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Phytochemical Analysis of *Cynanchum acutum* and Evaluated Its Effect on *Staphylococcus aureus*

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Abstract: Due to the great need in treating some diseases and the popularity of natural herbal medicine with lack of side effect. This study was conducted to analyze the chemical component and determine minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of ethanolic extract of the leaves and the stems of *C. acutum* against Gram-positive bacteria *Staphylococcus aureus*. The ethanolic extract of the leaves and the stems of the plant were analyzed, using gas mass spectroscopy (GC-MS) techniques. Ten compounds were identified for the leaves extract. It contains seven Flavonoids, (78.14%), and three Terpenes (21.87%). As for the stems extract, It was found contain twenty-four important peaks, fifteen of which were flavonoids, (83.31%), and six Terpene compounds, (11.47%) . The Steroid had one peak (2.93%) as well as the Tannin and the Resin (0.84%) and (1.38%) respectively. In this research, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the leaves and stems extract of *C. acutum* against *S. aureus* bacteria were determined using the microtiter plate method at different concentrations, of the plant extracts and resazurin dye. It was noted that a concentration of 125,000 µg /ml of leaves extract was lethal (MBC) for most of the isolates, with the exception of one isolate that was highly resistant, for which the lethal concentration reached 250,000 µg /ml. The lowest concentration following the lethal concentration is recorded as the minimum inhibitory concentration (MIC). As for the stems extract, the lethal concentration (MBC) reached 250,000 µg/ml for all isolates, which indicates that the leaves extract was more effective inhibitory than the stems extract. It was concluded that the type of bacteria was detected by various methods, including traditional and molecular methods, and the Vitek was used to increase accuracy in diagnosis.

Keywords: ***Cynanchum acutm, Staphylococcus aureus*, GC-MS, Medicinal plants.**

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Introduction

It is known that plants are wealthy in a large number of phytochemicals, which can be filtered and applied to treat many types of diseases in addition to their valuable nutritional benefits (1).

Phytochemicals are chemicals generated in plants during their regular metabolism. The phytochemical composition is often complex and varies depending on the stage of development and the origin of the plant. Higher plants have been used as a reservoir of beneficial phytochemicals in the pharmaceutical sector (2). Nowadays bacteria due to improper use of medicines are gaining resistance to antibiotics , some bioactive chemicals produced from plants are capable of reversing antibiotic resistance and enhancing the synergetic impact of existing antibiotics (3).

Cynanchum acutum the climbing vine that belongs to the Apocynaceae family. It is to Asia,

Africa and Europe. It is grows extremely in Egypt and Iraq and is known by the locals as Leech, Mod, or Labin. Insecticidal, antidiabetic, antioxidant, antibacterial, anti-cancer, anti-inflammatory, analgesic and antipyretic properties are attributed to the extracts of its leaves (4). *Staphylococcus aureus* is one of the most common bacterial infections in humans and is the causative agent of multiple human infections, including bacteremia, infectious endocarditis, skin and soft tissue infections, osteomyelitis, septic arthritis, prosthetic system infections, and lung infections (e. g, pneumonia and endocarditis. empyema), gastroenteritis, meningitis, toxic shock syndrome, and urinary tract infections (5). *S. aureus* possesses an astonishing array of virulence agents that allow to outrun harsh conditions in humans and enhance tissue inhabitation, tissue spoil, and consequent life-menacing systemic infections. This contain a broad ambit of toxins, immune crossing agents, and a broad ambit of protein and non-protein agents that ability host habitation during contagion (6). The plant contain many active compounds such as flavonoids, tannins, saponins, alkaloids and terpenes (7). The purpose of this research was to recognize the effective chemical compounds of ethanolic extracts of leaves and stems of *C. acutum* by gas chromatography (GC MS) method and to evaluate the antibacterial effect of its ethanolic extracts against *S. aureus*.

Materials and methods

Ethanolic extract of *Cynanchum acutum* leaves and stems

The leaves and stems of *C. acutum* were collected from the gardens of the Institute of Genetic Engineering at the University of Baghdad during October 2022, at the flowering stage. Classified at the College of Science, Department of Biology, University of Baghdad. The leaves and stems were washed with tap water, dried with air, crushed into a fine powder, then 100 g soaked in 1000 ml of 70% ethanol for 24 hours, with stirrer at 40°C on a hot plate. After a day, the materials were filtered using filter paper (8). This process was repeated twice, then activated charcoal were added to remove chlorophyll pigments, after that filtered using a Buechner funnel, the extract was evaporated using a rotary evaporator, then poured into Petri dishes and dried in a thermal oven at 40 ° C and kept at 4 ° C in a tightly closed dark glass container (9).

Gas Chromatography–Mass spectrometry (GC–MS) analysis

Injection samples were prepared by dissolving 0.025 g of leaves extract and 0.04 g of stems extract in 5 mL of pure methanol, mixed well, and then the suspension was filtered through a 0.22 µm filter before being sent to GC-MS for analysis (10). An analysis was performed for chemical components of the samples by :an Agilent (7820A) GC Mass Spectrometer. analytical Column: Agilent HP-5ms Ultra inlet (30 m length x 250 µm diameter x 0.25 µm inside diameter) Injection volume 1µl Pressure 11.933 psi. GC Inlet Line Temperature: 250 ˚C. Aux heaters Temperature 300˚C. Carrier Gas: He 99.99%. Injector Temperature: 250 ˚C Scan Range: m/z 25-1000 Injection Type: Splitless (11).

Time amounted to about 29 and 30 minutes. The chemical constituents of *C. acutum* extract were identified by comparing the results of the chromatogram and reference retention time using the (firmware\NIST11.L) library (12).

Isolates and identification of *Staphylococcus aureus*

Staphylococcus aureus isolates were obtained from the Ministry of Science and Technology and laboratories of the institute. The isolates were transferred to the biological laboratory under aseptic conditions, for cultivation on blood agar, nutrient agar, and mannitol salt agar. and incubated at 37°C for 24 hours for further confirmatory testing (13).

Molecular diagnosis

Bacterial DNA was extracted from the isolates using Monarch DNA extraction Kit according to the manufacturer's instructions (NEB®, USA)

Determining the concentration and purity of DNA

The Nanodrop spectrophotometer (Thermo Scientific/ USA) was used to determine the DNA concentration. At wavelengths of 260 nm and 280 nm, 1µl of extracted DNA was applied in nanodrop to measure the optical density (O.D) the ratio between the reading (OD_{260}/OD_{280}) was calculated to estimate the purity of the nucleic acids. Purity between 1.7 and 2.0 is accepted according to (14).

Primer

The origin of primer applied in this research was Macrogen® Korea. As shown in (Table 1).

Table (1): Primer applied for *Staphylococcus aureus* in this study

Primer for *S.aureus*	Primer sequence F (5'→3') R (5'→3')		Product size (bp)	Reference
16S rRNA	F	5'- ATACATGCAAGTCGAGCGAAC-3'	181 bp	These study
	R	5'-TAAGTGACAGCAAGACCGTCT - 3'		

R: Reverse primer, F: Forward primer, bp: base pair.

Molecular detection of the *16S rRNA* gene of *Staphylococcus aureus*

Molecular detection of the *16S rRNA* gene of *S. aureus* was performed to which 12.5 µl of OneTaq (NEB®) master mix, 3 µl of sample DNA, 1 µl of 10 pmol/µl of each primer and 7.5 µl of water were added. Nuclease free. The reaction was under optimized PCR conditions for the gene as shown in (Table 2).

Table (2): PCR conditions for the *16S rRNA* for *Staphylococcus aureus*

16S rRNA	Cycle No.	Stage	Temperature	Time
S. aureus	1	Initial Denaturation	94 ºC	5 mins
	38x	Denaturation	94 ºC	30 sec.
		Annealing	53ºC	45sec.
		Extension	72 ºC	45 sec.
	1	Final Extension	72 ºC	7 mins.

Determination of minimum inhibition concentration (MIC) and minimal bactericidal concentration (MBC) of leaves and stems extracts of *Cynanchum acutum*

The microtiter plate (MTP) method was adopted to evaluat the antibacterial activity of ethanolic extract of leaves and stems separately against Gram-positive bacteria *S. aureus*, MIC was determined on 96-well. Microtiter plate using the resazurin dye-assisted microdilution technique in Mueller-Hinton broth (MHB) as follows (15): 150 µL of broth medium was made into each well from 1 to 8, and then 150 µl

of the diluted 500.000 µg/ml ethanolic extracts transferred separately to the first well.

Then, for the 1-6 wells 150 µl were transferred from the first well to the next using two- fold dilution the concentrations become (250.000, 125.000, 62.500, 31.200, 15.600 and 7.800 µg/ml) of leaves and stems extract. Wells No. 7 and 8 were control (Muller Hinton Broth **(**MHB) and bacterial suspension) respectively, each well was inoculated with 15 µl of bacterial suspension equivalent to McFarland standard 0.5 (1.5×10^8 CFU\ml). The microtiter plate was incubated at 37°C for 24 h. 30 µl of resazurin (0.015%) was added to each well and incubated for 2 to 4 hours, to notice the color change. After completion of the incubation, rows with no color change (blue resazurin color remained unchanged) were scored as above the MIC (MBC) whereas the last blue well in the row was recorded as MIC and the next pink well recorded as sub-MIC.

Results and discussion

Cynanchum acutum ethanolic dry yield of the leaves extract was about 9.74 g, and for the stems was about 12.32 g.

Gas chromatography - Mass spectrometry (GC-MS) analysis

Figure (1) and Table (3) show the chemical compositions of the leaves extract using GC-MS. Ten compounds have been identified from *C. acutum* leaves. The percentage of flavonoids was 78.14%, which are in the form of compounds 3-Butyn-1-ol; Heptanedioic acid, dimethyl ester; Pyrimidine, 4,6-dimethoxy-5-nitro-; Dibutyl phthalate; Ethanol, 2-nitro-, propionate (ester), and their proportions are as follows (11.85%, 6.02%, 5.01%,23.72, 9.84%, and 12.68%) and three Terpenes which are Acetic acid, ethoxyhydroxy-, ethyl ester; 1,2,2,3,4-Butanepentacarbonitrile; Propanoic acid, 2-bromo-, methyl ester, 3,8-Dioxatricyclo [5.1.0.0(2,4)] octane, 4-ethenyl- The following are the percentages (8.84%, 8.10%, and 4.93%).

Total time 33 mint

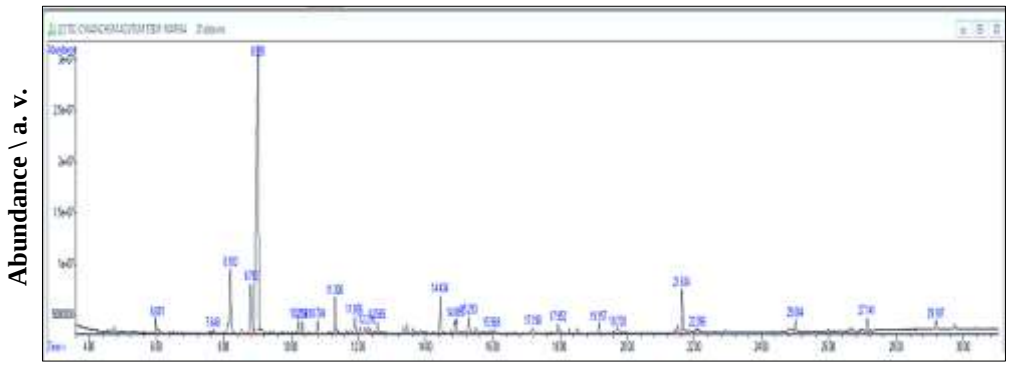
Figure (1): Typical GC-histogram of *Cynanchum autum* leaves extract, identified by :an Agilent (7820A) GC Mass Spectrometer. analytical Column: Agilent HP-5ms Ultra inlet (30 m length x 250 µm diameter x 0.25 µm inside diameter) Injection volume 1µl. Pressure 11.933 psi. GC Inlet Line Temperature: 250 ˚C. Aux heaters Temperature 300 ˚C. Carrier Gas: He 99.99%. Injector Temperature: 250 ˚C Scan Range: m/z 25-1000. Injection Type: Splitless

Table (3): Chemical composition of *Cynanchum acutum* leaves extract

No.	Compound	Classify	R. Time	Area%
1	Acetic acid, ethoxyhydroxy-, ethyl ester	Terpene	4.695	8.84
2	3-Butyn-1-ol	Flavonoids	5.327	11.85
3	3-Butyn-1-ol	Flavonoids	5.595	9.02
4	Heptanedioic acid, dimethyl ester	Flavonoids	6.763	6.02
5	1,2,2,3,4-Butanepentacarbonitrile	Terpene	13.532	8.10
6	Pyrimidine, 4,6-dimethoxy-5-nitro-	Flavonoids	18.716	5.01
7	Ethanol, 2-nitro-, propionate (ester)	Flavonoids	19.210	23.72
8	Dibutyl phthalate	Flavonoids	19.617	9.84
9	3,8-Dioxatricyclo[5.1.0.0(2,4)] octane, 4-ethenyl-	Terpene	21.512	4.93
10	Heptanedioic acid, dimethyl ester	Flavonoids	21.867	12.68

Twenty-four compounds were identified from the stem extract, the percentage of flavonoids reached 83.31%, and were in the form of cyclohexanol, 1-methyl-4-(1-methylethynyl)- cis-; Carbonic acid, dimethyl ester; Cyclohexane, 1-methylene-3-(1-meth ylethenyl)-, (R)-; Carveol; D-Carvone) and the percentage of each compound was as follows (1.89%, 1.00%, 8.89%, 6.98%,and 50.95%).

The percentage of Terpenes was 11.47%, and it was in the form of compounds, caryophyllene; Naphthalene, 1,2,4a,5,6,8a-hexahyd; Bicyclo [3.1.1] heptane, 6,6-dimethy l-3-methylene-; 6-Methyl-1,2,3,5,8,8a-hexahydronap hthalene; and N-Trifluoroacetylimidazole) the percentage of each compound was as follows (1.97%, 1.50%, 3.61%, 2.26%, and 1.13%). The percentage of steroids was 2.96% in the form of alpha.-Bourbonene, the percentage of resin was 1.39% in the form of Selina-3,7(11)-diene, and the percentage of tannin was 0.84% in the form of Bicyclo[4.4.0]dec-1-. ene, 2-isoprop yl-5-methyl-9-methylene-, shown in (Figure 2) and (Table 4).



Total time 33 mint

Figure (2): Typical GC-histogram of *Cynanchuum autum* stems extract, identified by :an Agilent (7820A) GC Mass Spectrometer. analytical Column: Agilent HP-5ms Ultra inlet (30 m length x 250 µm diameter x 0.25 µm inside diameter) Injection volume 1µl. Pressure 11.933 psi. GC Inlet Line Temperature: 250 ˚C. Aux heaters Temperature 300 ˚C. Carrier Gas: He 99.99%. Injector Temperature: 250 ˚C Scan Range: m/z 25-1000. Injection Type: Splitless

Table (4): The chemical composition of a plant stems extract *Cynanchum acutum*

No.	Compound	Classify	R. Time	Area%
1	Cyclohexanol, 1-methyl-4-(1-methyl ethenyl)-, cis-	Flavonoids	6.002	1.89
2	Carbonic acid, dimethyl ester	Flavonoids	7.646	1.00
3	Cyclohexane, 1-methylene-3-(1-meth ylethenyl)-, (R)-	Flavonoids	8.183	8.89
4	Carveol	Flavonoids	8.789	6.98
5	D-Carvone	Flavonoids	8.988	50.95
6	Dihydrocarvyl acetate	Flavonoids	10.217	1.00
7	2-Cyclohexen-1-ol, 2-methyl-5- ethylethenyl)-, acetate, (1R-cis)-	Flavonoids	10.338	0.87
8	trans-Carveyl acetate	Flavonoids	10.788	1.14
9	alpha.-Bourbonene	Steroid	11.307	2.93
10	Caryophyllene	Terpene	11.887	1.97
11	Naphthalene, 1,2,4a,5,6,8a-hexahyd	Terpene	12.268	1.50
12	Bicyclo[4.4.0]dec-1-ene, 2-isoprop yl-5-methyl-9-methylene-	Tannin	12.562	0.84
13	Bicyclo[3.1.1]heptane, 6,6-dimethy l-3-methylene-	Terpene	14.432	3.61
14	6-Methyl-1,2,3,5,8,8a-hexahydronap hthalene	Terpene	14.882	2.26
15	Selina-3,7(11)-diene	Resin	15.298	1.38
16	1,2-Cyclobutanedicarboxylic acid 3-methyl-, dimethyl ester	Flavonoids	17.202	1.03
17	N-Trifluoroacetylimidazole	Terpene	17.955	1.13
18	Hexadecanoic acid, methyl ester	Flavonoids	19.158	0.89
19	Pentadecanoic acid	Flavonoids	19.729	0.84
20	Phytol	Flavonoids	21.633	3.96
21	Spirohexan-4-one, 5,5-dimethyl-	Terpene	22.092	0.88
22	Phytol, acetate	Flavonoids	25.000	0.87
23	Phytol, acetate	Flavonoids	27.138	1.13
24	Phytol, acetate	Flavonoids	29.189	0.95

Isolates and identification of *staphylococcus aureus*

All isolates were identified based on conventional cultivation and morphological characteristics when cultivated on mannitol salt agar, blood agar media and nutrient agar to verify the presence of the target isolates in this study, as well as the following tests were used:

Morphological characteristics and Gram staining procedures (16). *S. aureus* shows its ability to aerobicly ferment mannitol and convert it from red to yellow color where yellow colonies appear (17). On blood agar, *S. aureus* usually displays a light to golden yellow pigment. On blood agar, *S. aureus* is usually betahemolytic that meaning the breakdown of red blood cells was considered as *S. aureus* (18). Golden colonies shown on nutrient agar (19) as shown in (Figure 3).

Figure (3): Colonies of *Staphylococcus aureus* growth on mannitol salt agar(A): blood agar(B): and nutrient agar(C) in 37 °C for 24 hr.

Molecular detection of the *16S rRNA* gene. The *16S rRNA* gene sequence is used to diagnosis bacterial strains, as it is considered a genetic marker, and is used in housekeeping due to its presence in almost all bacteria; Gene function did never alteration through years, indicates that random gradation alteration are a more delicate standard of time (development); The *16S rRNA* gene (1500 bp) is big sufficient for informatics aims (20). The DNA of all isolates was successfully extracted and the concentrations of the DNA samples showed acceptable integrity and the A260/A280 ratio was within the range of 1.7-1.9, indicating that the DNA samples were pure. (Figure 4) shows the result of DNA extracted from *S. aureus* isolates.

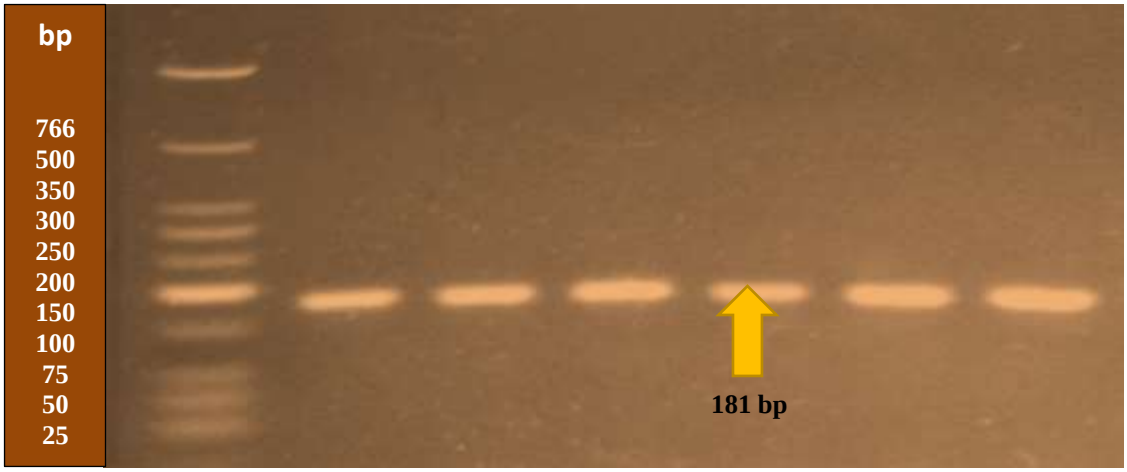


Figure (4): DNA bands on a 1.8% agarose gel at 70 V for 90 minutes. Genomic DNA extracted from *Staphylococcus aureus* samples.

Vitek-2 system was used for identity confirmation (21)**.**

Determination of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Clinical laboratories employ MICs primarily to confirm resistance; however, they are also used as a research tool to determine the activity of novel antibacterial agents as well as their MIC breakpoints (22). Comparison of the MIC of both extracts showed that the leaves extract was more efficient in inhibition against the candidate bacteria for this Study. It was found that the antibacterial activity of the leaves is greater than that of the stems, as evidenced by the color change at different concentrations as a result of bacterial development, which led to a reduction of the resazurin dye from blue to pink. Rows that did not change color (resazurin blue color remained unchanged) were scored as above MIC (MBC) while the last blue well in the row was scored as MIC and the next pink well was scored as Sub-MIC, as shown in (Figure 5).

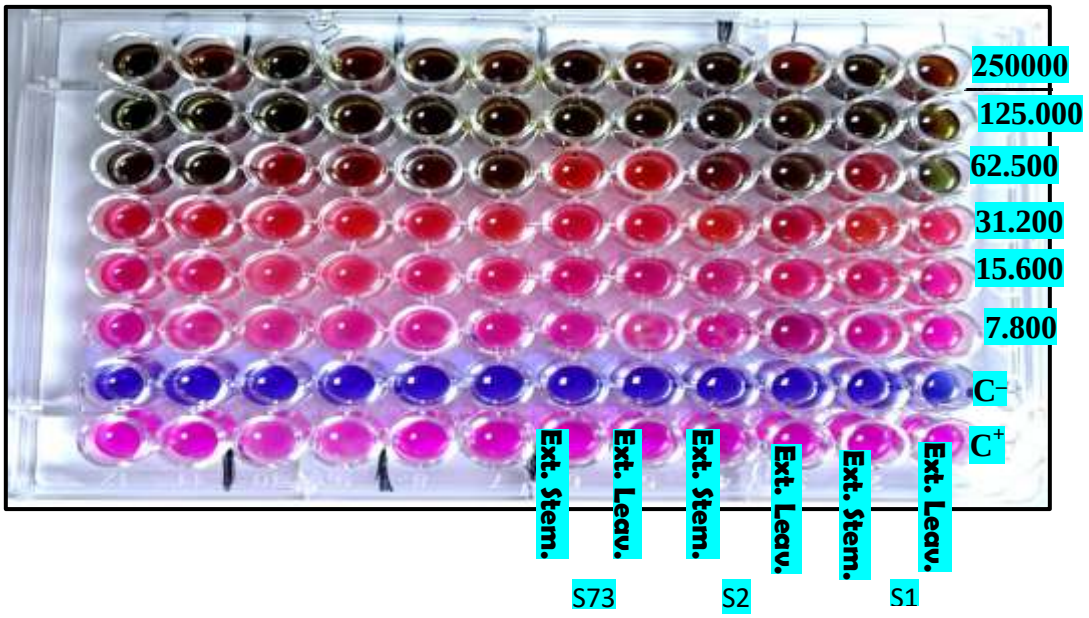


Figure (5): Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of leaves and stems ethanolic extract of *Cynanchum acutum* against *Staphylococcus aureus* isolates

The results showed that a concentration 125,000 µg/ml of leaves extract was lethal (MBC) to most isolates of *S. aureus* bacteria, with the exception of S73 isolate, which was more resistant. Therefore, the concentration of 250,000 µg/ml was the lethal concentration (MBC), and the concentration from 125,000 to 62,500 µg/ml was consider as the minimum inhibitory concentration (MIC), and concentrations from 62,500 to 31,200 µg/ml were relatively less effective (sub-MIC), these concentrations depend on the resistance of each isolate. In contrast, 250,000 µg/ml was the lethal concentration (MBC) in most isolates for stems extract and 125,000 µg/ml for inhibitors (MIC). The concentration of 62.500 µg/ml is considered the least effective inhibitory agent (Sub-MIC) as shown in (Table 5).

Table (5): Minimal Bactericidal Concentration (MBC): Minimum Inhibitory Concentration (MIC) and Sub-minimum Inhibitory Concentration (Sub-MIC) of *Cynanchum acutum* Leaves and Stems ethanolic extract against *Staphylococcus aureus* using the Resazurin-assisted microdilution method.

Isolates	Extract leaves MBC	Extract leaves MIC	Extract leaves Sub-MIC	Extract stems MBC	Extract stems MIC	Extract stems Sub-MIC
S1	125000	62.500	31.200	250000	125000	62.500
S2	125000	62.500	31.200	250000	125000	62.500
S73	250000	125000	62.500	250000	125000	62.500

The antibacterial activity was significantly different across concentrations, as the leaves extract was more effective than the stems extract in inhibiting *S. aureus* bacteria. The reason for that may be the quality of the flavonoids found in the leaves, and that they present in large quantities (78.14%), in addition to the terpenes that present in double quantities (21.87%) compared to the stems, which considered inhibitors of microbes (23).

Conclusion

Cynanchum acutum contains phytochemical compounds such as flavonoids, terpenes, steroids, tannins, and other resin compounds. It has been proven through many previous studies that the presence of these compounds may have an inhibitory effect on bacterial activity due to increased oxidative stress in microbial cells, and this causes damage to macromolecules within cells, leading to cell death.

References

1. Hassan, A.; Akmal, Z. and Khan, N. (2020). The phytochemical screening and antioxidants potential of Schoenoplectus triqueter L. Palla. Journal of Chemistry, 1-8.
2. Siddiqui, M. (2021). Phytochemical Analysis of some Medicinal Plants. Liaquat Medical Research Journal, 3(8):1–5.
3. Mickymaray, S. (2019). Efficacy and Mechanism of Traditional Medicinal Plants and Bioactive Compounds Against Clinically Important Pathogens. Antibiotics, 8(4): 257.
4. Abdelhameed, R. F.; Ibrahim, A. K.; Elfaky, M. A.; Habib, E. S.; Mahamed, M. I.; Mehanna, E. T., *et al*. (2021). Antioxidant and Anti-inflammatory Activity of *Cynanchum acutum* L. isolated flavonoids using experimentally induced type 2 diabetes mellitus: Biological and in silico investigation for nf-κb pathway/mir-146a expression modulation. Antioxidants, 10(11): 1713.
5. Tong, S. Y.; Davis, J. S.; Eichenberger, E.; Holland, T. L. and Fowler Jr, V. G. (2015). *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. Clinical Microbiology Reviews, 28 (3): 603-661.
6. Cheung, G. Y.; Bae, J. S. and Otto, M. (2021). Pathogenicity and virulence of *Staphylococcus aureus*. Virulence, 12(1): 547-569.
7. Balaure, P. C. and Grumezescu, A. M. (2020). Recent Advances in Surface Nanoengineering for Biofilm Prevention and Control. Part I: Molecular Basis of Biofilm Recalcitrance. Passive Anti-Biofouling Nanocoatings. Nanomaterials, 10: 1230.
8. Mahmod, W. S.; Al-Jumaili, E. F. A. and Mohamad, N. B. A. (2022). Qualitative and Quantitative Evaluation of the Extracted Flavonoids in Iraqi-Sumac (Rhus Coriaria L.): Iraqi Journal of Biotechnology, 21, (1): 50-60.
9. Mashhady, M. A.; Abkhoo, J.; Jahani, S.; Abyar, S. and Khosravani, F. (2016). Inhibitory Effects of Plant Extracts on *Pseudomonas aeruginosa* biofilm formation. International Journal of Infection, 3(4).
10. Brito, R. E.; González-Rodríguez, J.; Montoya, M. R. and Rodríguez Mellado, J. M. (2017). Comparison of the Volatile Antioxidant Contents in the Aqueous and Methanolic Extracts of set of commercial spices and condiments. European Food Research and Technology, 243(8): 1439–1445.
11. Ahmed, N. F.; Saadedin, S. M. K. and Rustam, H. Q. (2023). Gas Chromatography-Mass Analysis and Evaluation of Antibacteral Activity of Mint and Basil Essential Oil. Iraqi Journal of Biotechnology, (1): 200-207.
12. Amelia, B.; Saepudin, E.; Cahyana, A. H.; Rahayu, D. U.; Sulistyoningrum, A. S. and Haib, J. (2017). GC-MS Analysis of Clove (Syzygium aromaticum) Bud Essential Oil from Java and Manado. AIP Conference Proceedings, 1862: 030082-1–82-9.
13. Alagely, H. S. ; AL-hashimy, A. B. and Majeed, K. R. (2019). Characterization of Five Types of Staphylococcal Cassette Chromosomal mec Genes in Methicillin-Resistant Staphylococcus aureus (MRSA) Isolates from Iraqi Patients, Iraqi Journal of Biotechnology, 18(2): 289-299.
14. Sambrook, J.; Roberts, K.; Walter, P. and Green, M. R. (2015). Molecular Cloning. A Laboratory Manual Fourth Edition.
15. Elshikh, M.; Ahmed, S.; Funston, S.; Dunlop, P. and McGaw, M. (2016). Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. Biotechnology Letters, 38(6): 1015–1019.
16. Tripathi, N. and Sapra, A. (2020). Gram Staining. In StatPearls. StatPearls Publishing.
17. Thakur, P.; Nayyar, C.; Tak, V. and Saigal, K. (2017). Mannitol-fermenting and tube coagulase-negative staphylococcal isolates: unraveling the diagnostic dilemma. Journal of Laboratory Physicians, 9(01): 065-066.

18. Turista, D. D. R. and Puspitasari, E. (2019). The growth of *Staphylococcus aureus* in the blood agar plate media of sheep blood and human blood groups A, B, AB, and O. Journal Teknologi Laboratorium, 8(1): 1-7.
19. Missiakas, D. M. and Schneewind, O. (2013). Growth and laboratory maintenance of *Staphylococcus aureus*. Current Protocols in Microbiology, 28(1): 9C-11.
20. Patel J. B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Molecular diagnosis : a journal devoted to the understanding of human disease through the Clinical Application of Molecular Biology, 6(4): 313–321.
21. Simgamsetty, S.; Yarlagadda, P.; Yenigalla, B. M. and Myneni, R. B. (2016). Ease with VITEK 2 systems, bioMerieux in identification of no lactose fermenting bacteria including their antibiotic drug susceptibility: our experience. International Journal of Research in Medical Sciences, 4(3): 813-817.
22. Kowalska-Krochmal, B. and Dudek-Wicher, R. (2021). The minimum inhibitory concentration of antibiotics: methods, interpretation, clinical relevance. Pathogens, 10(2):165.
23. Abu Ziada, M. E.; Al Kraeeshi, N. K. and Alshami, M. (2016). Ecological Studies on Wild Medicinal Plants In Egypt. III-Cynanchum acutum L. Journal of Plant Production, 7(1): 53-60.