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GC-MS Analysis of Bioactive Compounds of *Gardenia thunbergia* Thumb. Leaves and Antibiofilm against *Staphylococcus aureus* Clinical Isolates

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ABSTRACT

The goal of this study was to utilize gas chromatography-mass spectrometry to characterize the bioactive phytochemicals found in the petroleum ether fraction of *Gardenia thunbergia* Thunb. leaves methanol extract. The GC-MS analysis yielded several peaks, indicating the existence of 23 compounds tentatively with various medicinal properties. Palmitic acid, butyl palmitate, acetyl tributyl citrate, methyl palmitate, cholesta-4,6-dien-3-ol, and ursolic acid methyl ester were found to be the most prevalent compounds. In this study, the antibacterial effect of total methanol extract and petroleum ether fraction against *Staphylococcus aureus* was evaluated and compared with the positive control (vancomycin) and the negative control (dimethyl sulfoxide). Twenty-two *S. aureus* clinical isolates were obtained for the experiment. The values of MICs were detected, and the effect of the tested extracts on the biofilm formation ability of *S. aureus* isolates was evaluated. The total methanol extract and the petroleum ether fraction have notable antibacterial activity against the tested *S. aureus* clinical isolates.

Keywords: Bioactive compounds, *Gardenia thunbergia*, GC-MS, Petroleum ether fraction, *Staphylococcus aureus*.

1. INTRODUCTION

Staphylococcus aureus has the ability to cause a wide array of infections that range from minor skin infections to severe life-threatening ones. This could be attributed to its multidrug resistance in addition to its multiple virulence factors. Recently, virulence suppression approaches have been developed to fight infections caused by *S. aureus*.[1] These strategies are based on the concept of prevention of infections via suppressing the action or inhibition of the production of the virulence factors instead of targeting the bacterial pathways. Biofilm formation is an important virulence factor of *S. aureus* that is considered a challenge for treating infections caused by such pathogenic bacteria.[2] So, there is a need to find novel compounds that can suppress biofilm formation by *S. aureus*.

Medicinal plants provide enormous benefits to individuals and societies as over 3.4 billion individuals in the developing world use traditional plant-based treatments. This equates to more than 88 percent of the world's population, with conventional medicine providing the majority of primary health care. The World Health Organization (WHO) supports

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traditional medicine if it can be proven effective and safe (WHO 1985). As a result, there is a need to validate herbal medicine's ethnomedicinal use and then isolate and characterize the chemicals that are likely to be added to the prospective drug list.[3]

Gardenia thunbergia Thunb. is a member of family Rubiaceae and is found across the African tropics, from Mozambique and Zimbabwe in the south to Ethiopia and Kenya in the east. It is now categorized as a pantropical weed, and cultivations are assumed to cause extensive dispersion.[4] The plant has been shown to have significant biological and pharmacological qualities, as well as being used in folk medicine to cure a number of ailments.[5] According to a survey, traditional healers in KwaZulu-Natal employ *G. thunbergia* to treat malaria.[6] In addition, one of the most potent naturally occurring molluscicides is 4-ethylcatechol, obtained from *G. thunbergia*.[7] Furthermore, The flavonoid, terpene, iridoid, and steroid chemicals found in the *Gardenia* family have a variety of medicinal uses, such as anti-inflammatory and analgesic properties as well as antioxidant effects.[8, 9] A previous study reported the isolation of nine compounds from *G. thunbergia*: astragalin, isoquercitrin, loliolide, 5,6-epoxy-3-hydroxymegastigm-7-en-9-one, scopoletin, lupeol, *β*-sitosterol, and stigmasterol, including a new triterpenoid saponin with antiplasmodial activity.[5] In addition, the *G. thunbergia* aerial parts demonstrated selective *in vitro* cytotoxic activities against HL-60 and HepG2 cells, according to a previous study.[10] Previously, it was reported that a methanol extract of *G. thunbergia* inhibited the *in vitro* growth of *Plasmodium falciparum* strain D10 (IC_{50} 14.36 mg/mL).[6] Because advances in biotechnology have enabled faster and more detailed investigation of natural compounds, leading to the isolation of bioactive compounds with health benefits, our research is focused on elucidating potential sources of ethnomedicinal plants using modern scientific analysis such as gas chromatography-mass spectrometry.

In recent years, GC-MS has solidified its position as a key technical platform for secondary metabolite profiling in both plant and non-plant species.[3] As a result, the goal of this study was to look into the possible chemical components by first preparing the saponifiable matter of the petroleum ether fraction of *G. thunbergia* leaves methanol extract and preparing methyl esters of fatty acids present in the saponifiable matter then identifying the compounds using GC-MS analysis. Also, we aimed to explore the antibacterial and antibiofilm potential of the total methanol extract and petroleum ether fraction against *S. aureus* clinical isolates.

2. METHODS

2.1. Collection and extraction of plant material

Plant material collection of *G. thunbergia* leaves portions were obtained from plants growing in Al Sadat City, Egypt. Prof. Dr. Ibrahim Abd El Rahim Mashaly, Professor of Plant Ecology and Flora, Botany Department, Faculty of Science, Mansoura University, certified the plant's legitimacy. The leaves were air-dried and then ground before use for this study. In March 2018, the voucher specimen (PG00415-M) was placed in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tanta University. *G. thunbergia* leaves were dried and powdered at room temperature, yielding 1.5 kg of dry powder. They were extracted three times, each time with 3 L of 95% methanol, and then concentrated using a rotary evaporator to provide a dry extract of (220 g) yield. A part of the methanol extract (175 g) was successively fractionated using petroleum ether, methylene chloride, ethyl acetate, and *n*-butanol. Petroleum ether fraction obtained from the leaves' methanolic extract was used to investigate the unsaponifiable matter.

2.2. Preparation of unsaponifiable and saponifiable matter

Two grams of petroleum ether fraction of the leaves were saponified by heating in 30 mL alcoholic KOH (10%) for 6 hours, using a boiling water bath with a reflux condenser. The evaporation of the majority of alcohol was performed by distilling the saponifiable matter, and then the aqueous liquid was diluted with 20 mL water before being extracted with ether till exhaustion. The combined ethereal extracts were washed with distilled water, dehydrated over anhydrous Na_2SO_4, and distilled off under a vacuum to yield the unsaponifiable matter (USM) of *G. thunbergia* leaves. After extracting USM, the alkaline aqueous solutions were acidified separately with conc. HCl and the released fatty acids were extracted with ether. The fatty acids were obtained by washing the ether extract with distilled water, dehydrating over anhydrous Na_2SO_4, and distilling off.

The ether extract was evaporated to dryness, and the residue was weighed and subjected to chromatographic analysis and separation. After dissolving 0.2 g of the fatty acid fraction of the leaves in 25 mL pure methanol, 3 mL sulfuric acid was added, and the mixture was refluxed for 2 hours. The methanol was removed, and the residue was extracted with increasing amounts of ether until they were exhausted. The ether was distilled out after the mixed ethereal extracts were washed with distilled water and dehydrated over anhydrous Na_2SO_4. GC-MS was used to analyze the fatty acid methyl esters residues. The GC-MS analysis was performed with a TRACE GC Ultra Gas Chromatographs (THERMO Scientific ISQ., USA), coupled with a thermo mass spectrometer detector (Thermo-Scientific Spectrometer) and MesloPC Acquisition as operator. The GC-MS system was equipped with a fused silica HP-5MS column (30 mL, 0.25 mm, 0.25 µm film thickness). Helium served as the carrier gas. The temperature was programed as 60 °C for 1 min; rising at 4 °C /min to 240 °C, and holding for 1 min. Diluted samples of 1 µL of the mixtures were always injected. The run time was 43.38 min. Electron ionization (EI) at 70 eV yielded mass spectra with a spectral range of *m/z* 30.0 -499.97.

2.3. Bacterial isolates

Twenty-two *S. aureus* clinical isolates were obtained from clinical samples from the laboratories of Tanta University Hospitals. The isolates were identified using the previously described biochemical tests.[11]

2.4. Antibacterial activity

S. aureus susceptibility to both the plant methanol extract and petroleum ether fraction was elucidated by the Kirby-Bauer method.[12] In brief, bacterial suspensions were spread onto Petri dishes containing Muller-Hinton agar. Then, after loading the tested agents (with a concentration of 1000 µg/mL) onto sterile filter paper discs, they were placed on the surface of MHA. The plates were left overnight at 37°C. A positive control (vancomycin) and a negative control (dimethyl sulfoxide) were used.

2.5. Minimum inhibitory concentrations (MICs)

The values of MICs were detected by broth microdilution method.[12] Briefly, the methanol extract and the petroleum ether fraction were serially two-fold diluted by Muller-Hinton broth in 96 well microtitration plates. Then, the bacterial suspensions were inoculated into the wells, and the plates were left overnight at 37 °C. As previously mentioned, the plates contained positive and negative controls. MIC values of the tested compounds were determined as the lowest concentrations, that resulted in an inhibition of the visual growth of the tested bacteria.

2.6. Crystal violet assay

The effect of the tested extracts (at 0.5 MIC values) on the biofilm formation ability of *S. aureus* isolates was evaluated using crystal violet test.[13] The values of the optical density (OD) were determined at 490 nm using an ELISA reader. Then, the capability of *S. aureus* isolates to form biofilm was grouped into four classes as follows:

Non-biofilm former isolates (ODc < OD < 2 ODc), weak biofilm former isolates (2 ODc < OD < 4 ODc), moderate biofilm former isolates (4 ODc < OD < 6 ODc), and strong biofilm former isolates (6 ODc < OD). The cut-off OD (ODc) was identified as the product of the addition of the mean OD to three standard deviations (SD) of the negative control. The biofilms formed in the microtitration plates wells were rinsed two times with phosphate-buffered saline (PBS) to remove the loosely attached cells. Then, the contents of the wells were scrapped using a pipette tip after adding PBS (200 µL). They were then serially diluted and cultured onto MHA plates. Finally, after overnight incubation at 37°C, the number of colony forming units (CFU/mL) were counted.

2.7. Statistical analysis

It was performed using GraphPad Prism 8 (USA). The obtained data were represented as mean ± standard deviation (SD), and they were analyzed using one-way analysis of variance (ANOVA).

3. RESULTS

The database of the Medical Research Institute (MRI) in Alexandria, Egypt, was used to interpret the mass spectrum of the GC-MS. The unknown components' mass spectra were compared to the known components' spectra kept in the Institute library. Principal components were determined using genuine standards and data from digital libraries. The materials' compound name, molecular formula, molecular weight, and probability were presented. By comparing the average peak area of each component to the total areas, the relative percentage quantity of each component was computed. **Figure 1, 2 and Table 1**, represent the results of the GC-MS analysis of the saponifiable matter of petroleum ether fraction of *G. thunbergia* leaves.

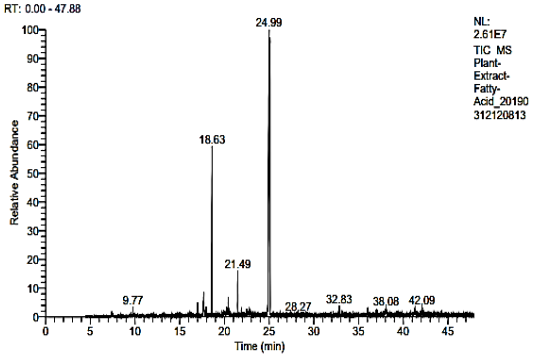


Figure 1: Mass spectrum of GC-MS Analysis of fatty acids methyl esters of *G. thunbergia*

3.1. Susceptibility of *S. aureus* clinical isolates to the total methanol extract and the petroleum ether fraction

The total methanol extract and petroleum ether fraction revealed antibacterial potential against the tested *S. aureus* isolates using Kirby-Bauer assay. Interestingly, the petroleum ether fraction had a lower MIC value than the total methanol extract, as shown in **Table 2**.

3.2. Antibiofilm activity

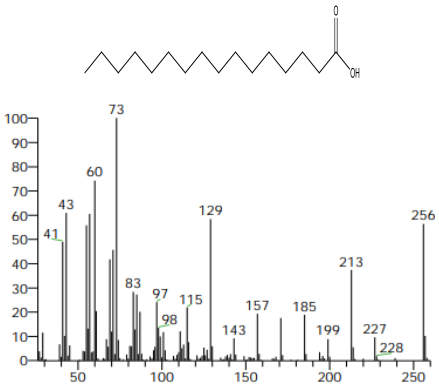
Petroleum ether fraction exhibited antibiofilm potential against the biofilm-forming *S. aureus* tested isolates. It caused a decline in the percentage of the isolates that strongly and moderately form biofilms from 45.45% to 13.64%, as presented in **Table 3**.

Table 1 shows the list of constituents. GC-MS analysis of *G. thunbergia* fatty acids methyl esters and other compounds with peak area percentage ≥ 1%, which revealed the presence of palmitic acid (35.74 %), butyl palmitate (13.64 %), acetyl tributyl citrate (4.29 %), methyl palmitate.

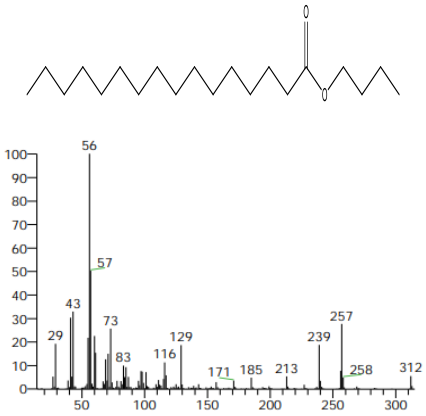
Table 1: GC-MS analysis of compounds detected in the saponifiable matter of petroleum ether fraction of *G. thunbergia* leaves

No.	R_t (min)	Peak area%	Identification Name	Molecular formula	$[M]^+$ *m/z*	Reported biological activity
1	12.10	1.20	Methyl-3,4-dimethoxybenzoate	$C_{10}H_{12}O_4$	196	Anti-filarial activity.[4]
2	16.37	1.67	Z-8-Methyl-9-tetradecenoic acid	$C_{15}H_{28}O_2$	240	Antibacterial activity.[14]
3	18.63	13.64	Butyl palmitate	$C_{20}H_{40}O_2$	312	Antioxidant activity.[15]
4	19.30	1.66	12,15-Octadecadienoic acid methyl ester	$C_{19}H_{34}O_2$	294	Anti-inflammatory activity.[16]
5	19.69	1.75	Methyl stearate	$C_{19}H_{38}O_2$	298	Antifungal and antioxidant activity.[17]
6	19.96	1.53	Linoleoyl chloride	$C_{18}H_{31}ClO$	298	Anti-inflammatory activity.[18]
7	20. 24	1.47	Stearic acid	$C_{18}H_{36}O_2$	284	Anti-microbial activity.[19]
8	20.43	1.99	Methyl palmitate	$C_{17}H_{34}O_2$	270	Anti-inflammatory activity.[16]
9	20.54	1.86	Ethyl stearate	$C_{20}H_{40}O_2$	312	Antioxidant effect.[20]
10	21.49	4.29	Acetyl tributyl citrate	$C_{20}H_{34}O_8$	402	Used as solvent, flavor ingredient, and in the manufacturing of pharmaceutical drugs.[16]
11	21.89	1.51	Ethyl palmitate	$C_{18}H_{36}O_2$	284	Antioxidant, Hemolytic, Hypo-cholesterolemic, Flavor, Nematicide, Anti-androgenic.[21]
12	22.51	1.41	4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	324	Supplementary ingredients in animal feed.[22]
13	22.82	1.81	Isobutyl stearate	$C_{22}H_{44}O_2$	340	Antioxidant activity.[23]
14	24.99	35.74	Palmitic acid	$C_{16}H_{32}O_2$	256	Antioxidant, hypercholesteremic, nematicide, and pesticide.[16]
15	31.89	1.24	2H-cyclopenta[a]phenanthrene-3,17-dione-16-(1,3-dimethyl-1H-pyrazol-4-ylmethylene)-10,13-dimethyl 1,6,7,8,9,10,11,12,13,14,15,16-Dodecahydro	$C_{25}H_{32}N_2O_2$	392	Antibacterial effect.[24]
16	32.84	1.93	Cholesta-4,6-dien-3-ol	$C_{27}H_{44}O$	384	Antimicrobial effect.[25]
17	33.19	1.42	Stigmasta-3,5-diene	$C_{29}H_{48}$	396	Antimicrobial activity.[26]
18	36.02	1.42	α'-Sitosterol	$C_{29}H_{50}O$	414	Antioxidant, anticancer, anti-diabetic, antimicrobial and immunomodulatory activities.[27]
19	37.96	1.26	Androstan-3-one-17-hydroxy-1,17-dimethyl	$C_{21}H_{34}O_2$	318	Food cosmetics, pharmaceuticals and bio-nano technological industries.[28]
20	38.08	1.72	Farnesyl bromide	$C_{15}H_{25}Br$	284	Used in synthesis of 3-Farnesyl Salicylic Acid, Antimicrobial agent.[29]
21	39.30	1.53	Cholest-4-en-3-one	$C_{27}H_{44}O$	384	Activity against obesity, keratinization, and hepatic diseases.[30]
22	41.28	1.82	Ursolic acid methyl ester	$C_{31}H_{50}O_3$	470	Anti-inflammatory, anticancer, antidiabetic, antioxidant and antibacterial activities.[31]
23	42.09	1.77	Androst-7-ene-6,17-dione-2,3,14-trihydroxy-(2α',3α',5α')	$C_{19}H_{26}O_5$	334	Cytotoxic activity.[32]

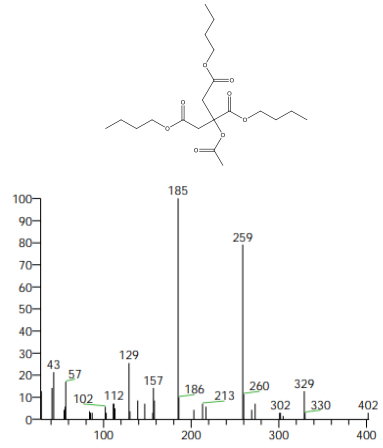
Palmitic acid



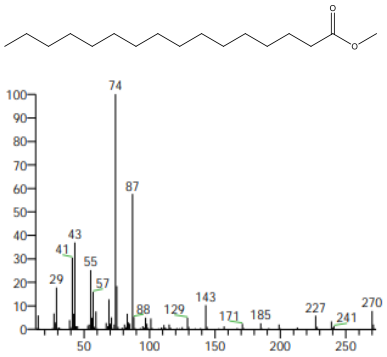
Butyl palmitate



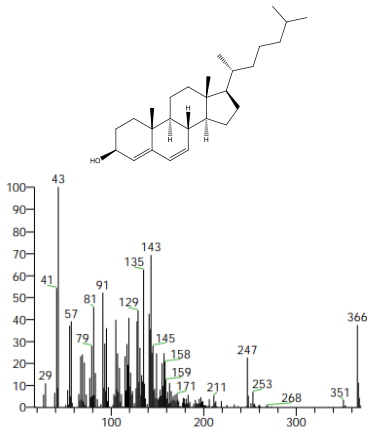
Acetyl tributyl citrate



Methyl palmitate



Cholesta-4,6-dien-3-ol



Ursolic acid methyl ester

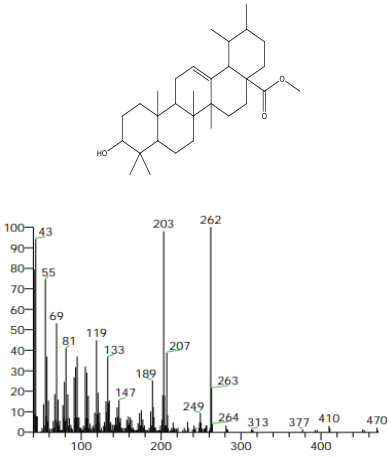


Figure 2: Structures and mass fragmentation pattern of major compounds identified by GC-MS

Figure 2 shows structures and mass fragmentation patterns of major compounds identified by GC-MS. The mass spectrometer detects the molecules eluted at various periods to detect their nature. The large compound splits into little fragments, causing peaks with varying *m/z* ratios to appear. These mass spectra are the compound's fingerprint, which can be identified using the library data.

Table 2: MIC values of the total methanol extract and the petroleum ether fraction of *G. thunbergia* against *S. aureus* clinical isolates.

Isolate code	MIC values (µg/mL)		Isolate code	MIC values (µg/mL)	
	Total methanol extract	petroleum ether fraction		Total methanol extract	petroleum ether fraction
S1	1024	32	S12	512	128
S2	1024	32	S13	512	64
S3	512	64	S14	256	128
S4	512	32	S15	1024	32
S5	1024	128	S16	1024	128
S6	1024	64	S17	512	64
S7	512	32	S18	512	32
S8	256	32	S19	256	64
S9	1024	64	S20	512	64
S10	256	64	S21	512	32
S11	256	32	S22	256	32

Table 3: Influence of petroleum ether fraction of *G. thunbergia* on the ability of *S. aureus* isolates to form biofilm.

Biofilm forming ability	Number of isolates before treatment	Number of isolates after treatment
Non-biofilm former	5	11
Weak biofilm former	7	8
Moderate biofilm former	4	1
Strong biofilm former	6	2

Regarding the number of CFU/mL, it significantly decreased ($p<0.05$) after treatment with petroleum ether fraction in 13.64 % of the isolates, as shown in **Figure 3**.

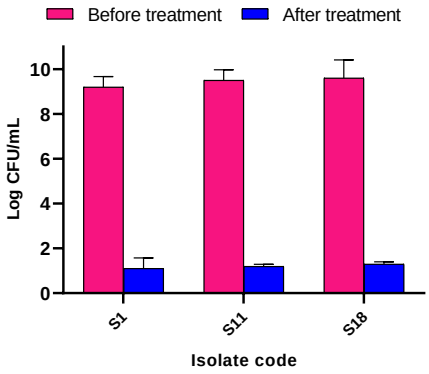


Figure 3: Bar chart revealing the effect of petroleum ether fraction *of G. thunbergia* on the decreasing of the number of CFU/mL of clinical isolates S1, S11, and S18 from total of 22 tested clinical isolates (13.64% of the tested clinical isolates).

4. DISCUSSION

Traditional medicinal expertise has provided insights into the identification of valuable drugs, despite the advantages of current high drug discovery and screening methodologies.[3] There is an increasing understanding of the relationship between phytochemical substances and biological activities.[3] By comparing retention indices and mass spectra fragmentation, GC-MS analysis of fatty acid methyl esters of petroleum ether fraction led to identifying twenty-three compounds from *G. thunbergia*.[33] Different compounds of saturated and unsaturated fatty acid methyl esters and other compounds were detected. **Table 1** demonstrated the compounds having peak area percentage ≥ 1%. They constituted 85.64% of the total. Saturated fatty acids composed 59.77% relative to the total, while unsaturated fatty acids formed 25.87%.

The phytochemicals showing the highest peak area percentage were palmitic acid, butyl palmitate, acetyl tributyl citrate, methyl palmitate, cholesta-4,6-dien-3-ol, and ursolic acid methyl ester. The High peak area percentage of palmitic acid (35.74 %) and butyl palmitate (13.64 %) could explain the antioxidant, antibacterial and antifungal effects.[34] Palmitic acid also exhibited hypercholesteremic, nematicide, and pesticide actions.[16] Acetyl tributyl citrate (ATBC) is an FDA-approved substance for use as a pharmaceutical excipient (the Food and Drug Administration).[35]

Methyl palmitate has anti-inflammatory and antifibrotic effects that could be through NF-kB inhibition. Thus methyl palmitate like molecule could be a promising anti-inflammatory and antifibrotic drug.[36] Cholesta-4,6-dien-3-ol (1.93 %) had a broad-spectrum antimicrobial activity, particularly strongly against *Escherichia coli, Salmonella enterica, Pasteurella multocida, and Physalospora piricola*.[25] Ursolic acid methyl ester (1.82 %) exhibited anti-osteoclastogenesis, anti-inflammatory, anticancer, antidiabetic, antioxidant and antibacterial activities.[31, 37]

Despite the availability of various antimicrobial agents, managing pathogenic bacteria that can form biofilm remains problematic. This could be attributed to hindering the penetration of antimicrobial agents in the formed biofilms. Also, the transfer of the antibiotic resistance gene among bacterial isolates is facilitated in the biofilms.[38] Therefore, many studies have been conducted focusing on finding out novel antibiofilm agents which could be synthetic or from a natural source. Plants are a rich source of bioactive phytochemical compounds that possess antibacterial and antibiofilm potentials.[39] Here, the total methanol extract and the petroleum ether fraction showed antibacterial activity against the tested *S. aureus* isolates. Remarkably, only petroleum ether fraction showed antibiofilm activity by decreasing the bacterial viability in the formed biofilm.

5. CONCLUSION

GC-MS analysis of the saponifiable matter of petroleum ether fraction obtained from the leaves methanol extract of *Gardenia thunbergia* revealed the presence of palmitic acid, butyl palmitate, acetyl tributyl citrate, methyl palmitate, cholesta-4,6-dien-3-ol, and ursolic acid methyl ester. They were the main constituents in the unsaponifiable matter. The petroleum ether fraction showed remarkable antibacterial and antibiofilm effects against *S. aureus* clinical isolates.

CONFLICT OF INTERST

The authors declare no conflicts of interest

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