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Antibacterial and Antibiofilm Activity of Phenolic Compounds Extracted from *Camellia sinensis* and Evaluate its Effect on the Gene Expression of *pelA Gene* in *Pseudomonas aeruginosa*

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Abstract: The purpose of this study to extract phenolic compounds from *Camellia sinensis*, investigate their antibacterial and anti-biofilm properties, and apply them to certain virulence genes that are involved in the production of biofilm in multidrug-resistant *Pseudomonas aeruginosa*. Twenty-five isolates of *P. aeruginosa* were collected from the Institute of Genetic Engineering and Biotechnology labs at Baghdad University. Using the VITEK-2 technology and growing the isolates on cetrimide agar, the diagnosis was verified. Extracts of *Camellia sinensis* leaves are created methanolic and aqueous, respectively, using the maceration technique and the Soxhlet equipment. The results indicated that, at concentrations of 128 and 256 mg/ml, As compared to the aqueous extract, the methanolic extract demonstrated greater efficacy, giving the highest inhibition zone values of 19.33 and 23.67 mm, respectively, when compared to the aqueous extract, which gave inhibition zones of 14.33 and 19.00 mm, respectively. All *P. aeruginosa* isolates had MIC of 4 mg/ml for the methanolic extract, with the exception of isolates No. 1, 2, and 10, which had MICs of 8 mg/ml and 16 mg/ml, respectively, the MIC of an aqueous extract for five *P. aeruginosa* isolates was 16 mg/ml, for four isolates it was 32 mg/ml, and for isolate No. 10, it was 64 mg/ml. The methanolic leaf extract of *C. sinensis* completely prevented *P. aeruginosa* from forming biofilms at a concentration of 8 mg/ml, whereas the aqueous extract completely inhibited *P. aeruginosa* isolates' ability to produce biofilms at a concentration of 16 mg/ml. Investigating the virulence gene *pelA*, which is responsible for *P. aeruginosa* biofilm formation, the gene expression data revealed reduced *pelA* gene levels following treating with the methanolic extract's sub-MIC in contrast to the untreated isolates.

Keywords: Antibacterial, Antibiofilm, *C. sinensis*, *P. aeruginosa*, *pelA*, Phenolic compounds.

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Introduction

Growing in a semi-tropical climate, *Camellia sinensis* is a shrub of to the *Theaceae* family that is typically cut to a height of 2-3 feet (1). The history of medicine in ancient societies is extensive. Plants were utilised as a source of many medical compounds many years ago, and they continue to be the basis for many contemporary pharmaceuticals. Although the more recent drug business was sparked by plant medicine, the *C. sinensis* these days is the development of drugs by synthetic means. Due to their vast ability to produce biosynthetic materials, plants continue to be a significant source of pharmaceuticals(2). Naturally occurring chemicals have lately attracted renewed attention due to the failure of alternative medication development strategies.

Catechins and their derived polyphenols are present in substantial levels in *C. sinensis*, with its anti-inflammatory, anticarcinogenic, antibacterial, antidiabetic, and antioxidant properties, polyphenols are medicines with therapeutic benefits (3). The bacterium *Pseudomonas aeruginosa* is rod-shaped, gram-negative, asporogenous, and monoflagellated (4). It is a multipurpose, adaptive bacterial species that is common in the environment, has significant medicinal importance, a wide range of virulence genes, and several inherent and potentially acquired antibiotic resistance features. *P. aeruginosa* has an epidemic-clonal population pattern and produces a wide range of illnesses. A number of its dominant global clones have amassed a diverse range of resistance genes, making them multi-drug resistant (MDR). This makes them particularly dangerous for vulnerable populations, such as immunocompromised patients, Caucasians with cystic fibrosis (CF), and surgical patients. *P. aeruginosa* presents unique challenges with AMR and MDR in particular, which greatly impede the effectiveness of antibiotic therapy (5). Various investigations have proposed the emergence of novel microbial strains with genes resistant to antibiotics (6). Another issue that has proven challenging to solve is biofilm development, which is the irreversible adhesion of a colony of microorganisms on the surface. Although the precise mechanism of resistance is yet unknown, a number of variables, including the protection provided by matrix polysaccharides (7) and the over-expression of the efflux pump (8), may be involved a favourable environment for sustained colonisation in host tissue is created via biofilm development, which also increases resistance to oxidative stress, Antibacterial stress, and other environmental challenges (9).

Materials and methods

Bacterial isolates

Twenty-five *P. aeruginosa* isolates were obtained from the Baghdad university / Institute of Genetic Engineering and Biotechnology These isolates had previously been taken from various hospitals in Baghdad and were identified by molecular and chemical testing. The isolates were cultured on cetrimide agar for *P. aeruginosa* for 18–24 hours at 42°C using the VITEK-2 System technology which validated the diagnosis. Re-culture on Nutrient agar medium activated the isolates, which were then incubated aerobically at 37°C for 24 hours.

Antibiotic susceptibility test

The WHO (10) described the Kirby-Bauer technique, which was used to conduct an antibiotic susceptibility test for ten distinct antibiotics. To create a bacterial suspension with a moderate turbidity relative to the standard turbidity solution created, one or two isolated colonies of bacteria from the original culture were selected and added to a test tube holding four millilitres of normal saline, about 1.5×10^8 CFU/ml is equivalent to this. Mueller-Hinton agar medium was lightly and uniformly covered with a portion of the bacterial suspension using a sterile cotton swab, and the mixture was allowed to sit for ten minutes. Next, using sterile forceps to push down forcefully to guarantee contact with the agar, the antimicrobial discs were put on the agar. The plates were subsequently turned over and incubated for 18 to 24 hours at 37°C. According to Clinical Laboratories Standards Institute guidelines (11), inhibition zones that formed around the discs were measured in millimetres (mm) using a metric ruler.

Assessment of biofilm formation

Pseudomonas aeruginosa biofilm development was quantified using microtiter plate method reported by Patel *et a*l. (12); In Brain Heart Infusion Broth, all isolates were cultered for entire night at 37°C. Pipetting was used to thoroughly combine each isolate with tryptic soy broth (TSB) containing 1% glucose. The suspension of bacterial isolate was adjusted to meet the No. 0.5 turbidity criterion of McFarland.

In triplicate, 200 µl of each isolate's culture was put to a sterile 96-well microtiter plate with a flat bottom. The plates were covered with lids and incubated for 24 hours at 37°C in aerobic conditions. Following incubation, to get rid of any residual bacteria, the planktonic cells were twice washed with distilled water. Each well's bacterial cells were fixed for 20 minutes at room temperature in 200 µl of methanol. To stain the adherent cells, each well received 200 µl of 0.1% crystal violet for 15 minutes. After the staining reaction was complete, washing with distilled water two to three times helped remove the extra discoloration. In order to make sure the plate was completely dry before using 33% acetic acid to remove the stain, it was let to stand at room temperature for around half an hour. At 630 nm in wavelength, optical density (OD) readings were obtained using an ELISA auto reader. All test results have been removed of the sterile medium's average optical density values. Additionally, to classify isolates as biofilm producers or not, the cut-off value (ODc) was determined. Three separate replications of each experiment were carried out in triplicate. In addition, a cut-off value (ODc) was established. It is defined as three standard deviations (SD) above the mean OD of the negative control: Odc = average OD of negative control + (3 × SD of negative control). The isolates were classified into the four following categories based upon the OD: non-biofilm producer (OD < ODc); weak-biofilm producer (ODc < OD < 2 × ODc); moderate-biofilm producer (2 × ODc < OD < 4 × ODc); strong-biofilm producer (4 × ODc < OD) (13).

Collection of *Camellia sinensis*

Camellia sinensis was acquired from local Iraqi markets and identified as (*C. sinensis*) by a professional from the University of Baghdad / College of Science / Dept. of Biology. Following a water wash and room-temperature drying period, the leaves were ground into a powder using a grinder, and kept at 4°C for future examination. In order to defat them, 400 g of *C. sinensis* leaf powder was macerated in two liters of petroleum ether as a solvent. After being gathered, the residue was air dried and split into two bathes. According to N'Guessan *et al*. (14) and AACC (15), respectively, each batch of defatted plant leaves was extracted separately using hot water and methanol to create aqueous and methanolic extracts.

High-Performance Liquid Chromatography (HPLC)

Using (HPLC), methanolic and aqueous formulations of *C. sinensis* extract have been found (16).

Study the antibacterial activity of *C. sinensis* extracts

Disc diffusion method

The disc diffusion technique for antibacterial activity was used in accordance with the standard procedure by Razmavar *et al*. (17) to evaluate the antibacterial activity of the methanolic and aqueous extracts of *C. sinensis*.

Muller Hinton agar plates were uniformly inoculated with the bacterial culture (adjusted to 0.5 McFarland

standard) using a sterile swab. Following a 15-minute drying period, the plates were utilised for the sensitivity test. To achieve a final concentration of 512 mg/ml, A stock of plant extract solution was created by dissolving 0.512 g of the extracts with 1 ml of each of their respective solvents (distilled water for an aqueous extract and dimethyl sulfoxide (DMSO) for a methanolic extract). Following that, the stock solution was diluted to provide extract concentrations of 128 and 256 mg/ml. Six mm diameter sterile blank discs were impregnated with 20 microliters of each dilution. As negative controls, DMSO discs and distilled water were employed. Prior to putting on Mueller Hinton agar, each disc was completely dry. For 18 to 24 hours, the plates were incubated at 37°C. The diameter of the inhibition zone surrounding the discs was measured to assess the antibacterial activity following the incubation. To guarantee dependability, the test was conducted three times.

Determination of Minimum Inhibitory Concentration (MIC) of *C. sinensis* extracts

The 96-well microtiter plate was utilised to calculate the (MIC) of the *C. sinensis* extracts utilising the broth microdilution technique. Plant extracts were made as a working solution at a concentration of 256 mg/ml in broth. To achieve the concentrations of 128-1 for methanolic and aqueous extracts, respectively, successive two-fold dilutions of the extract were prepared immediately on the plate. First wells in row A received 100 µl of the produced both of methanolic and aqueous extracts of *C. sinensis*. There was just 100 µl of broth in rows B–H in the columns. Micropipettes were used to do methodical double serial dilutions down the columns (from rows A-H). After removing, 100 microliters from row A starting concentrations, the 100 µl broth was transferred to the following row and well mixed. This process was repeated all the way to row (H), where the last 100 µl was disposed of. As a result, the final volume in each test well containing the extracts is now 100 µl, with the exception of the column, which included 200 µl of broth for sterility control. All the wells except the negative control received 100µl of the 1×10^8 CFU/ml bacterial inoculum. For 18 to 20 hours, microtiter plates were incubated at 37 °C. Following the incubation period, each well received 20 µl of resazurin dye, which was applied and incubated for 30 minutes to monitor any colour changes. The lowest extract concentrations at which the colour of the resazurin broth assay did not shift from blue to pink were identified visually in broth microdilutions as the MIC (18).

Study the antibiofilm activity of *C. sinensis* extracts

The antibiofilm activity of the *C. sinensis* extracts was assessed using a 96-well microtiter plate. In order to achieve the concentrations of 128 to 1 mg/ml for methanolic and aqueous extracts, repeated two-fold dilutions of the plant extracts were made directly on the plate, starting with a working solution of 256 mg/ml in broth. First wells in row A were loaded with 100 microliters of the produced methanolic and aqueous extract. There was just 100 µl of broth in rows B–H in the columns. Micropipettes were used to do two-fold serial dilutions methodically down the columns (from rows A-H). After removing 100 µl from the initial concentrations in row A, the 100 µl broth was moved to the following row and well mixed. This process was

repeated all the way to row (H), where the last 100 µl was disposed of. All the wells except the negative control received 100 µl of the 1×10^8 CFU/ml bacterial inoculum. The same process was followed, as the paragraph (**Assessment of biofilm development**) indicates.

Gene expression Analysis Using qRT PCR Technique

Before and after the methanolic extract treatment, the resistant isolates' gene expression rates were measured in order to evaluate the impact on the *pelA* gene expression in *P. aeruginosa* associated to biofilm formation. Bacterial growth was facilitated by using the methanolic extract at a sub-MIC concentration. TRIzolTM Reagent was used to extract RNA in accordance with the manufacturer's recommended technique. Table 1 lists the primers used to measure the *pelA* gene's expression, and Table 2 provides a summary of the reaction mixture. Additionally, the thermal cycler procedure was refined via several trials and is presented in Table (3).

As a direct comparison of the Ct values between the reference (housekeeping) gene and the target gene, the qRT-PCR data findings were computed.

Table (1): Primers utilized in this Study.

Primer name		Sequence (5´-3´)	Product Size	Reference
PelA	F	CCTTCAGCCATCCGTTCTTCT	118 bp	(19)
	R	TCGCGTACGAAGTCGACCTT		
16S rRNA	F	ACTCCTACGGGAGGCAGCAGT	180 bp	(20)
	R	TATTACCGCGGCTGCTGGC		
GyrA	F	TGTGCTTTATGCCATGAGCGA	806 bp	(21)
	R	TCCACCGAACCGAAGTTGC		

Table (2): Volumes and concentrations of qRT-PCR reaction mix.

Component	Volume (µl)
Luna Universal qPCR Master Mix	10
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Template DNA	5
Nuclease-free Water	3
Total	**20**

Table (3): qRT-PCR Cycling Program.

Cycle Step	Temperature	Time	Cycles No.
Initial Denaturation	95 °C	60 seconds	1
Denaturation	95 °C	15 seconds	40
Anneling	60 °C	30 seconds	
Melt Curve	60-95 °C	40 minutes	1

Statistical Analysis

The program SAS (2012), which stands for Statistical Analysis System, was utilised to find the impact of various variables on research parameters. In this study, the means were significantly compared using the least significant difference (LSD) test.

Results and Discussion

Antibiotic susceptibility test

Using the CLSI (11) recommendations and the disc diffusion technique, the antibiotic susceptibility of *P. aeruginosa* isolates was ascertained based on the diameter of the inhibitory zone (mm). Ten antibiotics

(Amoxicillin + Clavulanic acid, Azithromycin, Gentamycin, Ciprofloxacin, Amikacin, Meropenem, Piperacillin, Ceftriaxone, Carbenicillin, and Imipenem) were tested against all 25 isolates of *P. aeruginosa* in this experiment. The findings showed that the isolates of *P. aeruginosa* exhibit extremely high levels of resistance to the administered medicines. Eleven *P. aeruginosa* isolates with (90-100%) multi-drug resistance, three isolates with (80%) resistance, seven isolates with (60-70%) resistance, and four isolates with (50%) resistance were found to have antibiograms. They can either have this capacity naturally or by genetic material mutations or horizontal gene transfer (22).

The reason behind the bacterial resistance to these antibiotics is due to the production of β-lactamase enzymes, which acted to destroy the β-lactams ring, thereby leading to modification of antibiotics structure and spoilage of their effects (23). This is almost identical to Hasan and his research group that they found all the 40 clinical isolates of patients in their study were high resistance to beta-lactams (Augmentin, Amoxicillin, Ampicillin and Cefixime) (24).

Ten *P. aeruginosa* isolates that were more resistant to antibiotics were selected for this investigation in order to test the effectiveness of *C. sinensis* on isolates, as indicated in (Table 4).

Table (4): Antibiotic susceptibility test of *P. aeruginosa* isolates.

No.	MEM	PRL	CRO	AZM	CN	CIP	AK	CAR	IMI	AMC	Percentage of resistance
P1	R	S	R	S	R	S	R	R	S	S	**50%**
P2	I	R	R	R	R	I	R	R	I	R	**70%**
P3	R	R	R	R	R	R	R	R	R	R	**100%**
P4	R	S	R	S	R	R	S	R	R	R	**70%**
P5	S	R	R	R	R	R	R	R	R	R	**90%**
P6	R	R	R	S	R	S	R	S	S	R	**60%**
P7	R	R	R	R	R	R	R	R	S	R	**90%**
P8	S	R	R	R	R	R	I	R	R	R	**80%**
P9	R	R	R	R	R	R	R	R	R	R	**100%**
P10	S	R	S	R	S	R	R	S	R	S	**50%**
P11	R	R	R	R	R	R	R	R	R	R	**100%**
P12	R	S	R	S	R	R	S	R	R	R	**70%**
P13	S	R	R	R	R	R	R	R	S	R	**80%**
P14	R	R	S	R	R	S	R	S	S	S	**50%**
P15	R	R	R	R	R	R	R	R	R	R	**100%**
P16	R	S	R	S	R	S	R	S	R	R	**60%**
P17	R	R	R	R	R	R	R	R	R	R	**100%**
P18	R	S	R	R	S	R	S	R	S	S	**50%**
P19	S	R	R	S	R	S	R	R	I	R	**60%**
P20	R	R	R	R	R	R	R	R	R	R	**100%**
P21	R	I	R	R	R	R	R	R	R	R	**90%**
P22	S	R	R	R	R	R	R	R	I	R	**80%**
P23	R	R	R	R	R	R	R	R	R	R	**100%**
P24	R	S	R	S	R	R	S	R	R	R	**70%**
P25	I	R	R	R	R	R	R	R	R	R	**90%**

(P):*p. aeruginosa,* **(MEM):** Meropenem, **(PRL):** Pipercillin, **(CRO):** Ceftriaxone, **(AZM):** Azithromycin, **(CN):** Gentamycin, **(CIP):** Ciprofloxacin, **(AK):** Amikacin, **(CAR):** Carbenicillin, **(IMI):** Imipenem, **(AMC):** Amoxicillin+ Clavic acid.

Detection of biofilm formation

The method of microtiter plates was used to quantify the production of biofilms and evaluate the absorbance at 630 nm using an ELISA reader in order to assess the intensity of the biofilm. Within the range of 0.215 to 0.622, the isolates' mean optical density was 0.435 ± 0.287. As can be seen in Table 5, the findings demonstrated that every isolate had 100% robust biofilm development.

Bacterial biofilm is now modelled by *P. aeruginosa*. A lot of research has been done to determine how this particular bacterial species forms biofilms and how surface types affect bacterial adherence and biofilm development (25). Due to its high throughput screening capacities, ease of handling, and quicker and more accurate method of quantifying the contact cell attachment and biofilm formation of various bacterial strains, the microtiter plate has been the most popular and standard test for the detection of biofilm formation (26).

Prior research has indicated a relationship between *P. aeruginosa's* multi-drug resistance phenotype and its propensity to produce biofilms (27, 28). The role of biofilm and its relevance in the establishment of high antibiotic resistance by several bacterial species were emphasised by Mahdi and AL-Azawi (29). According to Ramos *et al*. (30), bacteria can adhere to host cells by forming a biofilm. The current study's outcomes corroborate those of Al-Nuaimi (31), who found that all *P. aeruginosa* isolates had the ability to produce biofilms.

High-performance liquid chromatography (HPLC)

Using the HPLC technique, individual phenolic contents of *C. sinensis* were examined according to Radovanovic *et al*. (16). Figures 1 and 2 of this study of both methanolic and aqueous extracts showed the presence of five flavonoid derivatives (apigenin, caffeine, chlorogenic acid, gallic acid, and tannic acid), respectively, in comparison to the standard compounds displayed in Figures (3, 4, 5, 6 and 7).

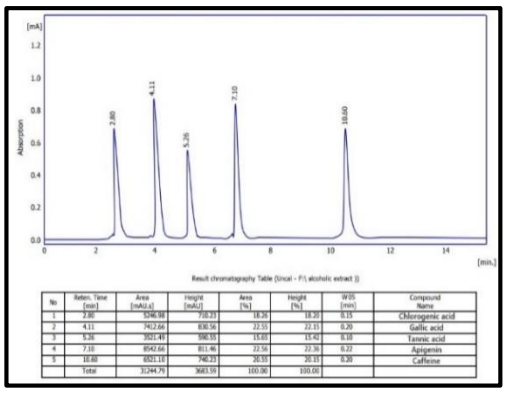


Figure (1): HPLC chromatogram of phenolic compounds in methanolic *C. sinensis* extract.

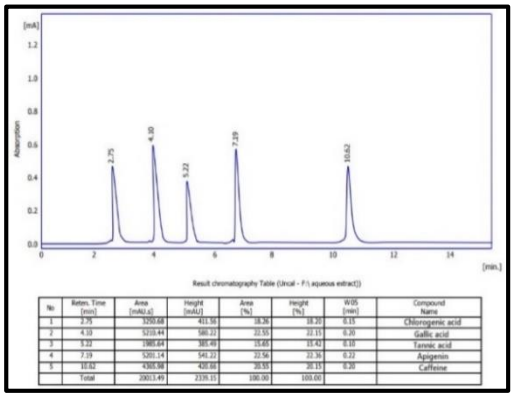


Figure (2): HPLC chromatogram of phenolic compounds in aqueous *C. sinensis* extract.

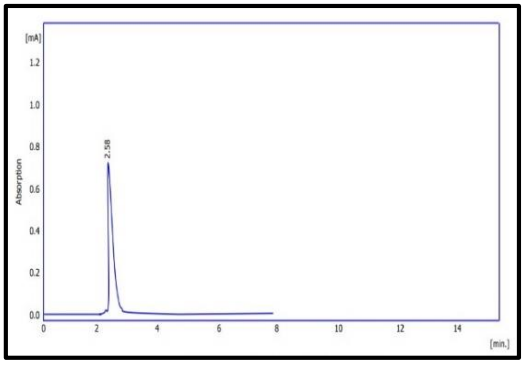


Figure (3): HPLC chromatogram of phenolic compound standard Chlorogenic acid

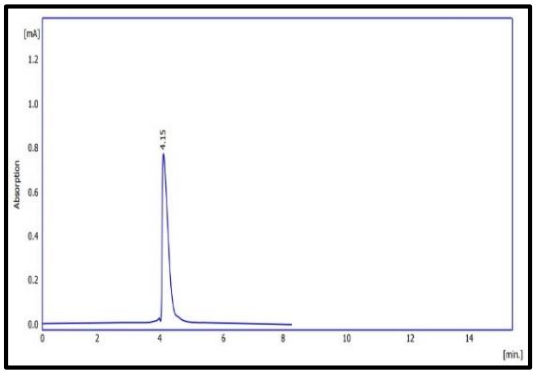


Figure (4): HPLC chromatogram of phenolic compound standard Gallic acid.

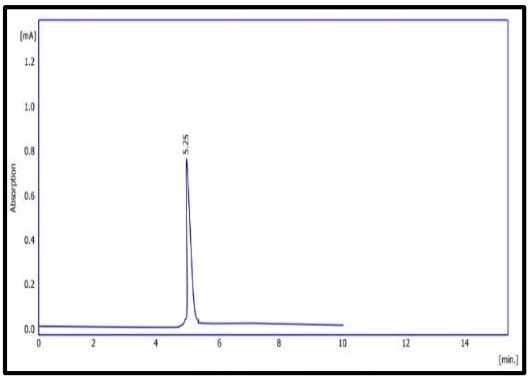


Figure (5): HPLC chromatogram of phenolic compound standard Tannic acid.

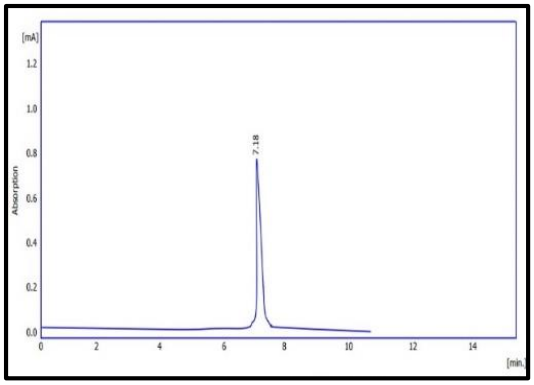


Figure (6): HPLC chromatogram of phenolic compound standard Apigenin.

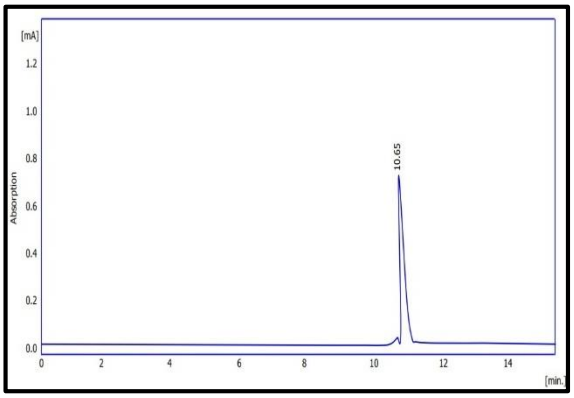


Figure (7): HPLC chromatogram of phenolic compound standard Caffeine

Antibacterial activity of *Camellia sinensis* extracts

Disk diffusion method

The disk-diffusion technique was used to determine the antibacterial activity of *C. sinensis* leaf extracts against isolates of *P. aeruginosa*. The results showed that the methanolic extract was more effective than the aqueous extract in concentrations of 128 and 256 mg/ml on *P. aeruginosa* isolates, giving the highest inhibition zone 19.33 ± 0.33 and 23.67 ± 0.33 mm, respectively in isolate (No. 9), when compared with the aqueous extract, which gave inhibition zone 14.33 ± 0.33 and 19.00 ± 0.00 mm respectively, as

seen in (Table 6) with a significant variance (P≤0.01).

The antibacterial activity in this study appears to be associated with the quantity of phenolic chemicals (apigenin, gallic acid, tannic acid, chlorogenic acid, and caffeine) that are found in the methanolic and aqueous extracts of *C. sinensis*.

The phenolic chemicals enter the bacterial cell and obstruct the metabolism of the cell. Additionally, they attach themselves to the cell's active sites of the enzymes, working to seal them off so that they can no longer connect to the building blocks. To prevent the function of ATP as an energy source, they either metabolically inhibit the enzymes and thereby nullify their work, or the phenolic compounds act as hydrogen ion carriers. This reduces the effectiveness of the microbial cell, whether it be bacterial or fungal, and ultimately results in its death (32).

Numerous phenolic compounds exhibited antibacterial properties against plant infections, suggesting that they might be useful in combating human diseases as well. Additionally, a number of derived phenolic compounds have antibacterial properties that differ from those of traditional medications and may thus play a significant role in improving antibacterial therapy (33). The current study's findings corroborated those of Barreira *et al*. (34), who found that methanolic extract of green tea was superior to aqueous extract in terms of effectiveness.

Table (5): Antibacterial activity of *Camellia sinensis* methanolic and aqueous extracts on *P. aeruginosa* isolates

No. of Isolate	Mean ± SE				LSD value
	Methanolic extract		Aqueous extract		
	128 mg/ml	256 mg/ml	128 mg/ml	256 mg/ml	
P_1	15.33 ± 0.33	18.33 ± 0.33	9.00 ± 0.00	13.33 ± 0.33	1.370**
P_2	15.67 ± 0.33	18.67 ± 0.33	10.33 ± 0.33	13.67 ± 0.33	1.582**
P_3	16.67 ± 0.33	21.33 ± 0.33	12.33 ± 0.33	16.33 ± 0.33	1.582**
P_4	17.33 ± 0.33	21.00 ± 0.00	13.33 ± 0.33	17.33 ± 0.33	1.370**
P_5	17.67 ± 0.33	21.67 ± 0.33	14.67 ± 0.33	18.67 ± 0.33	1.582**
P_6	14.33 ± 0.33	18.33 ± 0.33	11.67 ± 0.33	14.67 ± 0.33	1.582**
P_7	13.67 ± 0.33	17.33 ± 0.33	10.67 ± 0.33	15.67 ± 0.33	1.582**
P_8	13.67 ± 0.33	17.67 ± 0.33	11.33 ± 0.33	14.67 ± 0.33	1.582**
P_9	19.33 ± 0.33	23.67 ± 0.33	14.33 ± 0.33	19.00 ± 0.00	1.370**
P_{10}	10.67 ± 0.33	15.67 ± 0.33	8.67 ± 0.33	12.33 ± 0.33	1.582**
LSD value	1.341**	1.272**	1.272**	1.272**	-----
** (P≤0.01)					

(P): *P. aeruginosa*

The numbers in the table mention to inhibition zone measured in (mm)

Determination of the (MIC) of the *Camellia Sinensis* extracts

The MIC of the plant extracts was determined using the broth microdilution technique using a 96-well microtiter plate. The MIC results revealed that the methanolic extract was more efficient than the aqueous extract against *P. aeruginosa* isolates. For each isolate of *P. aeruginosa*, the methanolic

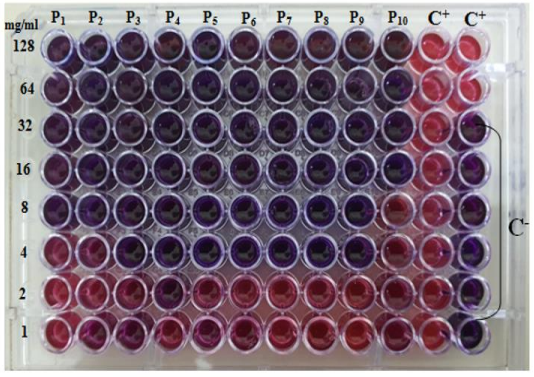
extract's mic values were 4 mg/ml, except for isolates 1 and 2, which were 8 mg/ml, and isolate No. 10, which was 16 mg/ml. The MIC of the aqueous extract on five *P. aeruginosa* isolates were 16 mg/ml, four isolates were 32 mg/ml, and isolate No. 10 was 64 mg/ml, as indicated in Table 7 and Figures (8 and 9).

This study's findings concurred with those of Latteef (35), who claimed that the methanolic extract of *Camellia sinensis* had a more potent inhibitory effect than the aqueous one. According to AL-Azawi (36), flavonoids have to be discovered as a strong antibacterial agent that can effectively combat a variety of pathogenic microorganisms in vitro.

Table (6): MIC of *Camellia sinensis* methanolic and aqueous extracts on *P. aeruginosa* isolates.

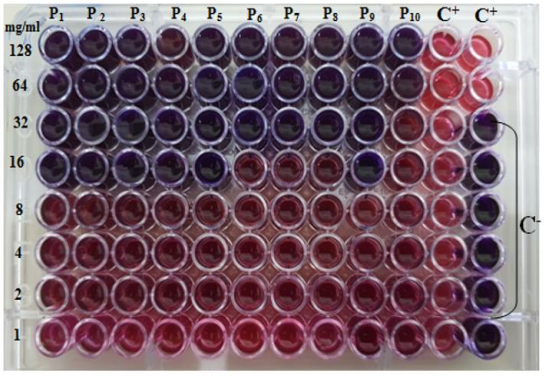
Isolate	Aqueous extract	Methanolic extract
	MIC (mg/ml)	MIC (mg/ml)
P_1	16	8
P_2	16	8
P_3	16	4
P_4	16	4
P_5	16	4
P_6	32	4
P_7	32	4
P_8	32	4
P_9	32	4
P_{10}	64	16

(P): *P. aeruginosa* isolate



(P): *P. aeruginosa* isolate, (C+): Control positive (Bacteria + Media), (C-): Control negative (Media only)

Figure (8): MIC of *Camellia sinensis* methanolic extract on *P. aeruginosa* isolates



(P): *P. aeruginosa* isolate, (C+): Control positive (Bacteria + Media), (C-): Control negative (Media only)

Figure (9): MIC of *Camellia sinensis* aqueous extract on *P. aeruginosa* isolates

Anti-Biofilm activity of *Camellia sinensis* extracts

Because of their propensity to develop multidrug resistance, elude host defences, and withstand a variety of stresses, bacteria and other microorganisms form complex structures called bacterial biofilms, which are linked to chronic bacterial infections that affect humans and other organisms and pose a major global health concern (37). One of the main

causes of nosocomial infections that do not go away after antibiotic therapy is *P. aeruginosa*. These bacteria can form biofilms on implanted and indwelling devices (38).

The biofilm development of *P. aeruginosa* was completely prevented by the *C. sinensis* methanolic leaf extract at 8 mg/ml, as indicated in Table(8). Conversely, the aqueous extract's antibiofilm efficacy on *P. aeruginosa* isolates was 100% suppressed at 16 mg/ml (Table 9).

By reducing or inhibiting the production of biofilms in a concentration-dependent manner, the flavonoids compounds demonstrated anti-biofilm efficacy. Based on Awolola *et al*. (39), it is believed that flavonoids cause bacterial aggregation by causing partial lysis, membrane fusion, and a decreased membrane surface that limits active nutrient absorption.

According to Zayed *et al*. (40), the produced alcoholic green tea extract was found to have antibiofilm action at a concentration lower than that of the aqueous extract. The reason for this could be attributed to the higher concentration of active ingredients in the alcoholic extract compared to the aqueous extract. Multiple studies have indicated that the polyphenolic components of green tea are responsible for the antibacterial action of the tea(41).

Table (7): Biofilm formation of *P. aeruginosa* isolates before and after treatment with *Camellia sinensis* methanolic extract.

No of isolates	Before treatment	After treatment with methanolic extract (mg/ml)							
		1	2	4	8	16	32	64	128
P_1	Strong	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_2	Strong	Weak	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_3	Strong	Moderate	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_4	Strong	Weak	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_5	Strong	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_6	Strong	Weak	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_7	Strong	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_8	Strong	Weak	Weak	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_9	Strong	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_{10}	Strong	Moderate	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm

Table (8): Biofilm formation of *P. aeruginosa* isolates before and after treatment with *Camellia sinensis* aqueous extract.

No of isolates	Before treatment	After treatment with aqueous extract (mg/ml)							
		1	2	4	8	16	32	64	128
P_1	Strong	Moderate	Weak	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_2	Strong	Moderate	Weak	Weak	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_3	Strong	Moderate	Moderate	Moderate	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_4	Strong	Moderate	Moderate	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_5	Strong	Moderate	Moderate	Moderate	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_6	Strong	Moderate	Weak	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_7	Strong	Moderate	Moderate	Weak	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_8	Strong	Moderate	Moderate	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_9	Strong	Moderate	Moderate	Moderate	Weak	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm
P_{10}	Strong	Moderate	Moderate	Moderate	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm	Non Biofilm

(P): *P. aeruginosa,* Control negative (cut off) = 0.13

The first step in the production of a biofilm is cell attachment. Nutrients and inorganic and organic molecules are adsorbed on a surface to create the film (surface conditioning). In addition to being essential for cell proliferation, surface conditioning frequently fosters bacterial attachment and encourages cell adherence to surfaces, which can result in infections. Thus, the possibility exists that the introduction of plant extracts into growth medium created an unfavourable environment that would prevent cell attachment or lessen surface adherence (42).

Gene expression of *pelA* gene

The mRNA expression of the *pelA* gene was examined in this work using the quantitative RT-PCR test by contrasting the isolates that were left untreated with those that were exposed to the sub MIC of *C. sinensis* methanolic leaf extracts (Table 10).

The accuracy of qRT-PCR results largely depends on the selected reference genes (43), the validity of which is a prerequisite for the correct application of qRT-PCR to analyze changes in target gene expression (44). Many previous local studies (22)(45) used the *16SrRNA*, *GyrA* genes as a housekeeping gene.

Cycle threshold, or Ct value, was used to record the amplification. strong Ct values signify low gene expression, whereas low Ct values signify strong gene expression. Because the housekeeping gene expresses itself consistently in cells or tissues under various settings, it is employed in molecular investigations (46). Methanolic extracts of *C. sinensis* demonstrated a significant reduction in *pelA* gene expression in this investigation.

Additional to anti-virulence strategies, flavonoids can demonstrate antibacterial activities by disrupting the cytoplasmic membrane, inhibiting the synthesis of nucleic acids, inhibiting

energy metabolism, inhibiting the synthesis of folic acids, and inhibiting the synthesis and function of cell membranes (47). Thus, All of these routes might be in charge of the phenolic extract's antibacterial and reducing properties, as demonstrated in this study.

Numerous scientists have investigated the impact of plant extracts on gene expression. Laith and AL-Azawi (48) indicated that the methanolic extract from plants had an impact on the virulence genes under investigation, which were shown to be considerably down-regulated in the samples.

Table (9): Gene expression results for *pelA* in *P. aeruginosa* before and after treatment with methanolic extract.

Group	Sample	Ct reference gene *GyrA*	Ct target gene *PelA*	Δct	ΔΔct	Fold gene of expression
Before treated (Control)	C1	17.17	17.4	0.23	0	1
	C2	16.94	15.52	-1.42	0	1
	C3	16.95	15.51	-1.44	0	1
	C4	16.98	15.3	-1.68	0	1
	C5	20.69	16.52	-4.17	0	1
After treated	P1	18.26	19.9	1.64	1.410	0.376
	P2	20	19.08	-0.92	0.500	0.707
	P3	20.05	19.11	-0.94	0.500	0.707
	P4	20.79	19.2	-1.59	0.090	0.940
	P5	20	20.03	0.03	4.200	0.054

(P): *P. aeruginosa* isolate, (C): Control.

Conclusion

This research found that even though the bacterial isolates formed significant *pelA* biofilms prior to treatment, the phenolic compounds isolated from *C. sinensis* leaves demonstrated high *P. aeruginosa* antibiofilm agent that has the ability to downregulate the gene.

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