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Original Article
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Chemical Composition and Quorum Sensing Inhibitory Effect of *Nepeta curviflora* Methanolic Extract against ESBL *Pseudomonas aeruginosa*

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Objectives: Bacterial biofilm is regarded as a significant threat to the production of safe food and the arise of antibiotic-resistant bacteria. The objective of this investigation is to evaluate the quorum sensing inhibitory effect of *Nepeta curviflora* methanolic extract.
Methods: The effectiveness of the leaves at sub-inhibitory concentrations of 2.5, 1.25, and 0.6 mg/mL on the virulence factors and biofilm formation of *P. aeruginosa* was evaluated. The effect of *N. curviflora* methanolic extract on the virulence factors of *P. aeruginosa*, including pyocyanin, rhamnolipid, protease, and chitinase, was evaluated. Other tests including the crystal violet assay, scanning electron microscopy (SEM), swarming motility, aggregation ability, hydrophobicity and exopolysaccharide production were conducted to assess the effect of the extract on the formation of biofilm. Insight into the mode of anti-quorum sensing action was evaluated by examining the effect of the extract on the activity of N-Acyl homoserine lactone (AHL) and the expression of pslA and pelA genes.
Results: The results showed a significant attenuation in the production of pyocyanin and rhamnolipid and in the activities of protease and chitinase enzymes at 2.5 and 1.25 mg/mL. In addition, *N. curviflora* methanolic extract significantly inhibited the formation of *P. aeruginosa* biofilm by decreasing aggregation, hydrophobicity, and swarming motility as well as the production of exopolysaccharide (EPS). A significant reduction in AHL secretion and pslA gene expression was observed, indicating that the extract inhibited quorum sensing by disrupting the quorum-sensing systems. The quorum-sensing inhibitory effect of *N. curviflora* extract appears to be attributed to the presence of kaempferol, quercetin, salicylic acid, rutin, and rosmarinic acid, as indicated by LCMS analysis.
Conclusion: The results of the present study provide insight into the potential of developing anti-quorum sensing agents using the extract and the identified compounds to treat infections resulting from quorum sensing-mediated bacterial pathogenesis.

Keywords: quorum sensing, antibiofilm, *P. aeruginosa*, *N. curviflora*

INTRODUCTION

Pseudomonas aeruginosa is a leading cause of potentially fatal nosocomial infections. In humans, it causes a wide range of systemic infections, including cystic fibrosis, urinary tract infections, burn infections, and several acute and chronic illnesses. Additionally, because of the elevated rate of antibiotic resistance and biofilm development, *P. aeruginosa* infections are challenging to treat [1]. Interestingly, some biofilm producers used in food production are human pathogens, which is especially important in the food industry. These organisms are able to form biofilm structures on a variety of common artificial substrates in food processing, such as stainless steel, glass, wood, polyethylene, and rubber [2]. Given its considerable metabolic diversity, short generation time, and capacity to grow at a wide range of temperatures (4-42℃), *P. aeruginosa* is widespread and, as a result, a prevalent food-borne pathogen [3].

P. aeruginosa develops a variety of virulence factors mediated

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by quorum-sensing (QS) systems. In *P. aeruginosa*, the quorum sensing system includes the lasI/R system, which controls the activity of the rhlI/R system. At specific conditions, *P. aeruginosa* produces three signal molecules, N-(3-oxododecanoyl)-L-homoserine lactone, N-butanoyl-L-homoserine lactone, and a quinolone-based signal, PQS. The two acyl homoserine lactone (AHL) molecules cause activation of the lasI/R and rhlI/R, initiating the virulence pathway. The las system controls the expression of protease and elastase, while the rhl system controls the expression of pyocyanin and rhamnolipids. The PQS is induced by 2-heptyl-3-hydroxy-4(1H)-quinolone and regulates virulence factor production, the formation of biofilm, and bacterial motility [4].

Due to the emergence of multidrug resistance, treating *P. aeruginosa* infections has become increasingly difficult [5]. Thus, scientists are searching for new antibiotics that can circumvent antibiotic resistance mechanisms with minimal host toxicity. Efficient QS inhibitors diminish the virulence and minimize the pathogenesis of *P. aeruginosa*, enabling the immune system to control the infection. However, focus should also be placed on anti-inflammatory therapies since the interaction of the host and pathogen during the infection poses the greatest threat to the persistence of the infection and elects harmful excessive immune responses such as edema, persistent fever, and organ dysfunction. Therefore, anti-inflammatory medications may halt the progression of chronic infections by attenuating the interaction cycle between infection and inflammation [6].

For millennia, a great variety of medications made from plants have been used extensively to treat bacterial infections and inflammation. They are deemed safe for therapeutic usage since they are thought to have minimal toxicity [7]. To reduce *P. aeruginosa* infections, plant products with no antibacterial activity and novel inhibitory potential of the QS system and inflammatory responses would be of the utmost therapeutic importance.

Nepeta curviflora is an annual herb that belongs to the Lamiaceae family. This 30-to-40-centimeter-tall aromatic plant is distinguished by its small leaves and violet-blue flowers. Traditionally, *N. curviflora* is used as a pain killer and for treating fever, respiratory illness, and intestinal worms [8]. Reports have shown that the nonpolar components (essential oils) extracted from *N. curviflora* possess broad-spectrum activities such as antioxidant, antiproliferative, and antimicrobial [9, 10]. In contrast, *N. curviflora* polar extracts (ethanol) have weak antibacterial activity [11]. Therefore, this study aimed to investigate the anti-QS and antibiofilm activities of *N. curviflora* methanolic extract against *P. aeruginosa*. The effect on the expression of QS genes, including pelA and pslA, was evaluated. Also, the chemical composition of *N. curviflora* methanolic extract was explored using liquid chromatography-mass spectrometry (LC-MS).

MATERIALS AND METHODS

1. Plant materials and extraction

N. curviflora was cultivated from the Al-Karak region of Jordan in March 2020. The taxonomic identification of the plant was achieved by Dr. Feryal Al-Khresat (Department of Biology, Mu'tah University, Al-Karak, Jordan). The leaves were dried, ground into a powder, and extracted using methanol at room temperature for 24 hours. After removing the solvents with a rotary evaporator, the crude methanol extract was collected and kept at 4℃.

2. Bacterial strain

P. aeruginosa was isolated from a urine sample of a patient with a urinary tract infection (AlKarak Government Hospital, Karak, Jordan) and identified using a Biomérieux VITEK® 2 system. It was characterized as Beta-Lactamase-producing *P. aeruginosa*. *Chromobacterium violaceum* ATCC 12472 was purchased from the American Type Culture Collection (ATCC).

3. The effect of *N. curviflora* methanolic extract on *P. aeruginosa* planktonic cell growth

A microdilution assay was used to determine the minimum inhibitory concentration (MIC) of *N. curviflora* methanolic extract against *P. aeruginosa* [12].

4. Effect of *N. curviflora* extract on the *P. aeruginosa* virulence factors

1) Violacein production

A violacein inhibition assay was performed using the well diffusion method described by Oliveira et al. [13]. In brief, 100 μL of *C. violaceum* culture adjusted to match 0.5 McFarland suspensions (1×10^8 CFU/mL) was combined with 30 mL of molted Mueller-Hinton agar. After solidification, a sterile

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pasture pipette was used to make a 6 mm well in the agar. The well was then filled with 100 μL of media containing 1 mg of *N. curviflora* extract. The tested plates were incubated at 37℃ for 24 hours, and the inhibition zone was measured as mm in diameter.

2) Pyocyanin production

The extraction and quantification of pyocyanin were performed according to [14] with some modifications. Briefly, a 24-hour-old *P. aeruginosa* culture treated with 0, 0.6, 1.25, and 2.5 mg/mL of the *N. curviflora* extract was centrifuged, and 7.5 mL of the supernatant was mixed with 4.5 mL of chloroform. Then, a portion of the chloroform layer was mixed with 0.2N HCl. The OD520 nm of the solution (pink) was measured using a spectrophotometer, and the percentage of pyocyanin production was calculated using the absorbance of the control sample.

3) Rhamnolipid assay

The rhamnolipid assay was performed using the orcinol method [15]. Briefly, a 24-hour-old *P. aeruginosa* culture treated with 0, 0.6, 1.25, and 2.5 mg/mL of the *N. curviflora* extract was centrifuged, and 1 mL of the supernatant was mixed with 3 mL of diethyl ether. The diethyl ether layer was separated and removed using a rotary evaporator. Then, 900 mL of 0.18% orcinol in 53% H_2SO_4 were transferred to the diethyl ether crude extract. The mixture was boiled and left to cool for 30 minutes. Then, the OD421 nm was measured using a spectrophotometer, and the percent of rhamnolipid production was calculated using the absorbance of the control sample.

4) LasA protease activity

The protease assay was performed according to [14] with some adjustments. Briefly, a 24-hour-old *P. aeruginosa* culture treated with 0, 0.6, 1.25, and 2.5 mg/mL of the *N. curviflora* extract was centrifuged, and 1 mL of the supernatant was mixed with 5 mL of a 0.65% casein solution. After 30 minutes of incubation at 37℃, 10% pre-chilled TCA (5 mL) was added, and the mixture was incubated at 35℃ for 30 minutes. After centrifugation (10,000 rpm, 5 min), a portion of the supernatant was mixed with Folin's reagent and sodium carbonate (0.5 M) and incubated at 35℃ for 30 minutes. The OD660 nm was measured using a spectrophotometer, and the percent of protease inhibition was calculated using the absorbance of the control sample.

5) Chitinase activity

A chitin azure assay was used to evaluate the chitinase activity [14]. Chitin azure solution was prepared in sodium phosphate buffer (1.3 mg of chitin azure to 130 mL 200 mM sodium phosphate buffer, pH 7.0). The prepared solution was incubated for seven days at 37℃ at an agitation rate of 150 rpm. Then, a 24-hour-old *P. aeruginosa* culture treated with 0, 0.6, 1.25, and 2.5 mg/mL of the *N. curviflora* extract was centrifuged, and 0.5 mL of the supernatant was mixed with 4.5 mL of the prepared chitin azure solution. The mixture was incubated for 24 hours at 37℃. Finally, the mixture was centrifuged at 16,000 for ten minutes, and the OD570 nm was measured. The percent of chitinase inhibition was calculated using the absorbance of the control sample.

5. Effect of *N. curviflora* extract on biofilm formation

The antibiofilm activity was measured using a crystal violet assay [16].

6. Effect of *N. curviflora* extract on *P. aeruginosa* viable cells in the biofilm matrix

The viable cell test was performed in a 96-well plate using tetrazolium salt 2,3,5-triphenyl-tetrazolium chloride (TTC) [17].

7. Swarming motility

The swarming motility assay was performed using swarm agar plates [18]. *N. curviflora* extract (mg/mL) was mixed with melted swarm agar media (glucose [1%], peptone [0.6%], yeast extract [0.2%], and agar [0.5%]). The media were left to solidify, and *P. aeruginosa* was grown in the center of the solidified plates. Swarming motility was measured in millimeters after incubating the plates at 37℃ for days.

8. Aggregation ability

The aggregation ability test was performed according to [19] with some modifications. After incubation at 37℃ for 24 hours, the OD600 nm of the *P. aeruginosa* culture treated with *N. curviflora* extract was measured (OD pre-vortex). After one minute of vertexing the culture, the OD600 nm was measured again (OD post-vortex). The aggregation percentage was calculated

using the formula:

$$\text{Aggregation\%} = \frac{\text{OD postvortex} - \text{OD prevortex}}{\text{OD postvortex}} \times 100\%$$

9. Surface hydrophobicity

The surface hydrophobicity was measured using n-hexadecane [20]. The *P. aeruginosa* culture treated with *N. curviflora* extract was incubated at 37℃. After two hours of incubation, the OD600 nm was measured as adherence initial (Ai). A portion from this culture was mixed with n-hexadecane in a proportion of 1:1. After 15 minutes of incubation at room temperature, the OD600 nm was measured as adherence final (Af). The hydrophobicity percentage was calculated using the following formula:

$$\text{FPc}(\%) \frac{\text{Ai} - \text{Af}}{\text{Ai}} \times 100\%$$

10. Exopolysaccharides (EPS)

The percent of exopolysaccharides (EPS) secretion was evaluated [21]. In this test, EPS from a 24-hour-old *P. aeruginosa* culture treated with 0, 0.6, 1.25, and 2.5 mg/mL of the *N. curviflora* extract was extracted using cold ethanol for 24 hours at 4℃. Then, the samples were centrifuged for 15 minutes at 10,000 rpm. The pellet was collected and resuspended in deionized water. The produced suspension was mixed with H_2SO_4 and ethanol in a proportion of 1:5:1, and the OD490 nm was measured using a spectrophotometer. The percent of EPS inhibition was calculated using the absorbance of the control sample.

11. N-Acyl homoserine lactone (AHL) activity

The N-Acyl homoserine lactone (AHL) activity was estimated [22]. AHL was extracted using ethyl acetate. After ten minutes of incubation at room temperature, the ethyl acetate layer was separated and removed using a rotary evaporator. A portion of the extracted AHL was mixed with hydroxyl amine (2M) and NaOH in a proportion of 1:1. At the same proportion, ferric chloride (10% in 4M HCl) and 95% ethanol was also added. The OD520 nm was measured, and the percent of AHL activity inhibition was calculated using the absorbance of the control sample.

12. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was performed to observe the effect of *N. curviflora* extract on the formation of *P. aeruginosa* biofilm [23]. In brief, sterile coverslips were placed in the bottom of a 24-well plate. Then, the *P. aeruginosa* cultures treated with 0 or 1.25 mg/mL of the *N. curviflora* extract were prepared in the wells. The plate was incubated at 37℃ for 24 hours without shaking. At the end of the incubation period, the coverslips were removed and washed gently with PBS. The coverslips were soaked in cold glutaraldehyde (5%). After 24 hours, the coverslips were dehydrated in increasing ethanol concentrations (10 to 100%, 10 min each). The dried samples were observed using a SEM.

13. The effect on the expression of PelA and PslA genes

The effect of *N. curviflora* extract on the expression of PelA and PslA genes was evaluated using rt-PCR. Total RNA was extracted from *P. aeruginosa* treated with 0 or 1.25 mg/mL of the *N. curviflora* extract using a Direct-zol™ RNA MiniPrep Kit. cDNA synthesis was performed using a cDNA Synthesis Kit (Bioline Reagents Ltd, UNITED KINGDOM). The primers listed in Table 1 were used to perform the real-time PCR using SYBR Green PCR Master Mix (Thermo Fischer Scientific, USA).

14. Liquid Chromatography – Mass Spectrometry (LCMS)

High-performance liquid chromatography (HPLC) separation was performed using the mobile phase containing solvent A and B in a gradient, where A was 0.1% (v/v) formic acid in water and B was 0.1% (v/v) formic acid in acetonitrile for the following gradients: 5% B for 5 minutes, 5-100% B for 15 minutes, and 100% for 5 minutes at a flow rate of 0.5 mL/min. The

Table 1. Sequences of primers for 16S rRNA, *PelA* and *Psl* gene

Gene		Primer sequence 5' - 3'	Reference
16S rRNA	Forward	CAAAACTACTGAGCTAGAGTACG	(Lenz et al. 2008)
	Reverse	TAAGATCTCAAGGATCCCAACGGCT	
PelA	Forward	CCTTCAGCCATCCGTTCTTCT	(Li et al. 2019)
	Reverse	TCGCGTACGAAGTCGACCTT	
PslA	Forward	AAGATCAAGAAACGCGTGGAAT	(Irie et al. 2012)
	Reverse	TGTAGAGGTCGAACCACACCG	

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column used was an Agilent Zorbax Eclipse XDB-C18 (2.1 × 150 mm × 3.5 um). The oven temperature used was 25℃, and the sample injection volume was 1 μL (18 mg/mL in methanol). The eluent was monitored by a Shimadzu LC-MS 8030 with an electrospray ion (ESI)-mass spectrometer (ESI-MS) in positive ion mode and scanned from 100 to 1,000 m/z. ESI was conducted using a fragmentor voltage of 125 V and a skimmer of 65 V. High-purity nitrogen (99.999%) was used as a drying gas at a flow rate of 10 L/min, a nebulizer at 45 psi and a capillary temperature of 350℃. In parallel, 0.1% formic acid was used as a blank.

Samples were injected into the mass detector using a Shimadzu CBM-20A system controller, LC-30AD pump, SIL-30AC autosampler with cooler, and CTO-30 column oven.

15. Statistical analysis

Results are reported as the mean, standard deviation, and percentage, which were calculated using Microsoft Excel 2009. Figures were prepared using GraphPad Prism (8.0). One-way ANOVA was used to determine the significant differences between groups based on p-values as follows: *p < 0.05, **p < 0.01, ***p < 0.001 compared to control untreated cells.

RESULTS

1. Effect of *N. curviflora* methanolic extracts on *P. aeruginosa* growth

The MIC of *N. curviflora* methanolic extracts against *P. aeruginosa* was found to be 10 mg/mL. Thus, the concentrations of 2.5 (1/4 MIC), 1.25 (1/8 MIC), and 0.6 (1/16 MIC) mg/mL were selected as the sub-MIC concentrations to be used in the following experiments.

2. Effect of *N. curviflora* methanolic extract on the *P. aeruginosa* virulence factors

1) Violacein inhibition assay

The inhibition of violacein by *C. violaceum* was determined using a well diffusion assay. The methanolic extract of *N. curviflora* (1 mg/disc) showed an inhibition zone of 12.5 ± 0.6 mm, indicating a remarkable anti-quorum sensing activity.

2) Pyocyanin production

Pyocyanin is released by *P. aeruginosa* as a blue-green pigment. Pyocyanin acts as a significant virulence factor. A significant reduction in pyocyanin production was found to be dose dependent with *N. curviflora* extract treatment (Fig. 1A). Pyo-

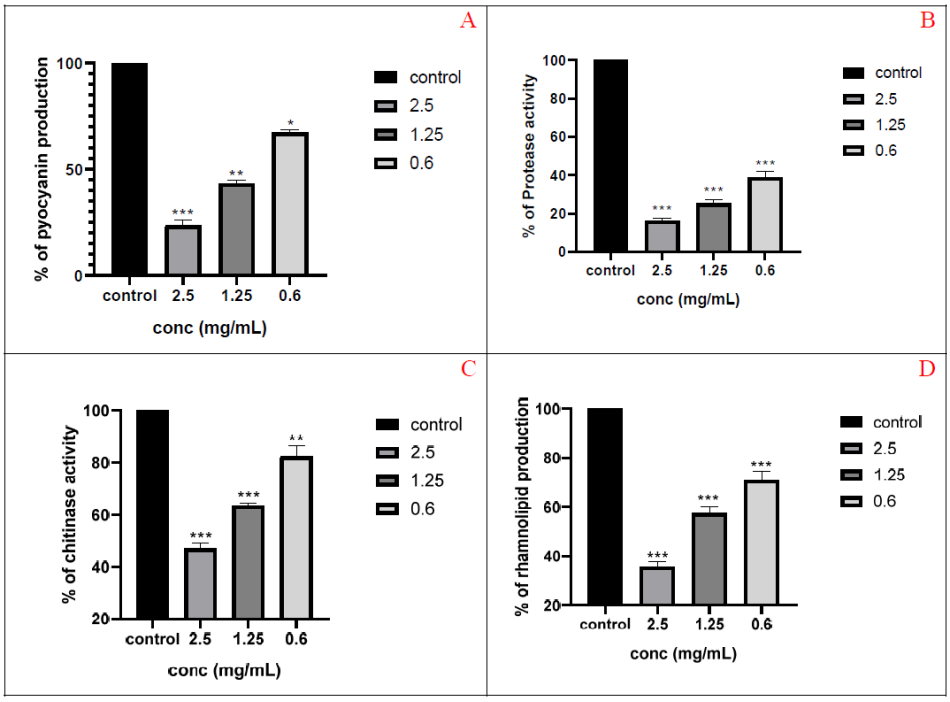

Figure 1. Effect of *N. curviflora* methanolic extract on the *P. aeruginosa* virulence factors. (A) Percent of pyocyanin production. (B) Percent of protease activity. (C) Percent of chitinase activity. (D) Percent of rhamnolipid production of *P. aeruginosa* treated with 0 (control), 0.6, 1.25, and 2.5 mg/mL *N. curviflora* methanolic extract. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control untreated cells.

cyanin production was reduced to 23.5, 43.3 and 67.4% when *P. aeruginosa* was treated with 2.5, 1.25, and 0.6 mg/mL of *N. curviflora* extract, respectively.

3) Protease activity

A significant reduction in protease activity was found with *N. curviflora* extract treatment (Fig. 1B). The significant reduction in protease activity at 0.6 mg/mL indicated a potent inhibitory effect of *N. curviflora* on protease activity.

4) Chitinase activity

An *N. curviflora* extract dose-dependent reduction in chitinase activity was found (Fig. 1C). The chitinase activity of *P. aeruginosa* treated with *N. curviflora* extract at concentrations of 2.5, 1.2, and 0.6 mg/mL was significantly reduced to 47.0, 63.5, and 82.3%, respectively.

5) Rhamnolipid production

A significant *N. curviflora* extract dose-dependent inhibition of rhamnolipid production was observed for all concentrations tested (Fig. 1D). The maximum reduction in rhamnolipid production from 100 to 35.7% was reported for the highest concentration tested (2.5 mg/mL), followed by 57.4 and 71.0% reductions when *P. aeruginosa* was treated with *N. curviflora* extract at 1.25 and 0.6 mg/mL, respectively.

3. Effect of *N. curviflora* extract on biofilm formation

1) Antibiofilm activity (MBIC)

The treatment of *P. aeruginosa* with *N. curviflora* methanolic extract led to the inhibition of biofilm formation in a dose-dependent manner (Fig. 2). Compared with the untreated cells, all the tested concentrations (2.5, 1.25, and 0.6 mg/mL) significantly reduced the formation of *P. aeruginosa* biofilm by 66.3, 37.7, and 18.3%, respectively. A TTC assay was performed to evaluate the effect of *N. curviflora* extract on the viability of cells in the formed biofilm. The selected sub-MIC concentrations possessed no significant inhibitory effects on the growth of *P. aeruginosa* after 24 hours (Fig. 3).

2) Swarming motility

The swarming motility pattern of the untreated *P. aeruginosa* reached 60.3 mm (Fig. 4A). A dose-dependent reduction in *P. aeruginosa* motility was observed when treated with *N. curviflora* methanolic extract. At *N. curviflora* extract concentrations of 2.5 and 1.25 mg/mL, the swarming motility diameter was significantly ($p < 0.001$) reduced to 23.0 and 42.8 mm, respectively.

3) Aggregation ability

Compared with the untreated cells, a significant ($p < 0.001$) reduction in the *P. aeruginosa* aggregation ability was detected (Fig. 4B). Due to the treatment with these concentrations, the aggregation ability was reduced from 73.6% to 25.13 and 48.7% when treated with 2.5 and 1.25 mg/mL of the *N. curviflora* extract, respectively.

4) Hydrophobicity

The treatment of *P. aeruginosa* with *N. curviflora* extract led to a significant dose-dependent reduction in the hydrophobicity (Fig. 4C). When *P. aeruginosa* was treated with 2.5 and 1.25 mg/mL of the *N. curviflora* extract, the hydrophobicity was

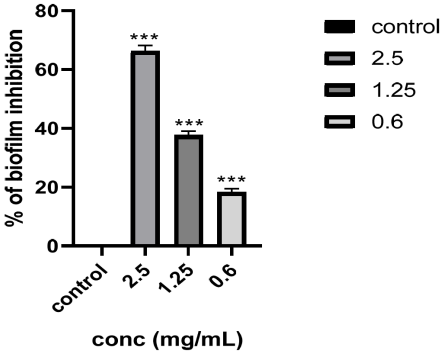

Figure 2. Percent of biofilm inhibition of *P. aeruginosa* treated with 0 (control), 0.6, 1.25, and 2.5 mg/mL *N. curviflora* methanolic extract. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control untreated cells.

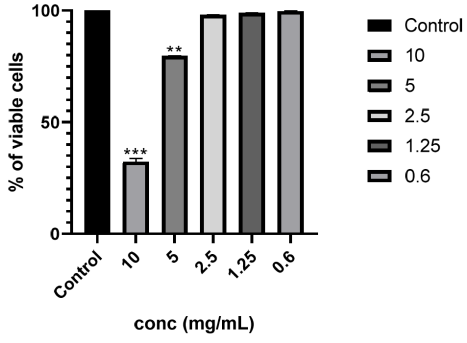

Figure 3. Percent of viable cells of *P. aeruginosa* treated with 0 (control), 0.6, 1.25, 2.5, 5.0 and 10 mg/mL *N. curviflora* methanolic extract. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control untreated cells.

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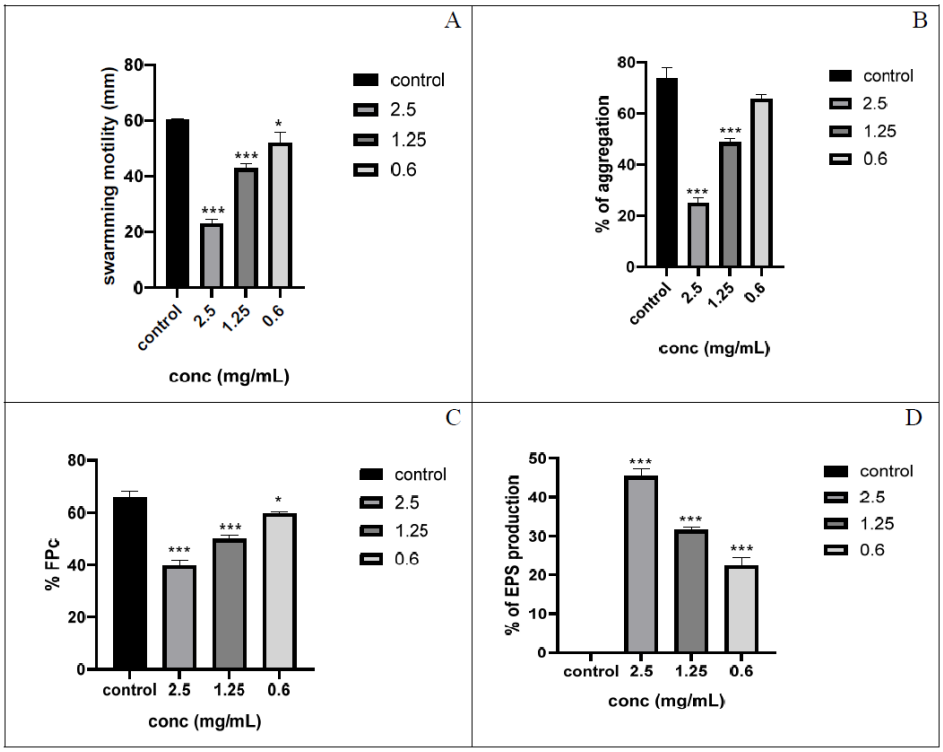

Figure 4. Effect of 0 (control), 0.6, 1.25 and 2.5 mg/mL *N. curviflora* methanolic extract on. (A) The swarming motility (mm). (B) Aggregation ability (%). (C) Surface hydrophobicity (%FPc). (D) EPS production (%) of *P. aeruginosa*. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control untreated cells.

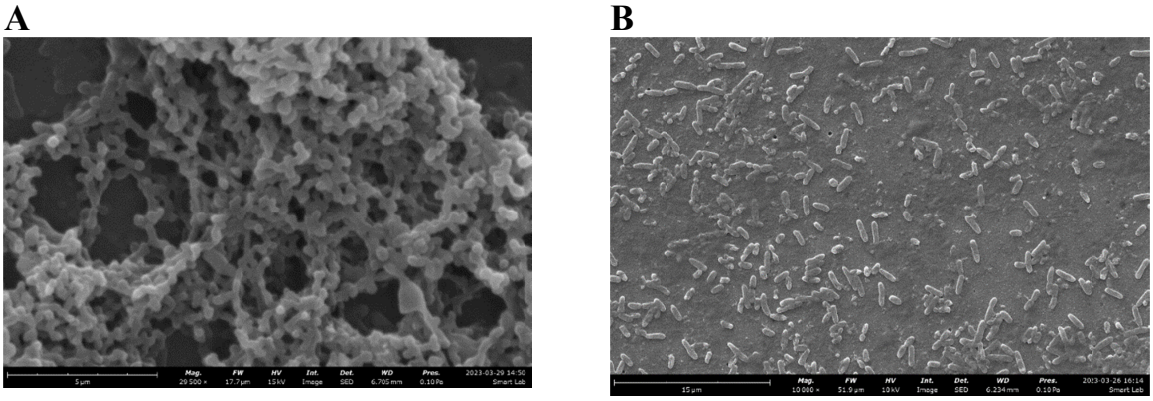

Figure 5. SEM images of *P. aeruginosa* treated with (A) 0 mg/mL (control) and (B) 1.25 mg/mL *N. curviflora* methanolic extract.

significantly reduced ($p < 0.001$) from 65.8% to 39.8 and 49.9%, respectively.

5) Exopolysaccharides production

A dose-dependent increase in EPS production was observed (Fig. 4D). Compared with the untreated cells, treatment of *P. aeruginosa* with 2.5, 1.25, and 0.6 mg/mL of the *N. curviflora* methanolic extract led to significant ($p < 0.001$) decreases in EPS production of 45.4, 31.5, and 22.4%, respectively.

6) SEM analysis

Untreated *P. aeruginosa* was observed to form a heavy biofilm layer with the adherent cells (Fig. 5A). In contrast, treated *P. aeruginosa* with 1.25 mg/mL of the *N. curviflora* extract displayed scattered, nonattached bacterial cells (Fig. 5B).

4. Mechanism of anti-quorum sensing activity

1) AHL production

A significant inhibition in AHL production was observed in a dose-dependent manner (Fig. 6). At all concentrations tested,

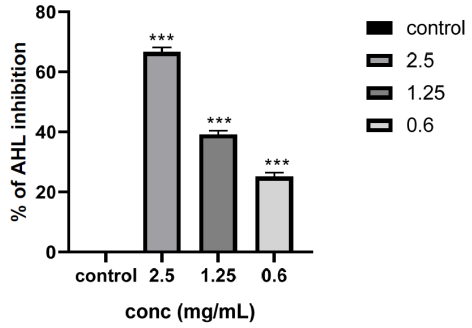

Figure 6. Percent of AHL inhibition of *P. aeruginosa* treated with 0 (control), 0.6, 1.25, 2.5, 5.0 and 10 mg/mL *N. curviflora* methanolic extract. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control untreated cells.

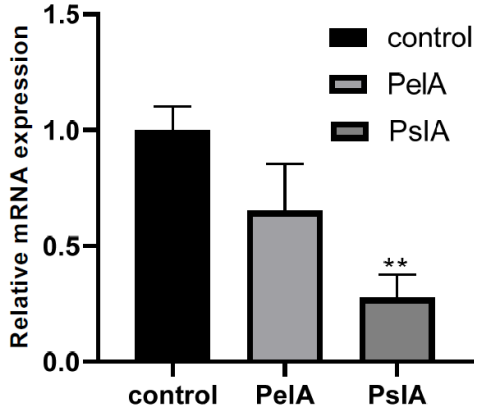

Figure 7. Effect of 1.2 mg/mL *N. curviflora* extract on the expression of PelA and PsIA genes in *P. aeruginosa*. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control untreated cells.

the treatment of *P. aeruginosa* with 2.5, 1.25, and 0.6 mg/mL of the *N. curviflora* methanolic extract caused a significant ($p < 0.001$) inhibition in AHL production of 66.6, 39.2, and 25.1%, respectively.

2) Effect on PelA and PsIA genes

The relative expression of two genes, PelA and PsIA, was determined in *P. aeruginosa* treated with 1.2 mg/mL of the *N. curviflora* extract and compared with the untreated *P. aeruginosa*. The results showed that the extract significantly reduced the expression level of PslA (Fig. 7). Also, there was a remarkable reduction in the expression level of PelA, but it was not significant.

Table 2. Chemical composition of *N. curviflora* methanolic extracts using LCMS

	RT	Compound	Percentage %
1	7.1	Rosmarinic acid	7.3
2	8.9	Rutin	8.1
3	10.3	Thymusin	8.7
4	11.5	Coniferin	1.4
5	14.2	Salicylic acid	10.5
6	14.8	Gallic acid	1.1
7	15.0	Syringic acid	0.5
8	16.3	Quercetin	15.3
9	17.9	Apigenin	1.5
10	20.4	Kaempferol	17.4
11	21.9	p-Coumaric acid	2.0
12	23.3	Salvigenin	0.4
13	25.1	Vanillic acid	0.9
14	28.6	Isothymusin	1.8
15	29.7	Genkwanin	0.8
16	32.1	Nepetonic acid	2.2
17	37.5	Chrysin	0.3
18	38.3	Ursolic acid	0.2
19	39.9	Luteolin	6.4
20	42.0	Beta-carotene	1.1
21	47.3	Acacetin	1.1
22	48.1	Nepetin	1.2
23	49.8	Ferulic acid	0.8
24	52.0	Caffeic acid	0.6
25	53.1	Chlorogenic acid	0.5
26	55.2	4-Hydroxybenzoic acid	0.5
		Total	92.6
		Flavonoid derivatives	52.5
		Phenol derivatives	26.1
		Others	14.0

5. Chemical composition of *N. curviflora* methanolic extracts using LCMS

A total of 26 compounds have been identified in *N. curviflora* extract, representing about 92.6% of the total compounds (Table 2). Most of the identified compounds belong to flavonoids (52.5) and phenolic (26.1) derivatives. Kaempferol (17.4%) was identified as a major component in *N. curviflora* methanolic extract, followed by quercetin (15.3%), salicylic acid (10.5%), thymosin (8.7%), rutin (8.1%), and rosmarinic acid (7.3%).

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DISCUSSION

P. aeruginosa is the most prevalent opportunistic bacterial species that can cause severe nosocomial infections, particularly in patients with compromised immune systems. *P. aeruginosa* produces extracellular virulence factors that are monitored by quorum sensing systems. Therefore, inhibiting quorum sensing is an appropriate strategy for combating *P. aeruginosa* infections [1]. This study demonstrated, for the first time, that an *N. curviflora* methanolic extract possesses an anti-quorum sensing potential. At subinhibitory concentrations (sub-MIC), *N. curviflora* extract reduces the production of virulence factors and the formation of biofilms in *P. aeruginosa* without altering its growth. Previous investigations have shown that anti-quorum sensing drugs should be considered when finding molecules that control cell signaling, biofilm formation, and virulence factors without influencing cell viability [24].

In this study, treating *P. aeruginosa* with *N. curviflora* extract led to a significant reduction in the formation of protease, chitinase, pyocyanin, and rhamnolipid. These molecules are produced extracellularly and are used as indicators to evaluate the quorum sensing system [25]. These factors have a significant role in the pathogenicity of *P. aeruginosa*. In particular, the Las system controls the release of elastase and protease, which are important for adhesion and colonization. The Rhl system controls the formation of pyocyanin and acts as a chelating agent for removing iron from transferrin. The Rhl system controls the production of rhamnolipids, a surfactant agent that mediates the induction of biofilm formation [26]. The significant reduction in the production of these virulence factors indicates that *N. curviflora* methanolic extract possesses novel anti-quorum sensing activity that could interfere with the pathogenicity of *P. aeruginosa* and the progress of the infection.

The association between biofilm formation and antimicrobial resistance makes *P. aeruginosa* difficult to treat. Thus, the search for powerful compounds that inhibit biofilm is of the utmost importance. Biofilm emerges in phases, commencing with the formation of the conditioning biofilm, subsequently followed by microbe mobility to the surface, adherence, replication, maturation, and dissociation [27]. Primary attachment and surface adhesion have been identified as the initial phase of biofilm development. In this study, the extract of *N. curviflora* significantly decreased bacterial hydrophobicity. This decrease in hydrophobicity may be due to the plant contents binding to adherence sites, thereby reducing the bacterial hydrophobicity. Hydrophobicity influences the initial adherent stage during biofilm development. The decrease in hydrophobicity could explain the reduction in biofilm formation by preventing adhesion. In addition, these findings demonstrate that the *N. curviflora* extract inhibited bacterial aggregation, thereby preventing the development of biofilms. Moreover, bacterial motility is one of the most important factors that can influence the initiation of biofilm formation. Initial attachment is greatly facilitated by swarming motility [28]. Swarming with the QS system functions to detect the bacterial population's produced signals. Sub-MIC *N. curviflora* extract had a dose-dependent effect that was on swarming motility in this study, indicating that it could be interfering with the QS system or effectively interacting with the flagellin protein of the flagellum. The addition of rhamnolipid can partially reverse the effect of the extracts on swarming motility. It appears that rhamnolipid is implicated in one of the mechanisms that contribute to swarming inhibition.

Biofilm architecture and microcolony formation require EPS production. EPS also confers antibiotic resistance to bacteria by acting as a barrier to prevent antibiotics from penetrating the bacteria [29]. In addition, EPS production results in changes in the biofilm structure that are associated with increased resistance to antibacterial drugs [29]. Thus, reducing the amount of EPS production will facilitate the eradication of biofilms by increasing their exposure to antimicrobials. In this study, *N. curviflora* extract was shown to substantially decrease EPS production. This decrease was also observed in the SEM analysis. Based on the SEM analysis, Walsh et al. [30] indicated that eugenol led to the rupture of the EPS matrix in *P. aeruginosa* biofilm. According to the research by Brackman et al. [31], Vibrio spp. release less EPS when exposed to cinnamaldehyde and its derivatives.

Treating *P. aeruginosa* with *N. curviflora* extract resulted in a significant reduction in AHL secretion. This finding indicates that the *N. curviflora* extract inhibited quorum sensing by disrupting the QS systems, including the las, rhl, and pqs systems [32]. Suppressing these QS systems reduces the synthesis of autoinducers, specifically N-3-oxododecanyol-L-homoserine lactone, N-butanoyl-L-homoserine lactone, and 2-heptyl-3-hydroxy-4-quinolone. This study observed a significant reduction in the pslA gene expression. Previous studies have reported that the QS significantly affects the expression of the pel and psl operons, which are involved in the formation of the two major matrix polysaccharides, Pel and Psl [33]. These secreted compounds are necessary for biofilm adhesion, structure, and

protection [34]. Sakuragi and Kolter [35] showed that the Las QS system regulates pel transcription by triggering the Rhl QS signaling system.

The LCMS analysis revealed that *N. curviflora* methanolic extract is rich in flavonoid and phenolic components. Among these, kaempferol (17.4%), quercetin (15.3%), salicylic acid (10.5%), thymusin (8.7%), rutin (8.1%), and rosmarinic acid (7.3%) were the most dominant. Rabee et al. [36] reported the isolation of several phenolic compounds from *N. curviflora* extract, including rosmarinic acid, caffeic acid, apigenin, and luteolin derivatives. In addition, other components such as b-sitosterol, palmitic acid, betulin, betulinic acid, ursolic acid, oleanolic acid, nepetalic acid methyl acetal, apigenin, b-sitosteryl glucoside, 5-deoxyloganic acid, 5-deoxyloganic acid, and apeginin-7-O-glucoside have been reported [37].

The QS inhibitory effect of the *N. curviflora* extract appears to be due to the occurrence of flavonoid and phenolic components at high concentrations. To a lesser extent, it might be due to the activity of kaempferol, quercetin, salicylic acid, rutin, or rosmarinic acid individually or in a synergistic manner [38-40].

CONCLUSION

The virulence factors of *P. aeruginosa* are inhibited by *N. curviflora* extract without influencing its viability, thus decreasing the possibility of developing antibiotic-resistant strains. The mode of action study revealed that the extract inhibits the adhesion process, the initial step in biofilm formation. Therefore, the extract can be regarded as a candidate anti-quorum sensing agent that can be developed to treat *P. aeruginosa* biofilm-associated conditions.

DATA AVAILABILITY

All data of this study are available upon request.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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