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Cynomorium coccineum extract inhibits biofilm formation and virulence factors and attenuates quorum sensing in *Pseudomonas aeruginosa*

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Abstract

This study aimed to evaluate the effectiveness of *Cynomorium coccineum* extract on several quorum sensing dependent mechanisms, including biofilm formation, the production of virulence factors, and the production of autoinducers in *Pseudomonas aeruginosa*. The impact on biofilm formation has been evaluated using a crystal violet assay and visualized using a light microscope and scanning electron microscope. The effect of the extract on swarming motility, aggregation, hydrophobicity, and exopolysaccharide production has been investigated. The ability of the extract to suppress the production of *P. aeruginosa* virulence factors, including pyocyanin, rhamnolipids, protease, and chitinase, has been evaluated. The antiquorum sensing mechanism was evaluated in *P. aeruginosa* by determining the effect on the production of acyl homoserine lactone, and *Chromobacterium violaceum*, to produce a violacein pigment. The chemical composition of the extract was analyzed using liquid chromatography mass spectrometry (LC-MS). The results of LC-MS analysis showed that *C. coccineum* extract is rich in quercetin and palmitic acid. The lowest concentration that inhibits 50% of *P. aeruginosa*'s ability to form biofilm (MBIC50) is 0.39 mg/mL. The extract at 0.78, 0.39, and 0.2 mg/mL exhibited a significant reduction in swarming motility, aggregation ability, surface hydrophobicity, and exopolysaccharide production. At these concentrations, the extract significantly suppressed the production of pyocyanin, rhamnolipids, protease, and chitinase. The ability to inhibit the production of violacein and acyl homoserine lactone indicated the anti-quorum sensing activity of *C. coccineum* extract. In conclusion, *C. coccineum* extract can be considered a promising candidate to be developed as an anti-infective drug.

Key words: Anti-infective, Quorum sensing, Biofilm, *Cynomorium coccineum*, *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa is one of the most common gram negative opportunistic bacteria. Its infections extend from local infections in immune-intact patients to severe, life-threatening infections in immune-impaired patients (Tapper & Armstrong, 1974). Once it adheres to a target tissue, *P. aeruginosa* recruits several virulence factors to invade the bloodstream and spread to several organs, causing serious or lethal health conditions such as septicemia, shock, and organ dysfunction. Those who suffer from bacteremia are usually subjected to prolonged, intensive antibiotic therapy. In some cases, finding effective antibiotics to treat *P. aeruginosa* infections is not an easy task due to their ability to resist a variety of antibiotic classes (Pang et al., 2019; Prevention, 2019). Therefore, infections due to *P. aeruginosa* are associated with a high persistent rate of mortality reaching 61% (Chatzinikolaou et al., 2000; Elmassry et al., 2023; Kang et al., 2003).

The ability of this bacteria to resist a variety of antibiotic classes and the use of multifactorial virulence factors that facilitate entrance, invasion, spread, and the overcome immune system make its infections difficult to treat. *P. aeruginosa* has been classified within the ESKAPE group; hence, attention has been given to the urgent need for effective antimicrobial agents to control their life-threatening infections (Pang et al., 2019; Shrivastava et al., 2018). Several strategies have been applied and modified, and newly developed agents have been adopted to minimize the antibiotic-resistant phenomenon. One of the most attractive strategies is to find anti-infective agents that attenuate the pathogenicity of the microbes and restrict their ability to cause disease, thus allowing the defense mechanisms to powerfully eliminate the infection (Ciofu & Tolker-Nielsen, 2019; Zloh, 2019).

As part of its pathogenicity, *P. aeruginosa* employs specific communication mechanisms to coordinate the release of the virulence factors. These regulatory mechanisms are known as quorum sensing. Bacteria are aggregated as a result of the secretion of specific molecules known as autoinducers. Once the cells reach a specific density, the autoinducer secretion increases, autoinducer diffuses into the cytoplasm, and binds with their specific receptors, which in turn activates several virulence factor-related genes (Duplantier et al., 2021; Z. Li & Nair, 2012; Naoun et al., 2022).

Finding an agent that can interfere with this quorum-sensing circuit could attenuate or inhibit the mechanism of pathogenicity. Due to their composition variability and traditional uses, plant natural products gain significant attention in medicinal chemistry. They were found to possess multiple modes of action against a broad range of biological processes. Previous reports showed that plants may act as a good source for finding anti-quorum sensing candidates (Camele et al., 2019).

Cynomorium coccineum (*C. coccineum*), whose Arabic name is tarthuth, is a non-photosynthetic parasitic plant that grows in salty, dry, or rocky soils. It can be found in the Mediterranean region and in central Asia, where it is traditionally used as an aphrodisiac to treat erectile dysfunction, immunomodulatory disorders and stomach ulcers. Scientific evidence about its broad biological activities, such as antioxidant, anticancer, antimicrobial, anti-tyrosinase, spermatogenesis and sperm motility, ovarian increase and folliculogenesis, and cardioprotective, has been confirmed previously (Zucca et al., 2019). The hydromethanolic extract of *C. coccineum* was found to inhibit the visible growth of *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, and *Escherichia coli*, as indicated by the minimum inhibitory concentration of 25 mg/mL, while *Pseudomonas aeruginosa* and *Enterococcus faecalis* were found to be resistant (Kadri & Nani, 2022). Zucca et al. (2016) showed that the aqueous extract of *C. coccineum* possesses narrow-spectrum antibacterial activity against gram-positive bacteria.

This study was conducted to investigate the effect of *C. coccineum* methanolic extract on *P. aeruginosa* quorum sensing. As part of this system, the effect on *P. aeruginosa* biofilm formation and virulence factors production was evaluated.

2. Materials and Methods

Plant materials collection and extraction

Cynomorium coccineum (Figure 1) was collected from southern Jordan, Lajjun, Karak, Jordan. This was in March and April 2022. The plant was identified by Dr. Feryal Al-Khresat (Biology department, Mutah University, Karak, Jordan). The voucher specimen (MU2022-03) was deposited in the same department.

The plant materials were washed, dried, grinded, and subjected to the extraction process. The extraction was made by soaking the plant materials in absolute methanol for 24h. The plant materials were removed by filtration and the solvent was collected. The solvent was removed using a rotary evaporator and the crude extract produced was collected and stored at 4C.

Figure 1. in situ image for *Cynomorium coccineum* collected from Lajjun, Karak, Jordan.

Bacterial species

Pseudomonas aeruginosa was obtained from Alkarak Government Hospital, Karak, Jordan. The bacterium was isolated from a urine sample and identified using standard methods. The identification was confirmed using bioMérieux VITEK 2 system. *Chromobacterium violaceum* ATCC 12472 was purchased from the American Type Culture Collection.

LC-MS

LC-MS analysis has been carried out to identify the chemical composition of *C. coccineum* methanolic extract. LC-MS-8030- (Tripple Quad Ms) coupled to LC-8030 MS/MS from Shimadzu LC (Japan) equipped with turbo ion spray (electrospray) ionization source (ESI). Chromatographic separation was achieved on Primesil RP-C18 column, (150-4.6mm, 5mm). The mobile phase A was water/acetonitrile (80:20% v/v) with 0.01% formic acid (pH 3), while the mobile phase B was acetonitrile with 0.01% formic acid(Merck). The injection volume of 10 µL, the mobile phase flow rate was 0.7 mL/min, and the column temperature was 25°C. The gradient elution started at 20% of mobile phase B, ramped to 50% B by 18 min, then to 100% B at 20 min, and held for 1 min. At 22 min, 20% B was re-adjusted and held constant for 2 min. Total run time was 31 min.

Antibacterial activity

The antibacterial activity of *C. coccineum* shoot methanolic extract has been evaluated using the disc diffusion method (ALrawashdeh et al., 2019). In brief, Muller Hinton agar was inoculated with 100 µL of *P. aeruginosa* culture containing 1x 10^8 CFU/mL (adjusted to 0.5 McFarland solution). Then, a disc loaded with 1 mg of the extract was placed on the surface of the agar plate and incubated at 37°C. After 24h, the inhibition zone formed was measured in mm.

To determine the minimum inhibitory concentration (MIC) of *C. coccineum* extract, a 96 well-plate was used (Jaafreh et al., 2019). Each well was filled with 200 µL of MHB and the extract was serially diluted to produce concentrations equal to 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2, and 0.1 mg/mL. Then, 10 µL of *P. aeruginosa* culture containing 10^4 CFU/mL was added to each test well. DMSO was prepared similarly as a negative control. Wells containing the extract without bacteria or bacteria without extract were also prepared. The plate was incubated for 24h at 37°C and the lowest concentration inhibiting the bacteria's visible growth was reported as the MIC.

Biofilm inhibitory effect

Antibiofilm activity

The ability of *C. coccineum* extract to prevent *P. aeruginosa* biofilm formation was evaluated using crystal violet (Qaralleh, 2023). Different concentrations of *C. coccineum* extract were prepared in a 96-well plate using MHB. Then, 10 µL of *P. aeruginosa* culture containing 10^4 CFU/mL was added to each test well. The plate was incubated for 24h at 37°C. Then, the plate contents were discarded, and the plate was washed three times with PBS. After that, the plate was filled with crystal violet and incubated for 15 min. Again, the plate contents were discarded, and the plate was washed three times with PBS. The plate was filled with ethanol as a decolorizing agent for 15 min. Then, the plate contents were transferred to a new 96-well plate, and the abs of 590 nm were recorded. The percentage of biofilm inhibition was calculated in reference to the untreated control group, and the concentration that caused a 50% inhibition in biofilm formation was reported as the minimum biofilm inhibitory concentration (MBIC50%).

Biofilm Viable cells

The tetrazolium salt 2,3,5-triphenyl-tetrazolium chloride (TTC) was used to determine the effect on the viable cells within the biofilm (Gordya et al., 2017). A 96-well plate was prepared as in the MIC and crystal violet protocols. The contents of the wells were discarded once the incubation period was completed (24 hours). Then, the wells were repeatedly washed, and TTC solution was added at a volume equal to 200 µL. This solution was prepared using both media, glucose (0.2%) and TTC (50 µL, 5 mg/mL). The prepared 96-well plate was then subjected to incubation under the following conditions: temperature 37°C, agitation 150 rpm, and a period of 6 hours. After that, the abs at 405 nm were measured, and the percent of viable cells was calculated.

Biofilm visualization using light microscope and scanning electron microscope

To microscopically observe the effect of *C. coccineum* extract on *P. aeruginosa* biofilm, bacteria treated with 0.78, 0.39, 0.2, and 0 (control) were cultured on 12 well-plate containing sterile coverslips. The prepared plate was cultured for 24h at 37°C. For light microscopy examination, the coverslips were removed, washed gently with PBS, and stained with crystal violet for 5 min. For SEM examination, the coverslips were removed, washed gently with PBS, and soaked in 2.5% glutaraldehyde for one hour. Then, the slips were subjected to the dehydration process using gradient concentrations of ethanol in water (10, 30, 50, 70, and 100%). The samples were then subjected to SEM analysis.

Effect on biofilm formation stages

Swarming motility

The effect of *C. coccineum* extract on *P. aeruginosa* swarming motility was evaluated using swarming agar (Sagar et al., 2022). In brief, swarming agar containing 0.78, 0.39, 0.2, and 0 mg/mL extract was prepared. Then, 1 µL of *P. aeruginosa* was cultured on the center of the prepared agar plates. After 48 hours of incubation at 37°C, the zone of motility was measured in mm.

Aggregation

The effect of *C. coccineum* extract on *P. aeruginosa* aggregation was determined according to Shanks et al. (2008). Briefly, *P. aeruginosa* treated with 0.78, 0.39, 0.2, and 0 mg/mL of extract was incubated for 24h at 37°C. The abs at 600 nm for these cultures were measured and recorded as the first absorbance. Then, the cultures were vortexed for 1 min, and the abs at 600 nm were measured as a second absorbance. The percentage of aggregation was calculated using the following formula:

$$percentage\ of\ aggregation = \frac{second\ abs - first\ abs}{second\ abs} \times 100\%$$

Hydrophobicity

The effect of *C. coccineum* extract on *P. aeruginosa* surface hydrophobicity was determined using n-hexadecane (Rosenberg et al., 1980). Briefly, *P. aeruginosa* treated with 0.78, 0.39, 0.2, and 0 mg/mL of extract was incubated for 24h at 37°C. The abs at 600 nm for these cultures were measured and recorded as the first absorbance. Then, 1.5 mL of the culture was mixed with 1.5 mL of n- hexadecane. After 15 min, the abs at 600 nm were measured as a second absorbance. The percentage of hydrophobicity was calculated using the following formula:

$$percentage\ of\ hydrophobicity = \frac{first\ abs - second\ abs}{first\ abs} \times 100\%$$

Exopolysaccharides (EPS) production

The phenol-sulfuric acid method was used to determine the effect of *C. coccineum* extract on the EPS production of *P. aeruginosa* (Razack et al., 2011). Briefly, *P. aeruginosa* treated with 0.78, 0.39, 0.2, and 0 mg/mL of extract was incubated for 24h at 37°C. A portion of this culture was added to cold ethanol. The mixture was then subjected to cold incubation at 4°C for 24h. After centrifugation, deionized water was added to the precipitated pellet, along with 5% cold phenol and concentrated H2SO4 in a proportion of 1:1:5. Finally, the abs at 490 nm of this solution was measured, and the percentage of EPS production was calculated.

Effect on virulence factors

Pyocyanin

The effect of the extract on pyocyanin production was evaluated based on the modified method of (Hossain et al., 2017). Briefly, *P. aeruginosa* treated with 0.78, 0.39, 0.2, and 0 mg/mL of extract was incubated for 24h at 37°C. A portion of this culture was centrifuged, and 7.5 mL of the blue-green supernatant was subjected to liquid-liquid extraction using 4.5 mL of chloroform. Then, 1.5 mL of hydrochloric acid (0.2N) was added. Finally, the abs at 520 nm of the developed pink solution was measured, and the percentage of pyocyanin inhibition was calculated.

Rhamnolipids

The effect of the extract on rhamnolipids production was evaluated using orcinol method (Luo et al., 2017). Briefly, *P. aeruginosa* treated with 0.78, 0.39, 0.2, and 0 mg/mL of extract was incubated for 24h at 37°C. A portion of this culture was centrifuged, and 1 mL of the supernatant was subjected to liquid-liquid extraction using 3 mL of diethyl ether. Then, the diethyl ether layer was collected and the solvent was removed using rotary evaporator. To the yielded materials, 200 µL of distilled water, and 900 mL of 0.18% orcinol (w/v) in 53% (v/v) H_2SO_4were added. The prepared solution was boiled for 30 min. Finally, the abs at 421 nm of this solution was measured, and the percentage of inhibition in rhamnolipids production was calculated.

LasA protease

The effect of the extract on Las protease activity was evaluated according to the modified method of (Hossain et al., 2017). Briefly, *P. aeruginosa* treated with 0.78, 0.39, 0.2, and 0 mg/mL of extract was incubated for 24h at 37°C. A portion of this culture was centrifuged, and 1 mL of the supernatant was transferred to tubes containing 5 mL of casein (0.65% in 50 mM Tris-HCl). The reaction was incubated for 30 min at 37°C. As a reaction terminator, pre-chilled trichloroacetic acid (5 mL, 10%) was added to the reaction tubes which were incubated at 35C for 30 min. After centrifugation, two reagents were added to the supernatant: sodium carbonate (5 mL, 0.5 M) and Folin's reagent (1 mL). The solutions were left at 37°C for 30 min and the abs at 660 nm was measured, and the percentage of inhibition in protease activity was calculated.

Chitinase activity

The effect of the extract on Chitinase activity was evaluated based on the modified method of (Hossain et al., 2017). The chitin azure solution was prepared by mixing 1.3 mg/mL with 130 mL of sodium phosphate buffer (200 mM, pH 7.0). The solution was then subjected to incubation for seven days at 37C and agitation rate of 150 rpm. *Pseudomonas aeruginosa* treated with 0.78, 0.39, 0.2, and 0 mg/mL of extract was incubated for 24h at 37°C. A portion of this culture was centrifuged, and 0.5 mL of the supernatant was combined with 4.5 mL of chitin azure solution. The reaction was left to proceed overnight at 37C. After centrifugation (16,000, 10 min), the abs at 570 nm of the supernatant was measured, and the percentage of inhibition in chitinase activity was calculated.

Quorum sensing inhibitory effect

Anti-quorum sensing against *C. violaceum*

The anti-quorum sensing activity of *C. coccineum* extract was evaluated based on the extraction and quantification of violacein pigment (Chen et al., 2022). To perform this test, a portion (1 mL) of *C. violaceum* treated with 0.78, 0.39, 0.2, and 0 (control) of the extract was centrifuged, and the supernatant was removed while the pellet was resuspended in DMSO (1 mL). The mixture was incubated at room temperature for 3 hours and then centrifuged. The DMSO containing violacein pigment was then collected, and the abs at 575 nm was recorded, and the percentage of violacein inhibition was calculated.

Acyl homoserine lactone

The effect of the extract on AHL production was evaluated based on the modified method of (Lahiri et al., 2021). *P. aeruginosa* treated with 0.78, 0.39, 0.2, and 0 mg/mL of extract was incubated for 24h at 37°C. A portion of this culture was centrifuged, and portion from this supernatant was combined with ethyl acetate. After 10 min, the solution was collected and concentrated using rotary evaporator. From this, 40 µL was combined with 50 µL of a 1:1 hydroxyl amine (2M): NaOH (3.5M) and 90 µL 1:1 mixture of ferric chloride (10% in 4M HCl): 95% ethanol. Finally, the abs at 520 nm were measured, and the percentage of inhibition in AHL production was calculated.

Statistical analysis

The significance difference between the tested group and the control untreated group was performed using GraphPad prism 8 and one-way ANOVA. The significant difference was stated as *, **, or *** based on the values of P of <0.05, <0.01, and <0.001, respectively.

3. Results

Chemical composition

Table 1. chemical composition of *C. coccineum* shoot methanolic extract using LC-MS

	Chemical class	Chemical compound	Chemical formula	%
1	Phenols	Gallic acid	$C_6H_2(OH)_3CO_2H$	1.1
2		Salicylic acid	HOC_6H_4COOH	0.7
3		Syringic acid	$C_9H_{10}O_5$	2.4
4	Flavonoids	Rutin	$C_{27}H_{30}O_{16}$	13.4
5		Procyanidin	$C_{30}H_{26}O_{13}$	12.1
6		Taxifolin	$C_{15}H_{12}O_7$	1.3
7		Quercetin	$C_{15}H_{10}O_7$	24.1
8	Fatty acids	Palmitic acid	$C_{16}H_{32}O_2$	18.1
9		Myristic acid	$C_{14}H_{28}O_2$	11.4
10		Oleic acid	$C_{18}H_{34}O_2$	0.8
11		Stearic acid	$C_{18}H_{36}O_2$	0.9
12	Triterpenoids	Betulinic acid	$C_{30}H_{48}O_3$	2.3
13		Ursolic acid	$C_{30}H_{48}O_3$	1.7
14	Sterols	β-Sitosterol	$C_{29}H_{50}O$	4.4
		Total		94.7

The chemical composition of *C. coccineum* shoot methanolic extract has been explored using LC-MS. As shown in Table 1, fourteen compounds have been identified, representing 94.7% of the total compounds. The results also showed that *C. coccineum* extracts are rich in flavonoids and fatty acids, accounting for 40.9 and 31.2%, respectively. The most dominant compounds were quercetin (24.1%), palmitic acid (18.1%), rutin (13.4%), procyanidin (12.1%), and myristic acid (11.4%). Other components, such as β-sitosterol (4.4%), syringic acid (2.4%), and betulinic acid (2.3%), have also been characterized.

Antibacterial activity

Table 2. inhibition zone and MIC of *C. coccineum* extract against *P. aeruginosa*

	Inhibition zone (mm)	MIC (mg/mL)
C. coccineum extract (1 mg/disc)	14.2±1.0	3.13
Cefotaxime (30 µg/disc)	17.75±2.15	-
DMSO	0.0±0.0	-

The inhibitory effect of *C. coccineum* methanolic extract on *P. aeruginosa* growth was identified based on the inhibition zone observed and the value of the MIC. As shown in Table 2, the inhibition zone of *C. coccineum* extract (1 mg/disc) against *P. aeruginosa* was 14.2 mm. However, complete inhibition of *P. aeruginosa* growth, as indicated by the MIC value, was found to be equal to 3.3 mg/mL. The negative control, DMSO, exhibited no inhibitory effect on *P. aeruginosa* growth while the positive control, Cefotaxime (30 µg/disc), displayed an inhibition zone of 17.75 mm.

Antibiofilm (crystal violet assay)

The ability of *C. coccineum* methanolic extract to prevent the formation of *P. aeruginosa* biofilm was evaluated using a crystal violet assay. The results showed that the extract inhibited

the formation of biofilm completely (more than 90%) at concentrations of 25, 12.5, 6.25, 3.13, and 1.56 mg/mL (Figure 2). At lower concentrations, a dose-dependent inhibition of biofilm formation was observed. The maximum percentage of biofilm inhibition was 85.2% at the treatment concentration of 0.78 mg/mL. The percentage of inhibition decreased as the concentration decreased to reach 49.2, 32.7, and 3.6% when the treatment was made using 0.39, 0.2, and 0.1 mg/mL, respectively. As shown in Figure 2, the lowest concentration that inhibits 50% of *P. aeruginosa*'s ability to form biofilm (MBIC50) is 0.39 mg/mL.

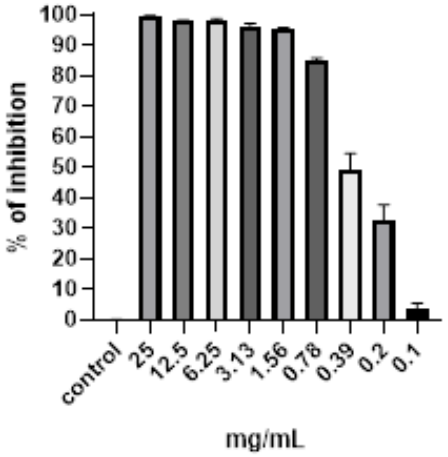

Figure 2. The effect of *C. coccineum* methanolic extract on the ability of *P. aeruginosa* to form biofilm. The result is expressed as a percent inhibition in biofilm formation. The percent inhibition for the control, untreated cells was calculated as 0.

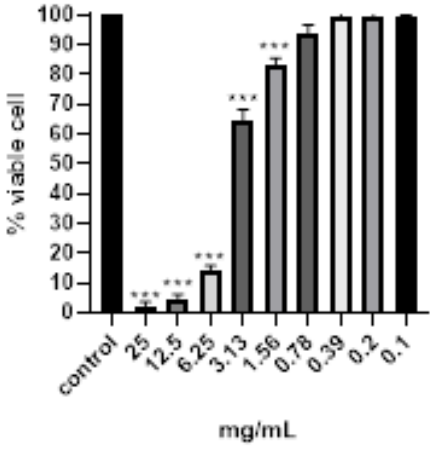

Figure 3. The effect of *C. coccineum* methanolic extract on *P. aeruginosa* biofilm viable cells. The result is expressed as a percent of viable cells. The percent of viable cells for the control, untreated cells, was calculated as 100. The significant difference between the treatment groups and the control group, untreated cells, was expressed as *** corresponding to the p-values of $p<0.001$.

Biofilm Viable cell

This test was performed to observe the effect of *C. coccineum* extract on the viable cells within the biofilm. As shown in Figure 3, a significant ($p<0.001$) reduction in *P. aeruginosa* viable cells was observed at the treatment concentrations of 25, 12.5, 6.25, 3.13, and 1.56 mg/mL. On the counter, at 0.78, 0.39, 0.2, and 0.1 mg/mL, there was no significant reduction in the viable cells. Based on the results of the crystal violet assay and the TTC assay, the concentrations of 0.78, 0.39, and 0.2 mg/mL have been selected for further investigation.

Biofilm visualization using light microscope and scanning electron microscope

As indicated by the light and SEM images (Figures 4 and 5), the cells within the normally grown biofilm (control) were clumped and aggregated. The cells appear as multilayer adherent cells, which indicates the formation of biofilm. However, as the concentration of treatment increases, the ability of the cells to aggregate and adhere to each other decreases. Due to the treatment with *C. coccineum* extract, the ability of *P. aeruginosa* to aggregate in a multilayered system has ceased, indicating the effectiveness of *C. coccineum* extract in preventing biofilm formation.

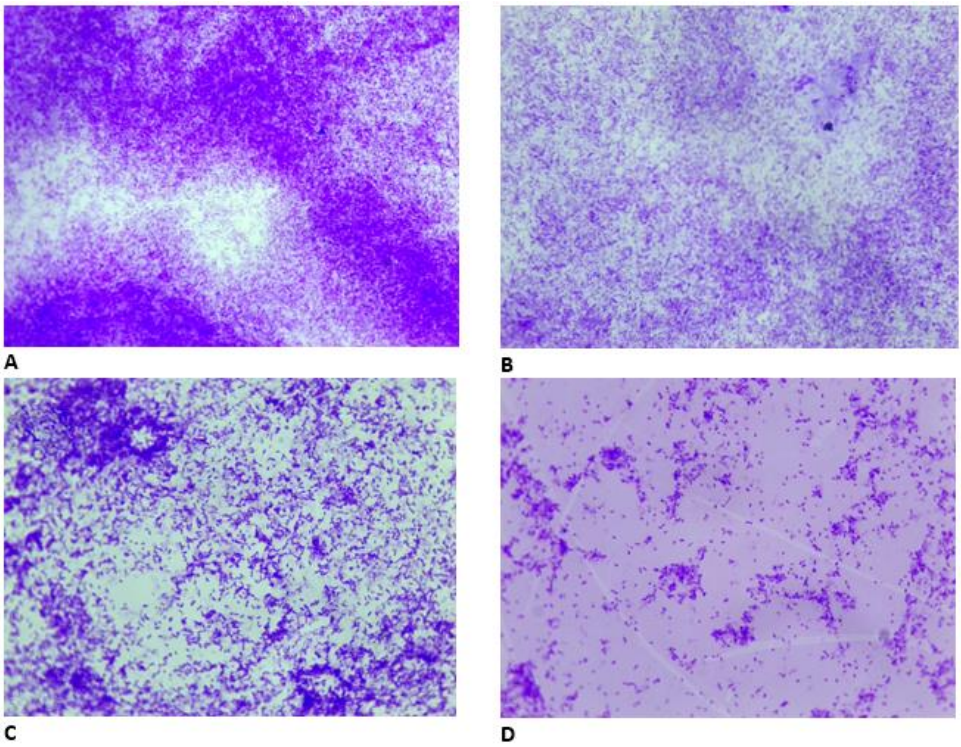

Figure 4. light microscope images of *P. aeruginosa* treated with 0 (A), 0.2 (B), 0.39 (C), and 0.78 (D) mg/mL *C. coccineum* extract.

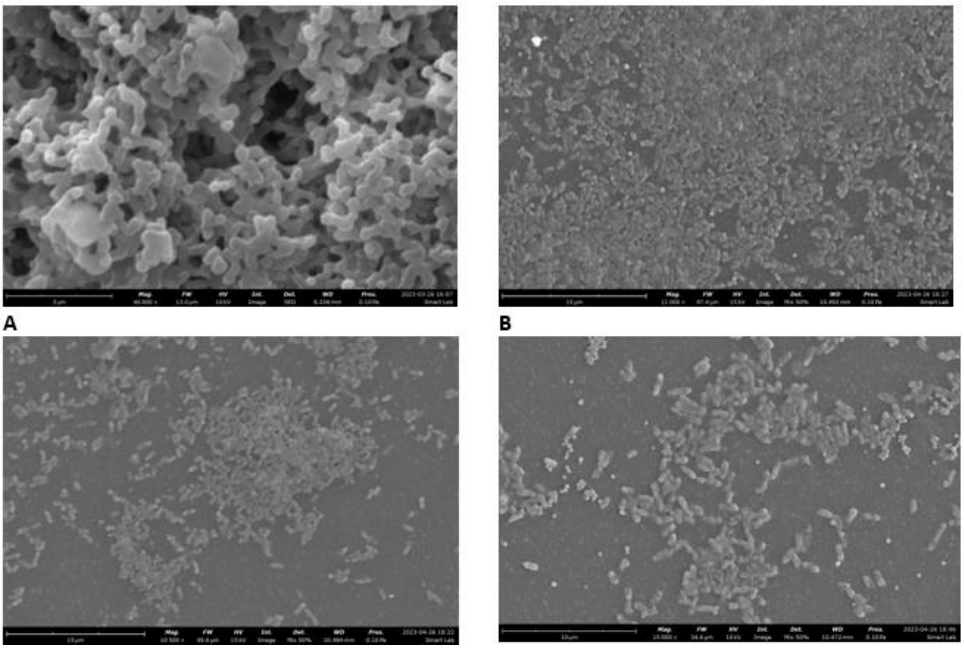

Figure 5. SEM images of *P. aeruginosa* treated with 0 (A), 0.2 (B), 0.39 (C), and 0.78 (D) mg/mL *C. coccineum* extract.

Effects on different stages of biofilm formation

Swarming motility

The inhibitory effect of *C. coccineum* extract on *P. aeruginosa* swarming motility is dose-dependent (Figure 6A). The results indicated the ability of 0.78, 0.39, and 0.2 *C. coccineum* extract to significantly reduce the swarming motility from 55 mm to 10.8, 16.5, and 26.7 mm, respectively.

Aggregation

A dose-dependent inhibition effect of the *C. coccineum* extract on the aggregation ability of *P. aeruginosa* was observed (Figure 6B). A significant ($p<0.001$) reduction in the percentage of aggregation from 55.1% to 10.5 and 18.6% was observed at concentrations of 0.78 and 0.39 mg/mL, respectively. There was no significant reduction in the aggregation at the treatment concentration of 0.2 mg/mL.

Hydrophobicity

A dose-dependent reduction in the surface hydrophobicity of *P. aeruginosa* was observed when treated with 0.78, 0.39, and 0.2 mg/mL of *C. coccineum* extract (figure 6C). However, the reduction was significant for the treatment concentrations of 0.78 and 0.39 mg/mL. The percentage of hydrophobicity was reduced from 82.8% to 51.2 and 58.2%, respectively. There was no significant reduction in the percentage of hydrophobicity at a concentration of 0.2 mg/mL.

Exopolysaccharides production

The production of EPS was decreased in a dose-dependent manner (Figure 6D). A significant ($p<0.01$) reduction of 68.3% in EPS production was observed at the treatment concentration of 0.78 mg/mL. The reduction due to the treatment with 0.39 mg/mL was also significant ($p<0.05$). There was no significant reduction in EPS production at 0.2 mg/mL.

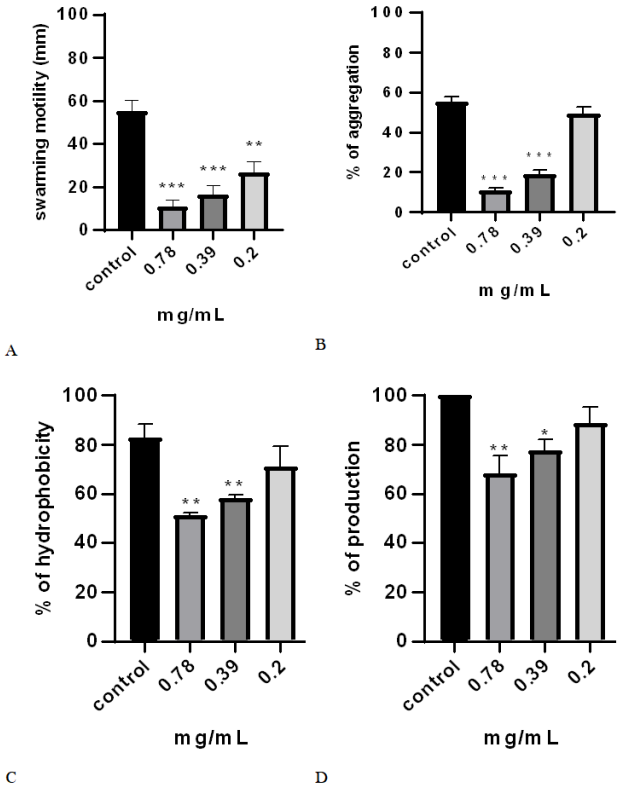

Figure 6. The effect of *C. coccineum* methanolic extract on different stages in biofilm formation, including swarming motility (A), aggregation (B), hydrophobicity (C), and EPS production.

Effect on the virulence factors

Pyocyanin

As shown in Figure 7A, *C. coccineum* extract exhibited a concentration dependent inhibition in pyocyanin production in *P. aeruginosa*. The percentage of pyocyanin inhibition was significant ($p<0.01$), reaching 48.4% when *P. aeruginosa* was treated with 0.78 mg/mL, whereas it was 27.7% when the treatment was at 0.39 mg/mL. There was no significant reduction in pyocyanin production at the treatment concentration of 0.2 mg/mL.

Rhamnolipids

As shown in Figure 7B, the production of rhamnolipids was reduced significantly as the concentration of the extract increases. At 0.78 and 0.39 mg/mL, *C. coccineum* extract inhibited rhamnolipids production significantly ($p<0.01$) by 46.2 and 36.9%, respectively. The percentage of inhibition of rhamnolipids production was also significant ($p<0.05$) when the treatment was at 0.2 mg/mL.

LasA protease

The effect of the extract on LasA protease activity as one of the *P. aeruginosa* quorum sensing factors was evaluated (figure 7C). A concentration-dependent inhibition in LasA protease activity was observed with *C. coccineum* extract treatment. However, a significant ($p<0.01$) reduction of 35.7% was observed at the treatment concentration of 0.78 mg/mL. At lower concentrations, there was no significant reduction in the percentage of inhibition of LasA protease activity.

Chitinase

Chitinase as a *P. aeruginosa* virulence factor has been investigated in this study. As shown in Figure 7D, a concentration-dependent inhibition pattern was observed with *C. coccineum* extract treatment. The maximum percentage of inhibition ($p<0.001$) in chitinase activity of 58.9% was observed when the treatment was at 0.78 mg/mL. Ranked second is the treatment at 0.39 mg/mL, which causes a significant reduction ($p<0.001$) in chitinase activity of 40.1%. At the lowest concentration (0.2 mg/mL), the reduction in the chitinase activity was also significant ($p<0.01$) reaching 29.3%.

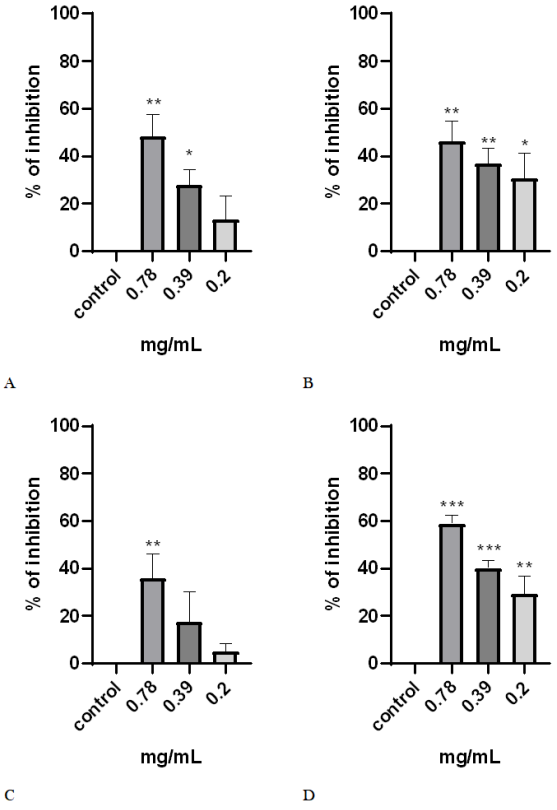

Figure 7. The effect of *C. coccineum* methanolic extract on *P. aeruginosa* virulence factors, including pyocyanin (A), rhamnolipids (B), LasA protease (C), and chitinase (D).

Quorum sensing inhibitory effect

Anti-quorum sensing activity

The anti-quorum sensing activity of *C. coccineum* extract has been evaluated using C. *violaceum*. As shown in Figure 8A, a dose-dependent inhibition in violacien production was

observed. However, there was a significant ($p< 0.001$) reduction in violacein production at concentrations of 0.78 and 0.39 mg/mL; the reduction at 0.2 mg/mL was not significant.

Effect on AHL production

As shown in Figure 8B, the reduction in acyl homoserine lactone (AHL) is in a dose-dependent manner. A significant reduction in AHL production was observed at concentrations as low as 0.2 mg/mL. The AHL production was reduced significantly ($p<0.001$) by 81.4 and 40.0% at the treatment concentrations of 0.78 and 0.39 mg/mL, respectively. The percentage of inhibition in AHL production of *P. aeruginosa* treated with 0.2 mg/mL of *C. coccineum* extract was also significant ($p<0.01$), reaching 13.3%.

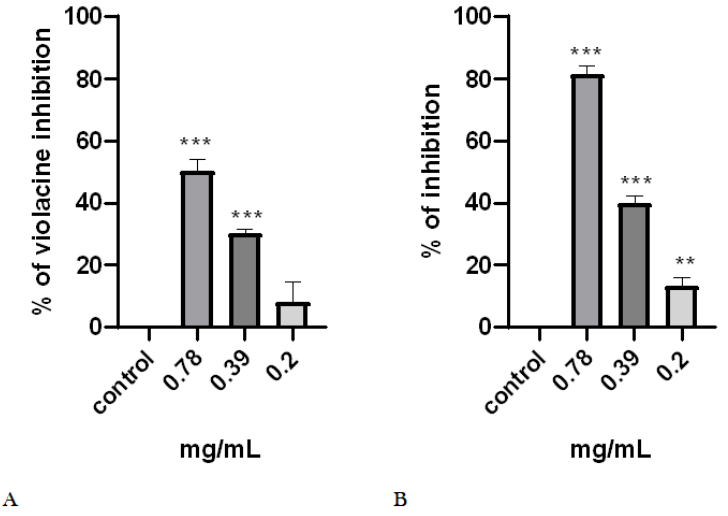

Figure 8. The effect of *C. coccineum* methanolic extract on the ability of *C. violaceum* to produce violacein pigment (A), and *P. aeruginosa* to produce AHL (B).

4. Discussion

Biofilms are considered one of the most important structural and functional factors that provide protection for bacteria against antibiotics and the ability to live inside the host. About 80% of the bacteria that make up biofilms can cause persistent infections, such as infection with *P. aeruginosa* in cystic fibrosis (Algburi et al., 2017). Due to the strength that biofilms give bacteria to resist antibiotics, many chemical, physical and biological agents have been employed to avoid the increase in the phenomenon of antibiotic resistance and to treat infections resulting from these bacteria, such as plant extracts, bio-surfactants, nanomaterials, ultrasound, and Photodynamic (Ceresa et al., 2023). Traditional folk use and scientific studies make plants important sources of antimicrobial and anti-infective compounds which can be developed as drugs to treat infections resulting from biofilm-forming bacteria (N. Singh et al., 2023; Yincharoen et al., 2021). Recently, many studies have shown the ability of plant extracts to inhibit quorum sensing and all quorum sensing related factors such as virulence factor production and biofilm formation (Qaralleh, 2023; Qaralleh et al., 2020, 2024). To the best of our knowledge, no data are available about the anti-quorum sensing, anti-virulence and antibiofilm activities of *C. coccineum* extract against *P. aeruginosa*. It is a parasitic plant with reported activities like antioxidant, anticancer, antimicrobial, anti-tyrosinase, spermatogenesis,

folliculogenesis, and cardioprotective (Zucca et al., 2019). In the current investigation, the antiquorum sensing potential of *C. coccineum* shoot methanolic extract against *P. aeruginosa* was evaluated. Also, the effect on the antibiofilm and antivirulence activities, as quorum sensing regulated factors, were demonstrated.

The chemical composition of the *C. coccineum* methanolic extract has been determined using LC-MS. The majority of the contents was flavonoids and fatty acids representing more than 73% of the total contents. Based on the literature, more than 76 compounds, belonging to different chemical classes such as flavonoids, terpenoids, steroids, and saccharides, have been identified previously in these plant extracts (Cui et al., 2018). The LC-MS analysis that has been conducted by (X. Li et al., 2020) showed that the hydroethanolic extract of *C. coccineum* possesses a diverse and complex mixture of triterpenoids, flavonoids, fatty acids, and phenolic acids. In Tunisia, the ethanolic extract has been reported with quercetin, palmitic acid, rutin, procyanidin, and myristic acid as the most dominant contents (Li et al., 2020). The variability in the chemical composition of plants is common and can be affected by several factors such as the weather and geographical conditions (Pant et al., 2021).
In this study, *C. coccineum* possesses moderate antibacterial activity against *P. aeruginosa*. This finding is in parallel to the finding of (Al-Mussawi, 2014) who evaluated the antibacterial activity of the ethanolic extract of *C. coccineum* against *P. aeruginosa* ATCC27853 using disc diffusion method and observed an inhibition zone of 14 mm. No antibacterial activity against *P. aeruginosa* and narrow spectrum activity against gram positive bacteria for the polar extract were observed (Kadri & Nani, 2022; Zucca et al., 2016).

This study also confirmed that *C. coccineum* extract can prevent biofilm formation as indicated by crystal violet assay, light microscope and SEM. The inhibitory effect at sub-MIC values that have no significant reduction in cell viability. A significant reduction in *P. aeruginosa* viable cells was observed at the treatment concentrations of 25, 12.5, 6.25, 3.13, and 1.56 mg/mL. This may suggest that these concentrations (25-1.56 mg/mL) exhibited a bacteriostatic effect, not an antibiofilm effect. But, at lower concentrations (0.78 - 0.1 mg/mL), the extract displayed antibiofilm activity, as indicated by the non-significant reduction in cell viability.

The process of biofilm formation unfolds through several stages, commencing with the initial layer formation, followed by microorganisms moving to the surface, attaching, reproducing, maturing, and ultimately detaching (Wang et al., 2022). The first step, primary attachment and surface adherence, primarily relies on factors such as the composition of the extracellular polymeric matrix, cell surface hydrophobicity, and flagellar motility to facilitate bacterial adherence (Zheng et al., 2021). In this study, it was observed that the *C. coccineum* extract effectively reduced bacterial hydrophobicity, likely due to its components binding to the adhesion site, thereby diminishing bacterial hydrophobicity. Since surface hydrophobicity influences the initial attachment phase of biofilm formation, reducing it may hinder adhesion and subsequently decrease biofilm growth. Additionally, the *C. coccineum* extract was found to impede bacterial aggregation, thus inhibiting biofilm formation. Bacterial motility, particularly swarming motion, significantly influences the onset of biofilm development (Worlitzer et al., 2022). The synthesis of rhamnolipid, a biosurfactant crucial for *P. aeruginosa* swarming movement, reduces surface tension and facilitates motility (Morin & Déziel, 2021). The study revealed that sub-MIC concentrations of *C. coccineum* extract inhibited swarming motility and rhamnolipid production in a dose-dependent manner. This suggests that one mechanism by which swarming motility is prevented is through the inhibition of rhamnolipid production. Moreover, it was reported that supplementing with rhamnolipids partially restores the inhibition of swarming motility caused by the extracts (O'May & Tufenkji, 2011). The production of EPS is crucial for building the structure of biofilms and forming microcolonies (Hooshdar et al., 2020). EPS acts as a protective barrier in bacteria, preventing antibiotics from entering bacterial cells and thereby contributing to antibiotic resistance. Additionally, EPS formation alters the biofilm structure, increasing resistance to antibacterial

drugs (S. Singh et al., 2021). Consequently, reducing EPS formation can improve biofilm elimination by enhancing contact with antimicrobial substances (Pinto et al., 2020). This study shows that the *C. coccineum* extract notably decreases EPS production.

An initial study revealed that the *C. coccineum* extract exhibits anti-QS activity, demonstrated by the decrease in violacein production in *C. violaceum*. Violacein, a purple pigment, is produced by *C. violaceum* through acyl homoserin lactone (Park et al., 2021). Inhibiting the production of this pigment suggests inhibition of the QS pathway, making a compound that hinders violacein production a potential candidate for anti-quorum sensing effects (Bouyahya et al., 2022).

QS serves as a critical regulatory mechanism utilized by bacteria, including the opportunistic pathogen *P. aeruginosa*, to coordinate gene expression based on population density (Wu et al., 2020). Once the cell density reaches a certain level, QS genes in *P. aeruginosa* become able to detect the produced signal molecules like N-acyl homoserine lactones. QS genes activation results in shifting in the bacterial behavior to produce virulence factors, and aggregate in a biofilm (Nag et al., 2023). It uses this aggregation to coordinately become more resistant to antibiotics. Together, these factors make *P. aeruginosa* more powerful to overcome immune response and to survive in the host (Prashanth et al., 2023). The extract showed a dose dependent inhibition in a set of *P. aeruginosa* virulence factors including pyocyanin, rhamnolipids, protease, and chitinase. These secreted factors are quorum sensing regulatory factors that have essential roles in the mechanism of pathogenicity. Protease is an important spreading and invading factor, facilitating the crossing of mechanical barriers, reaching surfaces, and mediating aggregation and colonization. Pyocyanin as an oxidative stressor, oxidize glutathione, thus, impairing its function as a cellular antioxidant agent. Rhamnolipids act as surfactants, facilitating the initiation of biofilm formation (Liao et al., 2022; Morin & Déziel, 2021). The notable decrease in the production of these harmful substances suggests that *C. coccineum* extract exhibits unique anti-QS activity, which may disrupt *P. aeruginosa*'s ability to cause disease and impede infection progression.

Cynomorium coccineum extract resulted in a significant decrease in the release of N-acyl-homoserine lactones, suggesting that it hindered QS by interfering with QS mechanisms, including the las, rhl, and pqs systems. Inhibiting these QS systems reduces the production of autoinducers (AIs) such as AHL (Rather et al., 2022). *Pseudomonas aeruginosa* operates at least four QS systems, with two controlled by N-acyl-homoserine lactones (HSL) known as Las and Rhl. The other two systems, regulated by quinolones and carbaldehyde, are the Pqs and Iqs QS systems, respectively (Brindhadevi et al., 2020). Inhibiting the Las system leads to the deactivate *lasB, lasA, aprA, psl* and *pel* genes and subsequently inhibits the production of elastase A, elastase B, alkaline protease, Psl and Pel exopolysaccharides, respectively (Ma'aitah 2024; Sánchez-Jiménez et al., 2023). This virulence factor production is also controlled by Rhl system which also controls the production of rhamnolipids. Exopolysaccharides and elastases production are also controlled by pqs system. This system regulation extends to control the production of pyocyanin, rhamnolipids, hydrogen cyanide, and lectins A and B (García-Reyes et al., 2020; Groleau et al., 2020).

5. Conclusion

This investigation provides scientific evidence about the biofilm, virulence factor, and QS inhibitory effects of *C. coccineum* extract against *P. aeruginosa*. The findings support the use of *C. coccineum* extract for treating infectious diseases. However, further investigations, such as isolating the active compounds, determining the exact mode of action, and evaluating the toxicity effect, are required.

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Conflict of interests

The authors declare that they have no competing interests.

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