Pak. j. life soc. Sci. (2024), 22(1): 2288-2304 E-ISSN: 2221-7630;P-ISSN: 1727-4915

Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

https://doi.org/10.57239/PJLSS-2024-22.1.00169

RESEARCH ARTICLE

Antibacterial and Antibiofilm Activity of Phenolic Compounds Extracted From Camellia Sinensis And Evaluate The Effect On The Gene Expression (Clfa) In Staphylococcus Aureus

Zeena F. Al-Aboudi [1], Ahmed H. AL-Azawi [2*]

[1,2]Institute of Genetic Engineering and Biotechnology for post graduate studies, University of Baghdad, Baghdad, Iraq

ARTICLE INFO

Received: May 28, 2024

Accepted: Jun 16, 2024

Keywords

Antibacterial

Antibiofilm

ClfA

C. sinensis

Phenolic compounds

S. aureus

***Corresponding Author:**

ahmed@ige.uobaghdad.edu.iq

ABSTRACT

The purpose of this study to extract phenolic compounds from Camellia sinensis, investigate their antibacterial and anti-biofilm properties and apply these chemicals to certain virulence genes that are involved in the production of biofilms in Staphylococcus aureus isolates. From laboratories of the Institute of Genetic Eng. and Biotech. at Baghdad university, twenty five isolates of S. aureus were obtained. VITEK-2 technology and growing isolates on Mannitol salt agar verified the diagnosis. Both methanolic and aqueous extracts of Camellia sinensis leaves were made using a Soxhlet equipment and maceration method, In addition to detecting the phenolic compound using high performance liquid chromatography (HPLC) technology. The results indicated that, at concentrations of 128 and 256 mg/ml, For every S. aureus isolate, the methanolic extract outperformed the aqueous extract in effectiveness. giving the highest inhibition zone of 20.33 and 24.67 mm, respectively, while the aqueous extract gave inhibition zones of 11.33 and 13.33 mm, respectively. All S. aureus isolates had a minimum inhibitory concentration (MIC) of 4 mg/ml for the methanolic extract, except isolates No. 7 and 8, which had MIC of 2 mg/ml, and isolate No.10, which had MIC of 8 mg/ml. However, all S. aureus isolates had MIC values of 16 mg/ml for aqueous extract. At 4 mg/ml, the methanolic leaf extract of C. sinensis completely prevented the biofilm formation of S. aureus, but at 16 mg/ml, the aqueous extract completely inhibited the antibiofilm activity of S. aureus isolates. Examining the virulence gene ClfA, which is in controlling S. aureus biofilm formation, the gene expression results showed the isolates treated with the methanolic extracts' sub-MIC had lower levels of the ClfA gene than the untreated isolates. Progress, rural women's participation in sports will face more opportunities.

INTRODUCTION

Since antimicrobial resistance has a major effect on the global economy, it is regarded as one of the most important public health issues. Antibacterial resistance is reducing the available treatment choices for treating infections, which increases the morbidity and mortality of bacterial infectious illnesses (1). Gram-positive *Staphylococcus aureus*, belonging to the *Stpohylococccceae* family, has a diameter of around 1 µm. It causes cutaneous infections and exhibits toxic shock syndrome. Additionally, it creates grape-like collections (2). *S. aureus* Multi-Drug Resistance is a serious problem. Patients with weak immune systems, diabetes, and weakened immune systems are more vulnerable to Staphylococcal infections, particularly those caused by *S. aureus*, which can lead to soft tissue and skin infections. When *S. aureus* overgrows in an infected area of the body, toxins are secreted, leading to the potentially lethal illness known as toxic shock syndrome, this enhances the severity of *S. aureus* infections (3). The method of creating drugs is using more and more natural components. According to Jafar-sales and Shadi-Dizaji (4), bioactive chemicals are employed not only as direct therapeutic medications but also as a

main component in the manufacture of drugs or as a foundation for the synthesis of new biologically active compounds. As a result, the use of natural therapies instead of pharmaceuticals and antibiotics was taken into consideration. Because they may protect against free radicals and delay the beginning of many chronic illnesses, finding plants with strong antioxidant capabilities has become increasingly relevant recently (5). The *camellia sinensis* is a shrub from to the *Theaceae* family that is often planted in semi-tropical climates and pruned to a height of 2-5 feet (6). The presence of chemical components in green tea that are directly linked to human health has been established by several researches. The components that are extracted and separated from green tea, such as tea polyphenols, caffeine, theanine, and tea polysaccharides, have been shown to have pharmacological activities, including anti-oxidant (7), anti-cancer (8), nervous system protection (9), and blood sugar-lowering (10). Furthermore, certain bacterio-ecophysiological factors indicate that plants could be a very valuable source of numerous additional anti-biofilm agents, with Epigallocatechin-3-gallate (EGCG), one of the main polyphenols, acting as a model for the identification and examination of action mechanisms (11).

MATERIALS AND METHODS

Bacterial isolates

Twenty-five *S. aureus* isolates were obtained from the Baghdad university / Institute of Genetic Eng. and Biotech. The isolates had previously been taken from various hospitals in Baghdad and were recently diagnosed using chemical and molecular tests. The isolates were then cultured on Mannitol salt agar for *S. aureus* for 18–24 hours at 42°C using the VITEK-2 System to confirm the diagnosis. Following their reconstitution on the Nutrient agar medium, the isolates had a 24-hour aerobic incubation period at 37°C.

Antibiotic susceptibility test

The antibiotic susceptibility test for ten distinct antibiotics was conducted using the Kirby-Bauer technique, according with guidelines of WHO (12). One or two isolated colonies of bacteria from the original culture were chosen, and they were then placed into a test tube with four milliliters of normal saline to create a bacterial suspension of moderate turbidity. This procedure was done in comparison to the standard turbidity solution that was prepared. This comes out to about 1.5×108 CFU/ml. A part of bacterial suspension was carefully and uniformly placed using a sterile cotton swab over Mueller-Hinton agar medium, and it was then incubated for ten minutes. The antimicrobial discs were then positioned on the agar and firmly pushed to make sure they made contact with the agar using sterile forceps. The plates were subsequently turned over and incubated for 18 to 24 hours at 37°C. In accordance with the Clinical Laboratories Standards Institute (13), inhibition zones that formed around the discs were measured in millimeters (mm) using a metric ruler.

Assessment of biofilm formation

According to Patel *et al.* (14), the quantification of *S. aureus* biofilm development was evaluated; all isolates were cultured overnight at 37°C in Brain Heart Infusion Broth. Tryptic soy broth (TSB) containing 1% glucose was added to each isolate, and the resulting mixture was carefully pipetted. The suspension of bacterial isolate was adjusted to meet the No. 0.5 turbidity criterion of McFarland.

A 200 µl amount of each isolate's culture was put, in triplicate, to a sterile, flat-bottomed, 96-well microtiter plate. After that, the plates were covered with lids and incubated for 24 hours at 37°C in aerobic conditions. To get rid of the bacteria that weren't adherent, the planktonic cells were washed twice with distilled water after incubation. 200 µl of 100% methanol was used to fix the adhering bacterial cells in each well for 20 minutes at room temperature. Each well was treated with 200 µl of 0.1% crystal violet for 15 minutes in order to stain the adhering cells. Following the completion of the staining process, washing with distilled water 2 or 3 times over removed the excess stain. After letting the plate at room temperature for about half an hour to make sure it was totally dry, 33% acetic acid was then used to cure the stain. At 630 nm in wavelength, optical density (OD) values were obtained with an ELISA auto reader. All test values were deducted from the sterile medium's average optical density (OD) values. 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) (15).

Collection of *Camellia sinensis*

Camellia sinensis plant were obtained from the nearby Iraqi markets, was recognized as such by an expert at the University of Baghdad, Department of Biology, College of Science. Following a water wash, room temperature drying, and grinder grinding, the leaves were placed in storage at 4oC for additional study. First, 400 g of powdered *C. sinensis* leaves were macerated with two liters of petroleum ether to defat the leaves. After being gathered, air drying, the residue was divided into two groups. N'Guessan et al. (16, 54, 55) and AACC (17) report that methanol and hot water were used individually to extract the defatted plant leaves into aqueous and methanolic extracts.

High-Performance Liquid Chromatography (HPLC)

Camellia sinensis Methanolic and aqueous extracts mixtures were found using (HPLC), according to Radovanovic *et al.* (18).

Study the antibacterial activity of *C. sinensis* extracts

Disc diffusion method

The disc diffusion method of antibacterial action was used in accordance with the standard procedure by Razmavar *et al.* (19) 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. 15 minutes were spent drying the dishes. In order 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. 6 mm 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 utilized to calculate the (MIC) of the *C. sinensis* extracts utilizing the broth microdilution process. Plant extracts were made as a working solution at a concentration of 256 mg/ml in broth. To achieve the concentrations of 128-1 mg/ml for methanolic and aqueous extracts, respectively, successive two-fold dilutions of the extract were prepared immediately on the plate. First wells in row A contained 100 µl of the produced methanolic and aqueous extracts of *C. sinensis*. In rows B through H of the columns, there were 100 µl of broth. Serial dilutions were performed twice carefully along the columns (from rows A-H) using a micropipette. The process was repeated up to the last row (H), when the last 100µl was disposed of. First, 100µl was taken out of the beginning concentrations in row A and transported to the following row with the 100µl broth, appropriately mixed. 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 1×108 CFU/ml bacterial inoculum. For 12 to 24 hours, microtiter plates were incubated at 37 °C (20). Following the duration of incubation, each well received 20 µl of resazurin dye, which was applied, and any color changes were observed for 30 minutes during incubation. The lowest

concentrations of extracts at which no color changed from blue to pink in the resazurin broth assay were identified visually in broth microdilutions as the Minimum Inhibitory Concentrations (21).

Study the antibiofilm activity of *C. sinensis* extracts

The methanolic extract of *C. sinensis* was tested for antibiofilm activity using a 96-well microtiter plate. Plant extracts were made as a working solution at a concentration of 256 mg/ml in broth. To make methanolic extract concentrations of 128-1 mg/ml, the extract was then instantly diluted twice and placed on a plate. The first wells in row A were filled with 100 µl of the produced methanolic extract. There was just 100 µl of broth in rows B–H in the columns. Methodically, two-fold serial dilutions were made down the columns (from rows A-H) using micropipettes. 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 1×108 CFU/ml bacterial inoculum. According to the paragraph **(Assessment of biofilm development)**, the same process was carried out.

Gene expression Analysis Using qRT PCR Technique

Before and after the methanolic extract treatment, the resistant isolates' gene expression levels were assessed. in order to evaluate the impact on the expression of *ClfA* in *S. aureus* 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 contains a list of primers, and Table 2 provides a summary of the reaction mixture. Additionally, the thermal cycler process has been improved via several trials and is shown in Tables 3.

To compare the Ct values directly between the reference (housekeeping) gene and the target gene, the qRT-PCR data findings were computed. Utilizing the ΔΔCt approach as outlined by Schmittgen *et al.* (22), the genes were subjected to relative measurement of gene expression levels (fold change).

Table 1: Primers utilized in this Study

Primer name		Sequence (5´-3´)	Size	Reference
ClfA	F	ATTGGCGTGGCTTCAGTGCT	280 bp	(23)
	R	CGTTTCTTCCGTAGTTGCATTTG		
16S rRNA	F	ACTCCTACGGGAGGCAGCAGT	180 bp	(24)
	R	TATTACCGCGGCTGCTGGC		
GyrB	F	GCGTCCGTTGATTGAAGCG	240 bp	(25)
	R	AACGTCACTTGCAACATCGC		

Table 2: Volumes and qRT-PCR reaction mix concentrations

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 software program SAS (2012), which stands for Statistical Analysis System, was utilized 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

Antibiotic susceptibility test

By using the disk diffusion technique in accordance with CLSI (2023) recommendations, the antibiotic susceptibility of *S. aureus* isolates was ascertained based on the inhibition zone diameter (mm). In this experiment, ten antibiotics were tested against all 25 *S. aureus* isolates (Amoxicillin+ Clavic acid, Azithromycin, Gentamycin, Chloramphenicol, Vancomycin, Meropenem, Pipercillin, Ceftriaxon, Trimethoprim and Nitrofuratin). The findings showed that the *S. aureus* isolates are extremely resistant to the prescribed antibiotics. The 10 antibiotics used in the sensitivity test for *S. aureus* showed varying degrees of resistance among the isolates. Ten *S. aureus* isolates (90–100%), eight isolates (80%), six isolates (60–70%), and one strain (50%) were multidrug resistant. According to Jabur and Kandala (26), all 25 bacterial isolates shown 100% resistance to the drug Gentamycin. Ten *S. aureus* 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 *S. aureus* isolates

No.	MEM	PRL	C	AZM	CN	VA	SXT	F	CRO	AMC	Percentage of resistance
S1	R	R	I	R	R	R	R	R	R	R	90%
S2	S	R	R	R	R	R	R	R	R	I	90%
S3	R	R	R	R	R	S	R	R	R	I	80%
S4	S	R	S	R	R	S	R	S	R	I	50%
S5	S	R	R	R	R	R	R	R	R	I	80%
S6	S	R	R	R	R	R	R	R	R	I	80%
S7	S	R	R	R	R	I	R	R	R	I	70%
S8	R	R	R	R	R	I	R	R	R	R	90%
S9	R	R	I	R	R	I	R	R	R	R	80%
S10	R	R	R	R	R	I	R	R	S	R	80%
S11	R	S	R	R	R	R	R	R	R	R	90%

S12	R	R	R	R	R	R	R	R	R	R	100%
S13	R	S	R	R	R	R	R	R	S	R	80%
S14	R	R	R	R	R	R	R	R	R	R	100%
S15	S	S	I	R	R	I	R	R	R	R	60%
S16	R	R	R	R	R	R	R	R	R	R	100%
S17	R	R	I	S	R	R	R	R	R	R	80%
S18	R	R	I	R	R	I	R	R	R	R	80%
S19	S	R	R	S	R	R	R	R	S	R	70%
S20	I	R	S	R	R	R	S	R	S	R	60%
S21	R	I	R	R	R	I	R	R	R	R	90%
S22	S	R	R	R	R	I	R	R	I	R	70%
S23	R	R	R	R	R	R	R	R	R	R	100%
S24	R	R	I	S	R	I	R	R	S	R	60%
S25	I	R	R	R	R	R	R	R	R	R	90%

S): *S.aureus*, **(MEM):** Meropenem, **(PRL):** Pipercillin **(C):** Chloramphenicol**, (AZM):** Azithromycin, **(CN):** Gentamycin**, (VA):** Vancomycin**, (SXT):** Trimethoprim, **(F):** Nitrofuratin, **(CRO):** Ceftriaxon, **(AMC):** Amoxicillin, **(AMC):** Amoxicillin+ Clavic acid**.**

Detection of biofilm formation

The method of microtiter plate, where the absorbance was measured at 630 nm by an ELISA reader, is used to detect the quantitative biofilm production and evaluate biofilm intensity. As can be seen in Table 5, the findings demonstrated that every isolate had 100% robust biofilm development.

Table 5: Biofilm forming of *S. aureus* isolates

S. aureus isolates	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}
Biofilm formation	Strong	Strong	Strong	Strong	Strong	Strong	Strong	Strong	Strong	Strong

***S*):** *S. aureus,* Control negative (cut off) = 0.31

High-performance liquid chromatography (HPLC)

The HPLC technique was utilized to examine the individual phenolic contents of *C. sinensis*, as reported by Radovanovic *et al*. (18). When compared with standard substances as indicated in Figures 3, 4, 5, 6 and 7, five flavonoids derivatives (apigenin, caffeine, chlorogenic acid, gallic acid, and tannic acid) were found in the methanolic and aqueous extracts Figure 1 and Figure 2, respectively.

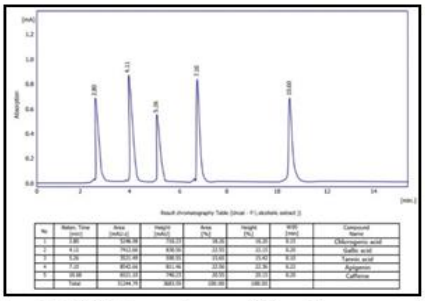

Figure 1: HPLC chromatogram of phenolic compounds in methanolic *C. sinensis* extract

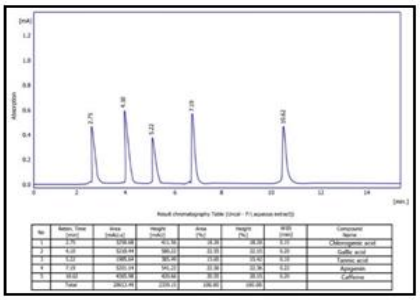

Figure 2: HPLC chromatogram of phenolic compounds in aqueous *C. sinensis* extract

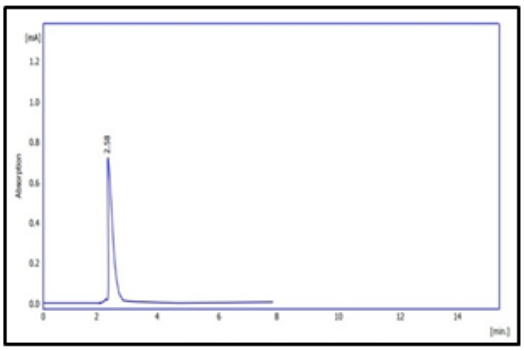

Figure 3: HPLC chromatogram of phenolic compound standard Chlorogenic acid

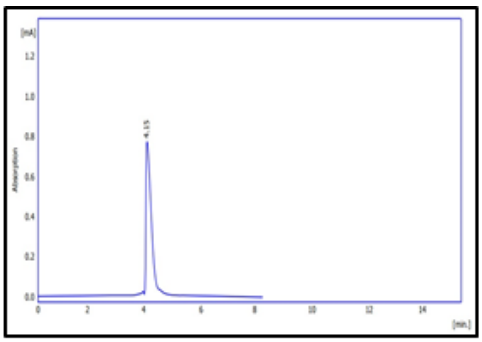

Figure 4: HPLC chromatogram of phenolic compound standard Gallic acid

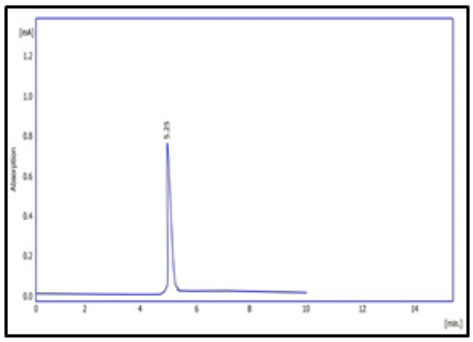

Figure 5: HPLC chromatogram of phenolic compound standard Tannic acid

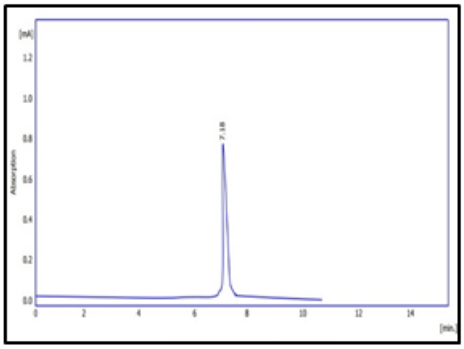

Figure 6: HPLC chromatogram of phenolic compound standard Apigenin

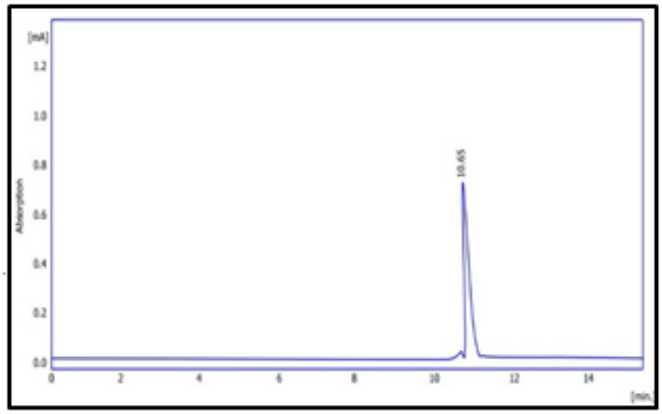

Figure 7: HPLC chromatogram of phenolic compound standard Caffeine

Antibacterial activity of *Camellia sinensis* extracts

Disk diffusion method

The disk-diffusion technique was utilized to evaluate the antibacterial activity of *C. sinensis* leaf extracts on *S. aureus* isolates. As demonstrated in Table 6, results showed that at doses of 128 and 256 mg/ml, the methanolic extract outperformed the aqueous extract in term of effectiveness against S.*aureus* isolates. The methanolic extract gave the highest inhibition zone value of 20.33 ± 0.33 and 24.67 ± 0.33 mm, respectively, in isolate (No. 9), when compared with the aqueous extract, which gave inhibition zone values of 11.33 ± 0.33 and 13.33 ± 0.33 mm, respectively.

Table 6: Antibacterial activity of *Camellia sinensis* methanolic and aqueous extracts on *S. aureus* isolates

No. of Isolate	Mean ± SE				LSD value
	Methanolic extract		Aqueous extract		
	128 mg/ml	256 mg/ml	128 mg/ml	256 mg/ml	
S_1	15.33 ± 0.33	20.33 ± 0.33	10.00 ± 0.00	12.33 ± 0.33	1.370**
S_2	12.33 ± 0.33	15.33 ± 0.33	10.33 ± 0.33	12.67 ± 0.33	1.582**
S_3	15.33 ± 0.33	18.33 ± 0.33	9.33 ± 0.33	12.33 ± 0.33	1.582**
S_4	11.67 ± 0.33	15.33 ± 0.33	8.33 ± 0.33	10.33 ± 0.33	1.582**
S_5	12.33 ± 0.33	16.67 ± 0.33	10.33 ± 0.33	12.33 ± 0.33	1.582**
S_6	14.33 ± 0.33	19.33 ± 0.33	9.33 ± 0.33	11.33 ± 0.33	1.582**
S_7	13.33 ± 0.33	17.33 ± 0.33	10.33 ± 0.33	12.33 ± 0.33	1.582**
S_8	15.33 ± 0.33	20.33 ± 0.33	10.67 ± 0.33	13.33 ± 0.33	1.582**
S_9	20.33 ± 0.33	24.67 ± 0.33	11.33 ± 0.33	13.33 ± 0.33	1.582**
S_{10}	15.33 ± 0.33	18.33 ± 0.33	10.33 ± 0.33	13.33 ± 0.33	1.582**
LSD value	1.341**	1.341**	1.272**	1.341**	----
** (P≤0.01)					

S): *S. aureus*The numbers in the table mention to inhibition zone measured in (mm)

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

The MIC result demonstrated that the methanolic extract superior to the aqueous extract in the way of efficacy when applied to *S. aureus* isolates. All *S. aureus* isolates had MIC values of 4 mg/ml for the methanolic extract, with the exception of isolates No. 7 and 8, which had MIC values of 2 mg/ml, and isolate No. 10, which had MIC values of 8 mg/ml. All *S. aureus* isolates had an aqueous extract minimum inhibitory concentration (MIC) of 16 mg/ml, as indicated by Table 7 and Figures 8 and 9.

Table 7: MIC of *Camellia sinensis* methanolic and aqueous extracts on *S. aureus* isolates

Isolate	Aqueous extract	Methanolic extract
	MIC (mg/ml)	MIC (mg/ml)
S_1	16	4
S_2	16	4
S_3	16	4
S_4	16	4
S_5	16	4
S_6	16	4
S_7	16	2
S_8	16	2
S_9	16	4
S_{10}	16	8

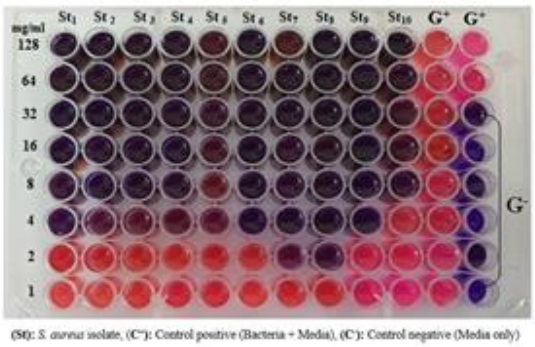

Figure 8: MIC of *Camellia sinensis* methanolic extract on *S. aureus* isolates

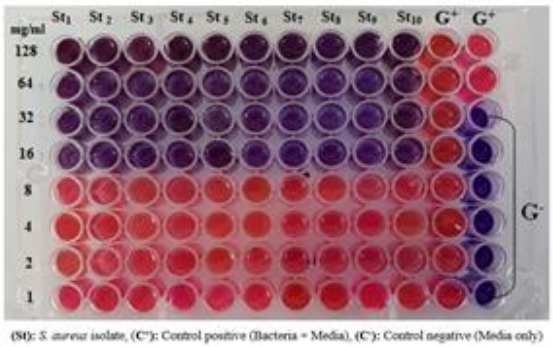

Figure 9: MIC of *Camellia sinensis* aqueous extract on *S. aureus* isolates

Anti-Biofilm activity of *Camellia sinensis* extracts

As shown in Table 8, the methanolic leaf extract of *Camellia sinensis* entirely inhibited *S. aureus* from forming biofilms at 4 mg/ml. In contrast, the aqueous extract's antibiofilm efficacy against *S. aureus* isolates was completely reduced at 16 mg/ml Table 9.

Table 8: Biofilm formation of *S. aureus* isolates before and after treatment with *C. sinensis* methanolic extract

No of isolates	Before treatment	After treatment with methanolic extract (mg/ml)							
		1	2	4	8	16	32	64	128
S_1	Strong	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_2	Strong	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_3	Strong	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_4	Strong	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_5	Strong	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_6	Strong	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_7	Strong	Moderate	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_8	Strong	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm

S_9	Strong	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_{10}	Strong	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm

Table 9: Biofilm formation of *S. aureus* 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
St_1	Strong	Moderate	Moderate	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_2	Strong	Moderate	Moderate	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_3	Strong	Moderate	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_4	Strong	Moderate	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_5	Strong	Weak	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_6	Strong	Moderate	Moderate	Moderate	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_7	Strong	Weak	Weak	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
S_8	Strong	Weak	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm

***S*9**	Strong	Moderate	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm	None Biofilm
***S*10**	Strong	Weak	Weak	Weak	Weak	None Biofilm	None Biofilm	None Biofilm	None Biofilm

***S*): *S. aureus*,** Control negative (cut off) = 0.27

Gene expression of *ClfA* gene

According to research, the methanolic extract outperformed the aqueous extract in terms of effectiveness. Therefore, the impact on *ClfA* gene expression was investigated using the methanolic extract. Through comparison of untreated isolates with isolates treated with the sub-MIC of *C. sinensis* methanolic leaf extracts, The *ClfA* gene's mRNA expression was examined in the quantitative RT-PCR experiment Table 10.

Table 10: Gene expression results for *ClfA* in *S. aureus* before and after treatment with methanolic extract

Group	Sample	Ct reference gene *GyrB*	Ct target gene *ClfA*	Δct	ΔΔct	Fold gene of expression
Before treated (Control)	**C1**	**20.26**	**22.74**	**2.48**	**0**	**1**
	C2	**20.88**	**23.11**	**2.23**	**0**	**1**
	C3	**20.48**	**23.1**	**2.62**	**0**	**1**
	C4	**21.19**	**23.33**	**2.14**	**0**	**1**
	C5	**21.46**	**24.04**	**2.58**	**0**	**1**
After treated	***S*1**	**20.24**	**23.88**	**3.64**	**1.160**	**0.448**
	***S*2**	**20.68**	**24.07**	**3.39**	**1.160**	**0.448**
	***S*3**	**20.43**	**23.83**	**3.4**	**0.780**	**0.582**
	***S*4**	**20.58**	**24.42**	**3.84**	**1.700**	**0.308**
	***S*5**	**19.95**	**24.87**	**4.92**	**2.340**	**0.198**

(*S*): *S. aureus* isolate

DISCUSSION

The results of this study revealed that *S. aureus* had a significant level of resistance to many β-lactam antibiotics; this resistance developed as a result of frequent usage of these antibiotics, including continuous, sporadic exposure and overuse at inappropriate dosages. The bacterial resistance to these antibiotics was caused by the production of β-lactamase enzymes, which broke down the β-lactam ring and changed the structure of the antibiotics, spoiling their effects (27). Additionally, the bacterial species that were effluent pumps and the outer membrane's decreased permeability also contributed to the resistance ratio's large increase (28).

Microtiter plate is a popular used technique, Because of its high throughput screening capacities and ease handling to detect biofilm formation of various bacterial strains (29).

The model organism for bacterial biofilms is *S. aureus*. Numerous studies have been conducted to determine how this particular bacterial species forms biofilms and how surface types affect bacterial adherence and biofilm development (30). Previous research (31) (32) showed a relationship between the multi-drug resistance phenotype of *S. aureus* and its capacity to generate biofilms. Mahdi and AL-Azawi (33) highlighted the importance of biofilm and its role in the development of high antibiotic resistance by various bacterial species. According to Ramos *et al*. (34), bacteria can adhere to host cells by forming a biofilm. The current study's findings concur with those of Setiabudy *et al*. (35), who found that all isolates of *S. aureus* developed biofilms in 100% of cases.

In current study, 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 compounds enter the bacterial cell and obstruct normal metabolic processes. Additionally, they attach to the cell's active sites of the enzymes and attempt to shut them so that the enzymes are unable to connect to the building blocks. As a result, they metabolically inhibit the enzymes and thereby neutralize their activity, or the phenolic compounds function as carriers of hydrogen ions, any reducing agents that bind with adenosine triphosphate, which stands for an oxidizing agent, and subsequently terminate the activity of ATP as an energy source, thereby weakening the energy within the microbial cell, be it fungal or bacterial, decreasing its efficacy, and ultimately causing its death (36). Numerous phenolic compounds have been shown to have antibacterial properties against plant diseases, suggesting that they may be useful in helping to fight human infections. Furthermore, many derived phenolic compounds have antibacterial properties that differ from those of traditional medications; as a result, they may be useful in boosting antibacterial treatment (37). The current study's findings corroborated those of Barreira *et al*. (38), who found that methanolic extract of green tea was superior to aqueous extract in terms of effectiveness. However, it differed with Mehta *et al.* (39) who found that the green tea aqueous extract had the most antibacterial activity against *S. aureus* out of all the tea extracts examined.

Furthermore, the broth microdilution method was used to determine the MIC of the plant extracts using the 96-well microtiter plate. The MIC of the antimicrobial agents against *S. aureus* has been ascertained by a technique that employs the oxidation-reduction colorimetric indicator resazurin. Resazurin is clearly visible to the unaided eye and may be used to assess the minimum inhibitory concentration (MIC) even in the absence of a spectrophotometer. It appears blue when oxidized and becomes pink when reduced by live cells (40). This study's findings agreed with those of Latteef (41), who claimed that the methanolic extract of *Camellia sinensis* had a more inhibitory effect than the aqueous one. According to Kiedrowski *et al*. (42), flavonoids have to be discovered as a strong antibacterial agent that can effectively combat a variety of pathogenic microorganisms.

Due to their capacity to develop multidrug resistance, elude host defenses, and withstand a variety of stresses, bacteria and other microorganisms form complex structures called bacterial biofilms, which are known to cause chronic bacterial infections in humans and other animals as well as to be a major global health concern (43). A common feature of respiratory infections in cystic fibrosis patients is the presence of *S. aureus* biofilm. Methicillin-resistant *S. aureus* (MRSA) has emerged as a result of these

infections being resistant to antibiotics (42). Finding novel, efficient compounds to prevent the development of bacterial biofilms is essential.

Through concentration-dependent inhibition or decrease of biofilm formation, the flavonoid compounds demonstrated antibiofilm action. According to Awolola *et al*. (44), By partly lysing the bacteria, flavonoids likely encourage bacterial aggregation. This decreases active nutrient absorption through a smaller membrane surface and causes membrane fusion.

According to Zayed *et al.* (45), the alcoholic green tea extract that was synthesized was found to have antibiofilm action, albeit at a lesser concentration than the aqueous extract. This may be because the alcoholic extract contains a greater number of active compounds than the aqueous extract does, specifically epicatechin and epigallocatechin-3-gallate (EGCG), which are the active constituents. Moreover, multiple studies have shown that the polyphenolic components of green tea are responsible for the antibacterial action of the tea (46). 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 (47). Thus, the possibility exists that the introduction of plant extracts into growth medium created an adverse environment that would prevent cell attachment or lessen surface adherence.

According to Huang *et al*. (48), the validity of the reference genes used has a significant impact on the accuracy of qRT-PCR findings. This is especially true when using qRT-PCR to examine changes in target gene expression, as Liu *et al.* (49) has shown. The housekeeping genes *16SrRNA* and *GyrB* were employed in several prior local research (50) (25). Cycle threshold, or Ct value, was used to record the amplification. high Ct values indicate low gene expression, whereas low Ct values indicate high gene expression. Because the housekeeping gene expresses itself consistently in cells or tissues under various settings, it is employed in molecular investigations (22).

In gene expression experiment, methanolic extracts of *C. sinensis* demonstrated a significant reduction in *ClfA* gene expression in this study. Besides 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 (51). Therefore, the antibacterial and down-regulation effects of phenolic extract in this study could be attributed to all these mechanisms. Laith and AL-Azawi (52) indicated that the methanolic extract from plants had an impact on the virulence genes, which were shown to be considerably down-regulated in the samples.

CONCLUSION

The results of this study showed that, even though the bacterial isolates had formed strong biofilms prior to treatment, the phenolic compounds that were extracted from *C. sinensis* leaves were able to downregulate the *ClfA* gene and exhibited strong antibacterial and anti-biofilm properties against *S. aureus*.

Conflict of Interest

None

REFERENCES

1. Mohamed IE, AL-Azawi AH. Evaluation of Antibacterial Activity of Laurus nobilis Leaves Extract against Escherichia coli Isolates, Iraqi Journal of Biotechnology. 2022; 21(2): 623-631.

2. Maddiboyina B, Roy H, Ramaiah M, Sarvesh CN, Kosuru SH, Nakkala RK, Nayak BS. Methicillin resistant *Staphylococcus aureus:* novel treatment approach breakthroughs. Bulletin of the National Research Centre. 2023; 47 (1): 95. doi:10.1186/s42269-023-01072-3

3. Mukherjee R, Priyadarshini A, Pandey RP, Raj VS. Antimicrobial resistance in *Staphylococcus aureus*. Insights into drug resistance in Staphylococcus aureus. 2021; 85. doi: 10.5772/intechopen.96888

4. Jafari-sales A, Shadi-Dizaji A. Evaluation of Inhibitory Effect of Methanol Extract of Allium Sativum in vitro on *Staphylococcus aureus* and *Escherichia coli*. *Scientific Journal of Nursing*, Midwifery and Paramedical Faculty. 2019; 5(1): 61-68.

5. Pruteanu L, Bailey LDS, Gradinaru AC, Jantschi L. The biochemistry and effectiveness of antioxidants in food, fruits, and marine algae. Antioxidants. 2023; 12: 860. doi: 10.3390/antiox12040860.

6. Goenka P, Sarawgi A, Karun V, Nigam AG, Dutta S, Marwah N. *Camellia sinensis* (Tea): Implications and role in preventing dental decay. Phcog Rev. 2013; 7: 152–6. doi: 10.4103/0973-7847.120515.

7. Lambert JD, Elias J. The antioxidant and pro-oxidant activities of green tea polyphenols: A role in cancer prevention. Arch. Biochem.Biophys. 2010; 50: 65–72. doi: 10.1016/j.abb.2010.06.013.

8. Balazi A, Sirotkin. AV, Foldesiova M, Makovicky P, Chrastinova L, Makovicky P, Chrenek P. Green tea can supress rabbit ovarian functions in vitro and in vivo. Theriogenology. 2019; 127: 72–79. doi: 10.1016/j.theriogenology.2019.01.010.

9. Yoneda Y, Kuramoto N, Kawada K. The role of glutamine in neurogenesis promoted by the green tea amino acid theanine in neural progenitor cells for brain health. Neurochem. Int. 2019; 129: 104505. doi: 10.1016/j.neuint.2019.104505.

10. De Amorim LMN, Vaz SR, Cesario G, Coelho ASG, Botelho PB. Effect of green tea extract on bone mass and body composition in individuals with diabetes. J. Funct. 2018; 40:589–594. doi.org/10.1016/j.jff.2017.11.039

11. Hengge R. Targeting bacterial biofilms by the green tea polyphenol EGCG. Molecules. 2019; 24 (13): 2403. doi: 10.3390/molecules24132403

12. World Health Organization (WHO). Basic laboratory procedures in clinical bacteriology. 2nd. Genev. 2003; Switzerland.

13. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptiblity Testing 33rd ed. CLSI supplement M100 (ISBN 978-1-68440-170-3 IPrintl: ISBN 978-1-68440-171-0 Electronich), Clinical and Laboratory Standards Institute. 2023; USA.

14. Patel FM, Goswami PN, Khara R. Detection of Biofilm formation in device associated clinical bacterial isolates in cancer patients. Sri Lankan Journal of Infectious Diseases. 2016; 6(1): 43-50. doi:10.4038/sljid.v6i1.8086

15. Kirmusaoglu S. The Methods for Detection of Biofilm and Screening Antibiofilm Activity of Agents. In Exopolysaccharides-Methods of Preparation and Application. In technology Open. 2019; 84411. doi: 10.5772/intechopen.84411

16. N'Guessan JD, Bidie AP, Lenta BN, Weniger B, Andre P, Guina F. *In vitro* assays for bioactivity-guided isolation of anti-salmonella and antioxidant compounds in *Thon ninja* sanguine flowers. African Journal of Biotechnology. 2007; 6: 1685-1689.

17. American Association of Cereal Chemists (AACC). Method 08-01. The Association St. Paul, M.N.1984.

18. Radovanovic B, Mladenovic J, Rdovanovic A, Pavlovic R, Nikolic V. Phenolic composition, antioxidant, antimicrobial and cytotoxic activites of *Allium porrum* L. (Serbia) Extracts. Journal of food and Nutrition Research. 2015; 3 (9): 564-569.

19. Razmavar S, Mahmood AA, Salmah BI, Pouya H. Antibacterial Activity of Leaf Extracts of *Baeckea frutescens* against Methicillin-Resistant *Staphylococcus aureus.* BioMedical Research International. 2014; Article ID 521287, 5. doi: 10.1155/2014/521287

20. Ohikhena FU, Wintola OA, Afolayan AJ. Evaluation of the Antibacterial and Antifungal Properties of *Phragmanthera capitata* (Sprengel) Balle (Loranthaceae), a Mistletoe Growing on Rubber Tree, Using the Dilution Techniques. The Scientific World Journal. 2017; Article ID 9658598. 8 pages doi.org/10.1155/2017/9658598.

21. Ohikhena FU, Wintola OA, Afolayan AJ. Evaluation of the Antibacterial and Antifungal Properties of *Phragmanthera capitata* (Sprengel) Balle (Loranthaceae), a Mistletoe Growing on Rubber Tree, Using the Dilution Techniques. The Scientific World Journal. 2017; Article ID 9658598: 8.

22. Schmittgen TD, Lee EJ, Jiang J, Sarkar A, Yang L, Elton T. Real-time PCR quantification of precursor and mature microRNA. Methods. 2008; 44 (1): 31-38.

23. Tristan A, Ying L, Bes M, Etienne J, Vandenesch F, Lina G. Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infections. J. Clin. Microbiol. 2003; 41: 4465-4467.

24. Clifford RJ, Milillo M Prestwood J, Quintero R, Zurawski DV, Kwak YL. Detection of bacterial *16SrRNA* and identification of four clinically important bacteria by real time PCR. PloS one. 2012; 7 (11): 48558. doi: 10.1371/journal.pone.0048558.

25. Crawford EC, Singh A, Metcalf D, Gibson TW, Weese SJ. Identification of appropriate reference genes for qPCR studies in *Staphylococcus pseudintermedius* and preliminary assessment of *icaA* gene expression in biofilm embedded bacteria. BMC research notes. 2014; 7 (1): 1-6. doi: 10.1186/1756-0500-7-451.

26. Jabur EQ Kandala N. The Production of Biofilm from Methicillin Resistant *Staphylococcus aureus* Isolated from PostSurgical Operation Inflammation, Iraqi Journal of Science. 2022; 63 (9): 3688-3702. Doi:10.24996/ijs.2022.63.9.3

27. Mora-Ochomogo M, Lohans CT. β-Lactam antibiotic targets and resistance mechanisms: from covalent inhibitors to substrates. RSC Medicinal Chemistry. 2021; 12 (10): 1623-1639.

28. Sun J, Rutherford ST, Silhavy TG, Huang KC. Physical properties of the bacterial outer membrane. Nature Reviews Microbiology. 2022; 20 (4): 236-248. doi: 10.1038/s41579-021-00638-0.

29. Senobar Tahaei SA, Stájer A, Barrak I, Ostorházi E, Szabó D, Gajdács M. Correlation between biofilm-formation and the antibiotic resistant phenotype in *Staphylococcus aureus* isolates: a laboratory-based study in Hungary and a review of the literature. Infection and drug resistance. 2021; 1155-1168. doi: 10.2147/IDR.S303992.

30. Idrees M, Sawant S. Karodia N, Rahman A. *Staphylococcus aureus* biofilm: Morphology, genetics, pathogenesis and treatment strategies. International Journal of Environmental Research and Public Health. 2021; 18 (14): 7602. doi: 10.3390/ijerph18147602.

31. Yasin SA, AL-Azawi AH. Antibacterial Activity of *Conocarpus Erectus* Leaves Extracts on Some Microorganisms Isolated From Patients With Burn Infection. Plant Archives. 2019; 19 (2): 583-589

32. Senobar Tahaei SA, Stajer A, Barrak I, Ostorhazi E, Szabo D, Gajdacs M. Correlation between biofilm-formation and the antibiotic resistant phenotype in *Staphylococcus aureus* isolates: a laboratory-based study in Hungary and a review of the literature. Infection and drug resistance. 2021; 1155-1168. doi: 10.2147/IDR.S303992.

33. Mahdi LF, AL-Azawi AH. Synergistic Effect of Conocarpus erectus Extract and some Antibiotics against Multi Drug Resistant *Pseudomonas aeruginosa*. Iraqi Journal of Biotechnology. 2022; 21 (2): 308-325

34. Ramos G, Rocha J, Tuon F. Seasonal humidity may influence *Pseudomonas aeruginosa* hospital acquired infection rates. International Journal of Infectious Diseases. 2013; 17: 757-76. doi: 10.1016/j.ijid.2013.03.002.

35. Setiabudy M, Adhiputra IKAI, Darwinata AE, Fatmawati NND. Correlation Between icaA/B/C/D Gene and Biofilm Formation in *Staphylococcus aureus* Infection. Jurnal Kedokteran: Media Informasi Ilmu Kedokteran dan Kesehatan. 2023; 9 (1): 15-22.

36. Fathima A, Rao JR. Selective toxicity of Catechin-a natural flavonoid towards bacteria. Applied Microbial Biotechnology. 2016; 100: 6395-6402. doi: 10.1007/s00253-016-7492-x.

37. Sanver D, Murray BS, Sadeghpour A, Rappolt M, Nelson AL. Experimental modeling of flavonoid bio- membrane interactions. Langmuir. 2016; 32: 13234-13243.

38. Barreira S, Moutinho C, Silva AM, Neves J, Seo EJ, Hegazy MEF. Phytochemical characterization and biological activities of green tea (*Camellia sinensis*) produced in the Azores, Portugal. Phytomedicine Plus. 2021; 1 (1): 100001.

39. Mehta A, Saxena G, Mani A. Comparative analysis of antibacterial activity of aqueous, ethanolic, methanolic and acetone extracts of commercial green tea and black tea against standard bacterial strains. Int J Curr Microbiol App Sci. 2016; 5 (11): 145-52. doi:10.20546/IJCMAS.2016.511.017

40. Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. African Journal of Biotechnology. 2008; 12 (7): 1797-1806. doi:10.4314/ajb.v7i12.58804

41. Latteef NS. Phytochemical, antibacterial and antioxidant activity of *Camellia sinensis* methanolic and aqueous extracts. IOSR J Pharm Biol Sci. 2016; 11: 113-119.

42. Kiedrowski MR, Gaston JR, Kocak BR, Coburn SL. Lee S, Pilewski JM. *Staphylococcus aureus* Biofilm Growth on Cystic Fibrosis Airway Epithelial Cells Is Enhanced during Respiratory Syncytial Virus Coinfection. mSphere. 2018; 3. doi: 10.1128/mSphere.00341-18.

43. Sharma D, Misba L, Khan AU. Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrob. Resist. Infect. Control. 2019; 8: 76. doi: 10.1186/s13756-019-0533-3.

44. Awolola GV, Koorbanally NA, Chenia H, Shode FO, Baijnath H. Antibacterial and anti-biofilm activity of flavonoids and triterpenes isolated from the extracts of Ficus Sansibarica warb. Subsp. Sansibarica (Moraceae) extracts. African Journal of Traditional, Complementary Alternative Medicines. 2014; 11:124-131. doi: 10.4314/ajtcam.v11i3.19.

45. Zayed SM, Aboulwafa MM, Hashem AM, Saleh SE. Biofilm formation by Streptococcus mutans and its inhibition by green tea extracts. AMB Express. 2021; 11 (1): 73. doi: 10.1186/s13568-021-01232-6.

46. Mohamed IE, AL-Azawi AH. Evaluation of the Antibiofilm Activity of *Laurus nobilis* Leaves Extract and Assessment of Its Effect on *fimA* and *papC* genes in *Escherichia coli* isolates*.* The Egyptian Journal of Hospital Medicine. 2023; 92: 5692-5700. doi:10.21608/ejhm.2023.307719

47. Pratiwi SUT, Lagendijk EL, Hertiani T, Weert SD, Hondel CA. Antimicrobial effects of Indonesian Medicinal Plants Extracts on Planktonic and Biofilm Growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Journal Horticulture. 2015; 2: 119. doi:10.4172/2376-0354.1000119

48. Huang ZC, Ouyang LJ, Zhang L, Sha YE, Zeng FH. Selection and evaluation of internal reference genes in Eucalyptus species. Journal of Northwest AF University (Nat. Sci). 2013; 41 (10): 67–72.

49. Liu J, Chen D, Peters BM, Li L, Li B Xu Z, Staphylococcal chromosomal cassettes mec (SCCmec): A mobile genetic element in methicillin-resistant *Staphylococcus aureus*. Microbial. Pathogenesis. 2016; 101: 56-67. doi: 10.1016/j.micpath.2016.10.028.

50. Meng L, Cao X, Li C, Li J, Xie H, Shi J, Liu C. Housekeeping gene stability in *Pesudomonas aeruginosa* PAO1 under the pressure of commonly used antibiotics in molecular microbiology assays. Frontiers in Microbiology. 2023; 14:1140515.

51. Al-Khafaji AR, AL-Azawi AH. The Phenolic Compounds Extracted From *Rosmarinus officinalis* L. and Effect of The Biofilm Genes in *Pseudomonas aeruginosa*. Ann. For. Res. 2022; 65(1): 1943-1958,

52. Laith FM, AL-Azawi AH. Antibiofilm Activity of *Conocarpus erectus* Leaves Extract and Assessment Its Effect on *pelA* and *algD* Genes on Multi-drug Resistant *Pseudomonas aeruginosa.* The Egyptian Journal of Hospital Medicine. 2022; 89 (2): 6961- 6969. doi: 10.21608/EJHM.2022.271919

53. Jam, F. A., Haq, I. U., & Fatima, T. (2012). Phychological contract and job outcomes: Mediating role of affective commitment. *Journal of Educational and Social Research*, *2*(4), 79-79.

54. Kanval, N., Ihsan, H., Irum, S., & Ambreen, I. (2024). Human Capital Formation, Foreign Direct Investment Inflows, and Economic Growth: A Way Forward to Achieve Sustainable Development. *Journal of Management Practices, Humanities and Social Sciences*, *8*(3), 48-61.