter plate method, following the procedure described in [6,8], for the extracts that exhibited antimicrobial activity in the agar well diffusion assay. In brief, two-fold serial dilutions of extracts (100 - 0.049 mg/mL) were prepared and inoculated with bacterial suspensions (1×10^6 CFU/mL). After incubation for 18 h at 37 °C, MIC was recorded as the lowest concentration at which no visible bacterial growth was observed.

Determination of minimum bactericidal concentration (MBC)

The MBC was determined by sub-culturing 15 µL of MIC broth wells with no visible growth onto nutrient agar plates. The MBC was recorded as the lowest concentration that is needed to kill the bacteria [8,9].

Determination of phytochemical compounds in *D. regia* extracts

The extracts (that showed antibacterial activity) were subjected to chromatographic analysis with High-Performance Liquid Chromatography (HPLC)/Mass spectrometry (Shimadzu Co., Japan). During the assessment, 1 mL of methanol was used to dissolve 25 mg of crude extract, and 10 µL of the prepared solution was

injected. Separations on ACE 5 C18 (250 x 4.6 mm; 5 µm) column were carried out at 30 °C with 0.8 mL/min flow rate. Gradient elution at 8 min was utilized for the analysis, with the mobile phase A: B: C (80:12:8), i.e., water with 0.1 % acetic acid (A), methanol with 0.1 % acetic acid (B), and acetonitrile with 0.1 % acetic acid (C). The polarity of the mobile phase was gradually increased by 25: 60: 15 at 40 - 45 min, then back to the initial ratio (80: 12: 8) to recondition the column for 5 min. Mass spectrometric detection was performed using electrospray ionization with nitrogen as the nebulizing (3 L/min) and drying gas (15 L/min), desolvation line at 300 °C, heat block at 400 °C, and probe voltage at + 4.5 kV. Compound identification was done by comparison with the NIST 2019 database. Before processing HPLC injections, extract samples and solvents for mobile phases were filtered via a 0.22 µm filtration system (Millipore Co., Billerica, MA).

Evaluation of the antibiofilm activity against *Pseudomonas aeruginosa*

The ability of methanolic flower extract of *D. regia* to reduce biofilm formation by *P. aeruginosa* was evaluated using microtiter plate assay as described by [8]. *P. aeruginosa* was particularly chosen as it is a well-known biofilm producer. Bacterial suspensions (10^8 CFU/mL) were incubated with 12 serial dilutions of the stock concentration of the extract (200 mg/mL) in 96-well plates at 37 °C for 24 h. After washing, biofilms were stained with 1% crystal violet, and absorbance was measured at 600 nm. Percentage biofilm inhibition was calculated relative to untreated controls. The mean % reduction of biofilm formation was determined for each concentration in triplicate using Eq 1.

$$\text{Reduction (\%)} = \{(A_c - A_m)/A_c\}100 \dots (1)$$

Where A_c is the absorbance of the control and A_m is the absorbance of methanolic extract

Scanning Electron Microscopy (SEM) analysis

The modified methods were used to evaluate the effect of *D. regia* methanolic flower extract on *P. aeruginosa* biofilm morphology [10]. Four glass slides were prepared: a control slide (untreated), a slide treated with the extract at zero time (simultaneously with bacterial inoculation), a slide treated after 24 h, and a slide treated after 48 h.

For each slide, 200 µL of bacterial suspension (10^8 CFU/mL) was added, and in the treated

groups, 200 µL of the extract was applied according to the respective time points. Nutrient broth (200 µL) was added daily to each slide. After 72 h, the slides were washed, fixed with 2.5 % glutaraldehyde, then dehydrated using a series of graded ethanol. The slides were air-dried to remove ethanol residues, and the areas containing biofilms were carefully cut with a glass cutter and mounted for carbon coating. The biofilm structure and morphological changes of the samples were subsequently examined using SEM.

Statistical analysis

All experiments were performed in triplicate, and the results, where applicable, are presented as mean ± standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance (ANOVA) to compare the differences between treated groups and the untreated control. A $p < 0.05$ was considered statistically significant. Statistical analyses were performed using Microsoft Excel 2021.

RESULTS

Extraction yields of *D. regia* using different solvents

Methanol extraction of *D. regia* leaves and flowers exhibited the highest extraction yield compared to chloroform and water (Table 1). However, the extraction of *D. regia* flowers with chloroform showed the lowest extraction yield.

Table 1: Extraction yield (%) of crude *D. regia* leaves and flowers extracts

	<i>Chloroform</i>	<i>Methanol</i>	<i>Water</i>
Flower	3.1±0.3	35.8±2.0	6.8±2.7
Leaf	12.1±4.7	34.4±1.1	11.6±0.7

Yield (%) mean ± SD, n=3

Antibacterial activity of extracts

D. regia water and chloroform extracts were suspended in 10 % DMSO in distilled water (100 mg/mL). The solution of 10 % DMSO in water was set as control and showed no zones of inhibition against all the tested bacteria (Figure 1, Table 2).

The methanolic extract of the flowers was active against all bacterial strains tested. While the methanolic and aqueous extracts of the leaves showed zones of inhibition against *B. cereus* and *S. aureus*, only the aqueous extract of the flowers was active against *B. cereus* and *E. coli*.

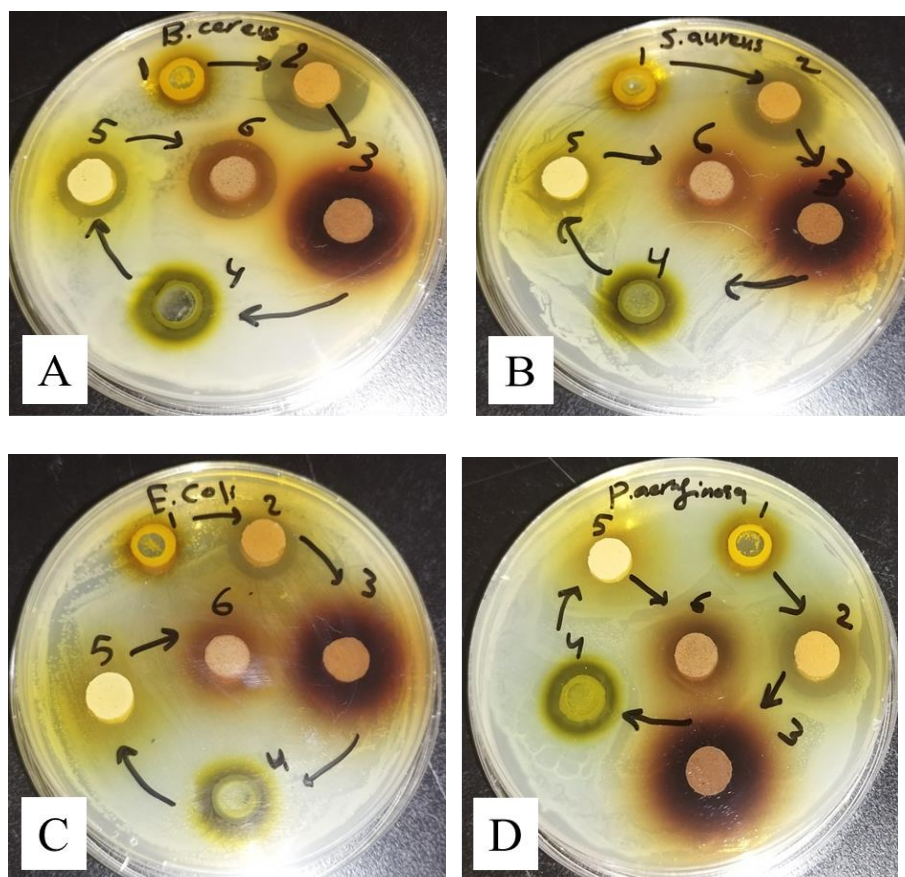


Figure 1: Antibacterial activity of *D. regia* extracts against the growth of *B. cereus* (A), *S. aureus* (B), *E. coli* (C), and *P. aeruginosa* (D) in terms of zone of inhibition utilizing chloroform flowers extract (1), methanolic flowers extract (2), aqueous flowers extract (3) chloroform leaves extract (4), methanolic leaves extract (5), aqueous leaves extract (6)

Table 2: Antibacterial activity of *D. regia* extracts using agar well diffusion method $\pm$ SD

Extract	<i>B. cereus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
CEF	No activity	No activity	No activity	No activity
MEF	2.06 $\pm$ 0.05	1.8 $\pm$ 0.16	1.36 $\pm$ 0.05	1.46 $\pm$ 0.05
WEF	2.2 $\pm$ 0.08	No activity	1.56 $\pm$ 0.05	No activity
CEL	No activity	No activity	No activity	No activity
MEL	1.33 $\pm$ 0.05	1.13 $\pm$ 0.05	No activity	No activity
WEL	1.5 $\pm$ 0.08	1.43 $\pm$ 0.05	No activity	No activity

Inhibition zone in cm $\pm$ SD. CEF: Chloroform extract of flower. MEF: Methanol extract of flower. WEF: Water extract of flower. CEL: Chloroform extract of leaf. MEL: Methanol leaf extract. WEL: Water extract leaf

Table 3: MIC values (mg/mL) of *D. regia* extracts against *B. cereus*, *S. aureus*, *E. coli* and *P. aeruginosa*

Extract	<i>B. cereus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
Methanol extract of flower	6.25	12.5	25	12.5
Aqueous extract of flower	50	>100	50	>100
Methanol leaf extract	50	25	>100	>100
Aqueous extract leaf	100	100	>100	>100
DMSO 10%	No effect	No effect	No effect	No effect

Effect of *D. regia* extract on minimum inhibitory concentration (MIC)

Methanolic extract of *D. regia* flowers recorded the lowest MIC against *B. cereus*. while the highest MIC value against all tested extracts was

recorded for water extract of *D. regia* leaves. The MIC results are presented in Table 3.

Effect of *D. regia* extract on Minimum Bactericidal Concentration (MBC)

Only the methanolic flower extract had killing effect against all tested bacteria, with the highest bactericidal concentration against *S. aureus*, *E. coli* and *P. aeruginosa* being (50 mg/mL). While for *B. cereus*, the MBC value was 6.25 mg/mL. The other extracts showed no bactericidal activity against the tested bacteria.

Chemical composition of *D. regia* flowers and leaves extracts

The phytochemical composition of *D. regia* water and methanol extracts of the flowers and leaves is presented in Tables 4 - 7. The phytochemical analysis revealed all extracts shared an identical phytochemical profile containing phenolic acids, terpenoids, flavonoids, sterols, carotenoids, anthocyanins, phenylpropanoids, lipids and amino acids. Meanwhile, myricetin was only detected in the aqueous and methanolic leaf extracts. The identified compounds had retention times ranging from 1 min to 5.6 min, and area percentages varying from 0.7 to 9 %.

Antibiofilm activity of *D. regia* methanolic flower extract against *P. aeruginosa*

The percentage (%) reduction of biofilm formation of *P. aeruginosa* strain by *D. regia* methanolic flower extract is presented in Figure 1. It was observed that the minimum biofilm inhibitory concentration is 25 mg/mL. Also, the concentrations (100, 50, and 25 mg/mL) significantly ($p < 0.05$) reduced biofilm formation by more than 80% compared to the lower concentrations.

Effect of extracts on biofilm formation under scanning electron microscopy (SEM)

The effect of the methanolic extract on biofilm formation under scanning electron microscope is presented in Figure 2 (A - H). Control group exhibited dense *P. aeruginosa* biofilm layer and normal bacterial morphology with widespread presence (Figure 2 a and b). Treatment of the bacterial inoculum at zero time profoundly affected the biofilm formation, which appeared discrete with much lower prevalence of bacterial cells than control group (Figure 2 c and d).

Table 4: Phytochemical compounds in *D. regia* methanolic flower extract

Compound	Molecular formula	Molecular weight	Area (%)	RT (Min)
Aspartic acid	C ₄ H ₇ NO ₄	133.1	1.5	1.0
Lysine	C ₆ H ₁₄ N ₂ O ₂	146.2	1.8	1.1
Cinnamic acid	C ₉ H ₈ O ₂	148.2	0.8	1.2
Gallic Acid	C ₇ H ₆ O ₅	170.1	3.1	1.3
Caffeic acid	C ₉ H ₈ O ₄	180.2	5.2	1.4
Ferulic acid	C ₁₀ H ₁₀ O ₄	194.2	3.2	1.5
Germacrene D	C ₁₅ H ₂₄	204.4	1.2	1.6
Myristic acid	C ₁₄ H ₂₈ O ₂	228.4	0.9	1.7
Palmitic acid	C ₁₆ H ₃₂ O ₂	256.4	0.7	1.7
Linolenic acid	C ₁₈ H ₃₀ O ₂	278.4	1.0	1.8
Linoleic acid	C ₁₈ H ₃₂ O ₂	280.4	2.1	1.9
Oleic acid	C ₁₈ H ₃₄ O ₂	282.5	2.0	2.0
Stearic acid	C ₁₈ H ₃₆ O ₂	284.5	3.1	2.1
Luteolin	C ₁₅ H ₁₀ O ₆	286.2	4.1	2.3
Ricinoleic acid	C ₁₈ H ₃₄ O ₃	298.5	5.2	2.3
Quercetin	C ₁₅ H ₁₀ O ₇	302.2	4.2	2.4
Erucic acid	C ₂₂ H ₄₂ O ₂	338.6	5.1	2.7
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	354.3	5.0	2.8
Stigmasterol	C ₂₉ H ₄₈ O	412.7	4.2	2.9
β-sitosterol	C ₂₉ H ₅₀ O	414.7	8.2	3.1
Lupeol	C ₃₀ H ₅₀ O	426.7	7.0	3.2
Epilupeol	C ₃₀ H ₅₀ O	426.7	4.2	3.3
Carotene	C ₄₀ H ₅₆	536.9	3.2	3.5
Rubixanthin	C ₄₀ H ₅₆ O	552.9	1.3	3.65
β-Cryptoxanthin	C ₄₀ H ₅₆ O	552.9	1.2	3.8
Lutein	C ₄₀ H ₅₆ O ₂	568.9	2.0	4.0
Zeaxanthin	C ₄₀ H ₅₆ O ₂	568.9	2.1	4.2
Pelargonidine 3-o-rutinoside	C ₂₇ H ₃₀ O ₁₄	578.5	2.4	4.4
Cyanidine 3-o-rutinoside	C ₂₇ H ₃₁ O ₁₅ ⁺	595.5	2.0	4.6
Astaxanthin	C ₄₀ H ₅₂ O ₄	596.8	3.0	4.9
Rutin	C ₂₇ H ₃₀ O ₁₆	610.5	9.0	5.2

Table 5: Phytochemical compounds detected in *D. regia* aqueous flower extract

Compound	Molecular formula	Molecular weight	Area (%)	RT (Min)
Aspartic acid	C ₄ H ₇ NO ₄	133.1	2.0	1.0
Lysine	C ₆ H ₁₄ N ₂ O ₂	146.2	2.1	1.1
Cinnamic acid	C ₉ H ₈ O ₂	148.2	1.1	1.2
Gallic Acid	C ₇ H ₆ O ₅	170.1	3.0	1.3
Caffeic acid	C ₉ H ₈ O ₄	180.2	5.0	1.4
Ferulic acid	C ₁₀ H ₁₀ O ₄	194.2	3.0	1.5
Germacrene D	C ₁₅ H ₂₄	204.4	1.0	1.6
Myristic acid	C ₁₄ H ₂₈ O ₂	228.4	0.5	1.7
Palmitic acid	C ₁₆ H ₃₂ O ₂	256.4	0.4	1.7
Linolenic acid	C ₁₈ H ₃₀ O ₂	278.4	0.6	1.8
Linoleic acid	C ₁₈ H ₃₂ O ₂	280.4	1.6	1.9
Oleic acid	C ₁₈ H ₃₄ O ₂	282.5	1.2	2.0
Stearic acid	C ₁₈ H ₃₆ O ₂	284.5	2.0	2.1
Luteolin	C ₁₅ H ₁₀ O ₆	286.24	4.0	2.3
Ricinoic acid	C ₁₈ H ₃₄ O ₃	298.5	5.0	2.3
Quercetin	C ₁₅ H ₁₀ O ₇	302.2	3.5	2.4
Erucic acid	C ₂₂ H ₄₂ O ₂	338.6	4.2	2.7
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	354.31	4.0	2.8
Stigmasterol	C ₂₉ H ₄₈ O	412.7	5.1	2.9
β-sitosterol	C ₂₉ H ₅₀ O	414.7	8.0	3.1
Lupeol	C ₃₀ H ₅₀ O	426.7	6.8	3.2
Epilupeol	C ₃₀ H ₅₀ O	426.7	4.0	3.3
Carotene	C ₄₀ H ₅₆	536.9	3.0	3.5
Rubixanthin	C ₄₀ H ₅₆ O	552.9	2.0	3.7
β-Cryptoxanthin	C ₄₀ H ₅₆ O	552.9	2.2	3.8
Lutein	C ₄₀ H ₅₆ O ₂	568.9	1.8	4.0
Zeaxanthin	C ₄₀ H ₅₆ O ₂	568.9	2.0	4.2
Pelargonidine 3-o-rutinoside	C ₂₇ H ₃₀ O ₁₄	578.5	2.0	4.4
Cyanidine 3-o-rutinoside	C ₂₇ H ₃₁ O ₁₅₊	595.5	1.5	4.6
Astaxanthin	C ₄₀ H ₅₂ O ₄	596.8	3.2	4.9
Rutin	C ₂₇ H ₃₀ O ₁₆	610.5	8.2	5.2

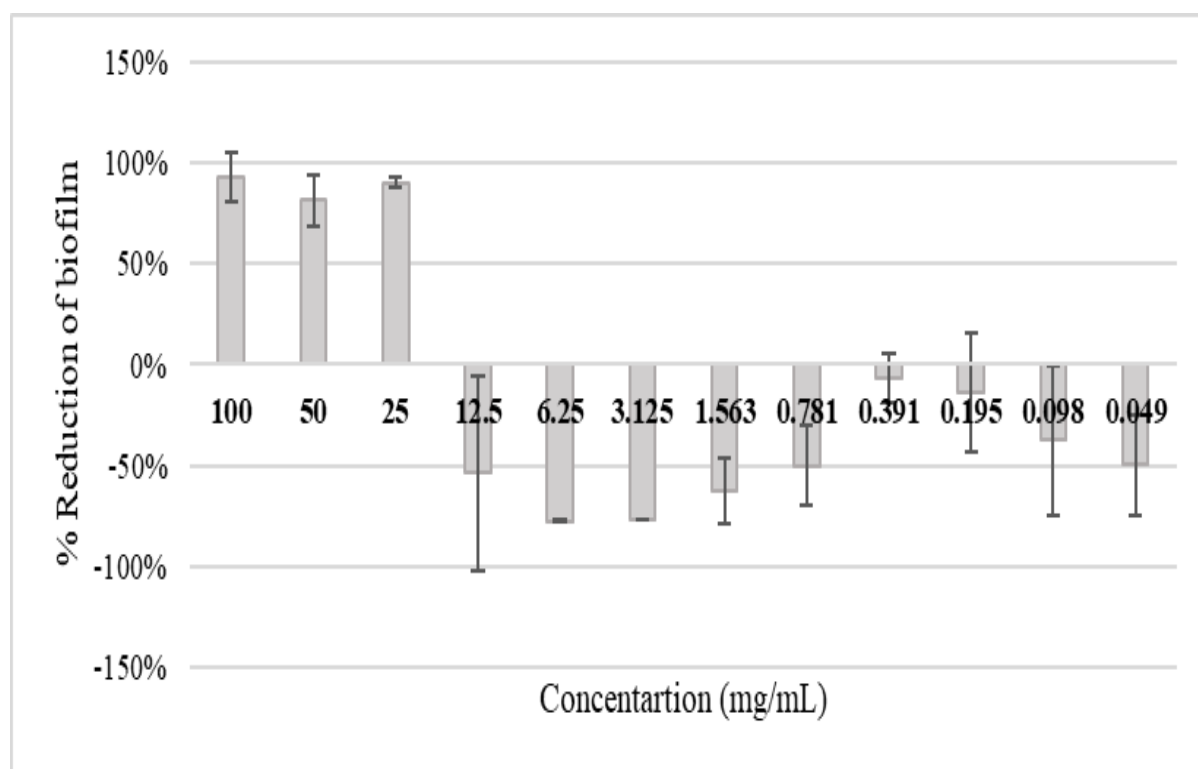
**Figure 1:** Biofilm inhibition activity of methanolic flower extract against *P. aeruginosa* biofilm

Table 6: Phytochemical compounds detected in *D. regia* methanolic leaves extract

Compound	Molecular formula	Molecular weight	Area (%)	RT (Min)
Aspartic acid	C ₄ H ₇ NO ₄	133.1	2.5	1.0
Lysine	C ₆ H ₁₄ N ₂ O ₂	146.2	1.4	1.1
Cinnamic acid	C ₉ H ₈ O ₂	148.2	3.2	1.2
Gallic Acid	C ₇ H ₆ O ₅	170.1	4.4	1.3
Caffeic acid	C ₉ H ₈ O ₄	180.16	3.2	1.4
Ferulic acid	C ₁₀ H ₁₀ O ₄	194.18	6.1	1.5
Germacrene D	C ₁₅ H ₂₄	204.4	3.0	1.6
Myristic acid	C ₁₄ H ₂₈ O ₂	228.4	4.3	1.7
Palmitic acid	C ₁₆ H ₃₂ O ₂	256.4	1.5	1.7
Linolenic acid	C ₁₈ H ₃₀ O ₂	278.4	5.6	1.8
Linoleic acid	C ₁₈ H ₃₂ O ₂	280.4	6.2	1.9
Oleic acid	C ₁₈ H ₃₄ O ₂	282.5	0.9	2.0
Stearic acid	C ₁₈ H ₃₆ O ₂	284.5	3.1	2.1
Luteolin	C ₁₅ H ₁₀ O ₆	286.2	5.5	2.3
Ricinoleic acid	C ₁₈ H ₃₄ O ₃	298.5	2.0	2.3
Quercetin	C ₁₅ H ₁₀ O ₇	302.2	4.2	2.4
Myricetin	C ₁₅ H ₁₀ O ₈	318.2	7.2	2.6
Erucic acid	C ₂₂ H ₄₂ O ₂	338.6	1.0	2.8
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	354.3	2.5	2.9
Stigmasterol	C ₂₉ H ₄₈ O	412.7	2.2	3.1
β-sitosterol	C ₂₉ H ₅₀ O	414.7	3.0	3.2
Lupeol	C ₃₀ H ₅₀ O	426.7	1.9	3.3
Epilupeol	C ₃₀ H ₅₀ O	426.7	2.1	3.5
Carotene	C ₄₀ H ₅₆	536.9	2.4	3.7
Rubixanthin	C ₄₀ H ₅₆ O	552.9	1.5	3.8
β-Cryptoxanthin	C ₄₀ H ₅₆ O	552.9	2.0	4.0
Lutein	C ₄₀ H ₅₆ O ₂	568.9	6.2	4.2
Zeaxanthin	C ₄₀ H ₅₆ O ₂	568.9	3.0	4.4
Pelargonidine 3-o-rutinoside	C ₂₇ H ₃₀ O ₁₄	578.5	2.1	4.6
Cyanidine 3-o-rutinoside	C ₂₇ H ₃₁ O ₁₅ ⁺	595.5	0.7	4.9
Astaxanthin	C ₄₀ H ₅₂ O ₄	596.8	1.1	5.2
Rutin	C ₂₇ H ₃₀ O ₁₆	610.5	4.0	5.6

Treatment at 24 and 48 h showed more pronounced biofilm compared with the 0 h treatment group. However, the biofilm observed in the treatment groups at 24 and 48 h lacked the consistency observed in the control group (Figure 2 e - h). Meanwhile, all the treatment groups, compared to the control group, showed lower presence of cell culture with morphological changes of bacterial cell walls.

DISCUSSION

D. regia was previously evaluated for antimicrobial activity on a variety of microbial strains. The studies found that the extracts of the leaves, stems, roots, bark, and flowers, and *D. regia* pigment extract (DRPE) demonstrated antimicrobial activity against bacterial (*Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella Typhimurium*) and fungal (*Aspergillus niger* and *Fusarium solani* and *Candida albicans*) strains [3,4].

To further explore the antibacterial activity of *D. regia*, three solvents were utilized to extract its flowers and leaves. The extraction yields obtained from *D. regia* indicate markedly higher yields with methanol for both flowers and leaves relative to water and chloroform. This implies that *D. regia* flowers and leaves are rich in polar and partially polar constituents. Water, being more polar than methanol, extracted only highly polar compounds.

To analyze the antibacterial activity of *D. regia* extracts, the well diffusion test was employed against the following bacterial strains: *B. cereus*, *S. aureus*, *E. coli*, and *P. aeruginosa*. The antibacterial activity of *D. regia* extracts showed that neither the chloroform flower extract nor the chloroform leaves extract showed antibacterial activity against any of the tested bacterial strains, suggesting a lack of antibacterial activity. Moreover, the extraction yield obtained with chloroform exhibited the lowest yield for both flowers and leaves compared to the other two solvents. Hence, the chloroform extract was excluded from the phytochemical screening of bioactive compounds.

Table 7: Phytochemical compounds detected in *D. regia* aqueous leaves extract

Compound	Molecular formula	Molecular weight	Area (%)	RT (Min)
Aspartic acid	C ₄ H ₇ NO ₄	133.1	2.3	1.0
Lysine	C ₆ H ₁₄ N ₂ O ₂	146.2	1.4	1.1
Cinnamic acid	C ₉ H ₈ O ₂	148.2	3.0	1.2
Gallic Acid	C ₇ H ₆ O ₅	170.1	4.1	1.3
Caffeic acid	C ₉ H ₈ O ₄	180.2	3.0	1.4
Ferulic acid	C ₁₀ H ₁₀ O ₄	194.2	6.0	1.5
Germacrene D	C ₁₅ H ₂₄	204.4	3.0	1.6
Myristic acid	C ₁₄ H ₂₈ O ₂	228.4	4.0	1.7
Palmitic acid	C ₁₆ H ₃₂ O ₂	256.4	1.3	1.7
Linolenic acid	C ₁₈ H ₃₀ O ₂	278.4	5.2	1.8
Linoleic acid	C ₁₈ H ₃₂ O ₂	280.4	6.0	1.9
Oleic acid	C ₁₈ H ₃₄ O ₂	282.5	0.6	2.0
Stearic acid	C ₁₈ H ₃₆ O ₂	284.5	3.0	2.1
Luteolin	C ₁₅ H ₁₀ O ₆	286.2	5.1	2.3
Ricinoleic acid	C ₁₈ H ₃₄ O ₃	298.5	1.6	2.3
Quercetin	C ₁₅ H ₁₀ O ₇	302.2	4.0	2.4
Myricetin	C ₁₅ H ₁₀ O ₈	318.2	8.2	2.6
Erucic acid	C ₂₂ H ₄₂ O ₂	338.6	0.8	2.8
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	354.3	2.2	2.9
Stigmasterol	C ₂₉ H ₄₈ O	412.7	2.0	3.1
β-sitosterol	C ₂₉ H ₅₀ O	414.7	2.6	3.2
Lupeol	C ₃₀ H ₅₀ O	426.7	1.6	3.3
Epilupeol	C ₃₀ H ₅₀ O	426.7	2.0	3.5
Carotene	C ₄₀ H ₅₆	536.9	3.0	3.7
Rubixanthin	C ₄₀ H ₅₆ O	552.9	1.2	3.8
β-Cryptoxanthin	C ₄₀ H ₅₆ O	552.9	2.0	4.0
Lutein	C ₄₀ H ₅₆ O ₂	568.9	6.0	4.2
Zeaxanthin	C ₄₀ H ₅₆ O ₂	568.9	2.8	4.4
Pelargonidine 3-o-rutinoside	C ₂₇ H ₃₀ O ₁₄	578.5	2.0	4.6
Cyanidine 3-o-rutinoside	C ₂₇ H ₃₁ O ₁₅ ⁺	595.5	0.5	4.9
Astaxanthin	C ₄₀ H ₅₂ O ₄	596.8	1.0	5.2
Rutin	C ₂₇ H ₃₀ O ₁₆	610.5	3.5	5.6

Aqueous extract of the flowers was highly active against *B. cereus* and *E. coli*. The aqueous extract of the leaves showed zones of inhibition only against *B. cereus* (high activity) and *S. aureus* (moderate activity), respectively. The methanolic extract of the flowers was active against all bacterial strains tested. This extract showed high activity against *B. cereus* and *S. aureus*, and moderate activity against *E. coli* and *P. aeruginosa*. This high activity of the methanolic flower extract may be linked to the solubility of the flavonoids, presence of rutin, lupeol, and β-sitosterol. The methanolic extract of the leaves exhibited zones of inhibition against *B. cereus* and *S. aureus*, indicating moderate activity. Moreover, the methanolic extract of the flowers exhibited the lowest MIC values, especially against *B. cereus*. The obtained MIC was equal to the MBC for *B. cereus*.

Phytochemical screening of the more active *D. regia* water and methanol extracts was performed using HPLC-MS. Notably, in the flower methanolic extract, rutin was the most abundant compound, followed by β-sitosterol and Lupeol. The three compounds, which accounted

together for 24 % of the extract, could be responsible for the observed broad antibacterial activity of the methanolic flower extract. Rutin is a natural flavonoid glycoside [11]. Rutin has a variety of pharmacological effects, such as antioxidant, anti-inflammatory, antihypertensive, maintaining vascular elasticity and neuroprotection activity [11]. It can inhibit the growth of *Escherichia coli*, *Pseudomonas vulgaris*, *Shigella sonnei*, *Klebsiella spp.*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* [12]. *Nepeta curviflora* methanolic extract was previously reported to have antibiofilm activity against *Pseudomonas aeruginosa* biofilm [13]. The quorum-sensing inhibitory effect of *Nepeta curviflora* extract was attributed to the presence of kaempferol, quercetin, salicylic acid, rutin, and rosmarinic acid [13]. β-sitosterol, the second most abundant component in methanolic flower extract, is a member of phytosterols that are characterized by a fundamental four-ring steroid [14]. It exhibits antioxidant, anti-inflammatory, anticancer, antimicrobial, angiogenic, immunomodulatory, and antidiabetic activities [15].

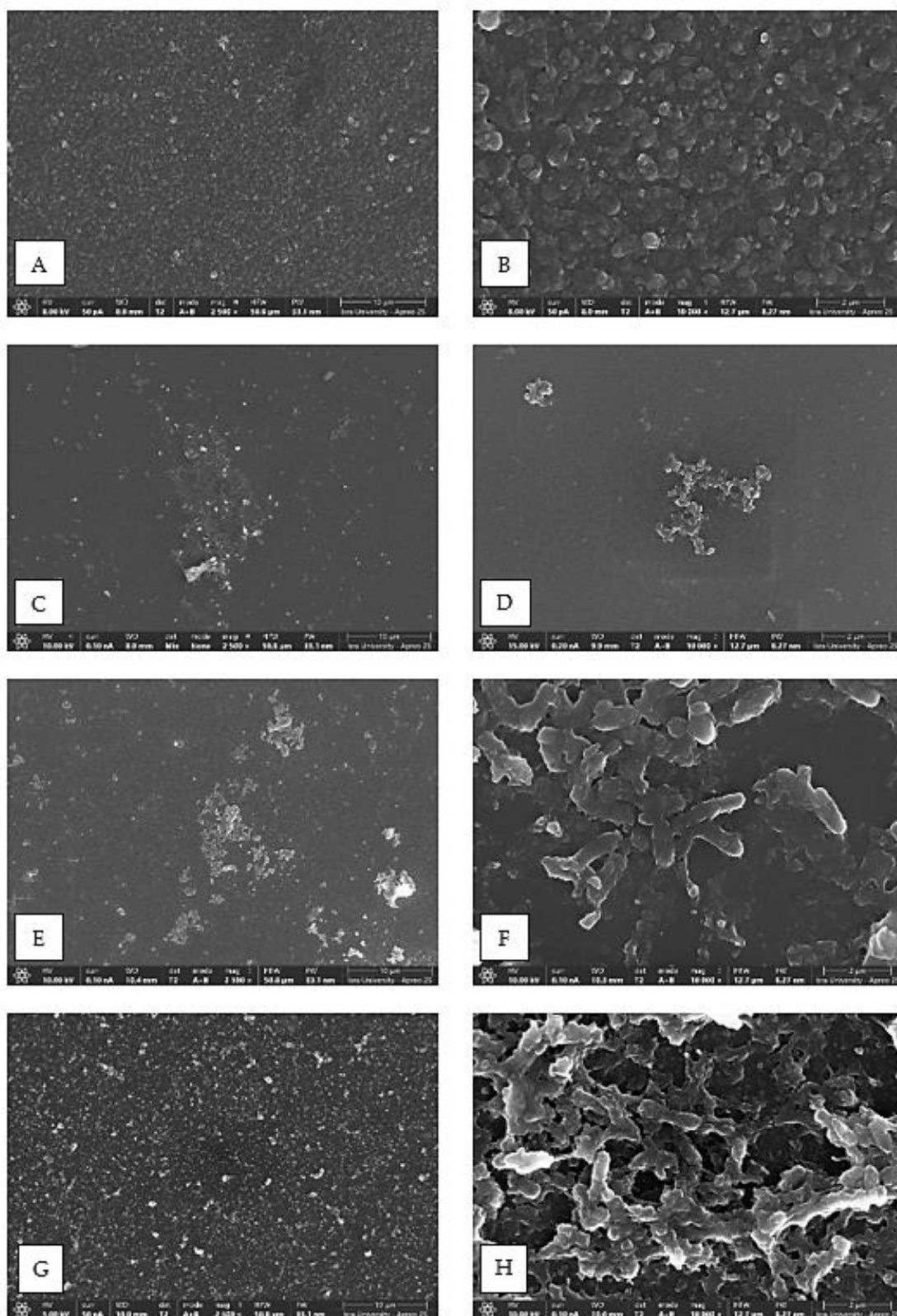


Figure 2: Scanning electron microscopy images of *P. aeruginosa* biofilm cultured on glass slides after 72 h, magnification x2500 (A) and x10000 (B) *P. aeruginosa* biofilm cultured on glass slides after 72 h, treated with *D. regia* methanolic flower extract, magnification x2500, (C) and x10000, (D) *P. aeruginosa* biofilm cultured on glass slides after 72 h, treated with *D. regia* methanolic flower extract at 24 h, magnification x2500, (E) and x10000, (F) *P. aeruginosa* biofilm cultured on glass slides after 72 h, treated with *D. regia* methanolic flower extract at 48 h, magnification x2500 (G) and x10000 (H)

The mechanism of the antibacterial activity was previously explained to be through inhibiting bacterial cell surface protein, “sortase,” thus preventing transpeptidation [16]. Lupeol, a dietary triterpene, also has anti-inflammatory, antioxidant, anticancer, and antibacterial properties [17]. Those compounds and other bioactive compounds that were found in the phytochemical analysis of the methanolic flower extract could be responsible for the observed antibacterial activity.

Methanolic extract of the flowers is the only extract that exhibited antibacterial activity against *Pseudomonas aeruginosa*. Hence, the study was taken one step further by determining the effect of the methanolic extract of *D. regia* flowers against *Pseudomonas aeruginosa* biofilm formation. It was observed that the concentrations 100, 50, and 25 mg/mL of the flowers’ methanolic extract significantly ($p < 0.5$) reduced biofilm formation. Overall, the activity is attributed to the richness of the extract with phytoconstituents having antimicrobial activity. Although the active concentrations appeared high for systemic use, they are fully appropriate for topical wound applications and antimicrobial coatings, where higher local doses are acceptable [18].

CONCLUSION

The methanolic flower extract of *D. regia* possesses strong antibacterial and antibiofilm activities, supported by a rich phytochemical profile. The lowest concentration exhibited biofilm reduction was 25 mg/mL, and the highest tested concentration was 100 mg/mL. The SEM examination of the biofilm showed a significant reduction in the formed biofilm, mainly when the extract was added before the initial biofilm phase. This study is the first to integrate the chemical composition of *D. regia* flowers and leaves extracts using HPLC–MS analysis, as well as testing their antibacterial, antibiofilm, and SEM properties against *P. aeruginosa*. These findings highlight the potential of *D. regia* as a promising candidate for topical antibacterial formulations and biofilm-preventive applications.

DECLARATIONS

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Ethical approval

Not applicable.

Use of Artificial intelligence/Large language models

The authors confirm that AI-based tools were used solely for language editing and manuscript polishing purposes (e.g., grammar, phrasing, and clarity). No AI tools were used to generate content, interpret data, or influence study design, results, or conclusions. All intellectual contributions, study analyses, and interpretations reported in the manuscript are the original work of the authors.

Availability of data and materials

The datasets used and/or analyzed during the current study are available within the manuscript.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Conceptualization, Suha Abudoleh and Ahmad Talhouni; methodology, Suha Abudoleh, Dima Lafi and Zead Abudayeh; formal analysis, Nisreen Dahshan; writing—original draft preparation, Nisreen Dahshan; writing—review and editing, Amjad Abuirmeleh and Juhaina Abuirshaid; supervision, Suha Abudoleh. All authors have read and agreed to the publication of this manuscript.

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