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Evaluate the Prevalence of the Virulence Genes (*lptD* & *lptE*) among *Pseudomonas aeruginosa* Isolated from Different Infectious Sources

***Ali S. mohalhil*[1*]; *Mohsen H. Risan*[2]**

[1,2] **Plant Biotechnology, College of Biotechnology, Al-Nahrian University, Baghdad, Iraq**
*Corresponding Author
E. mail: ali.sadun135@gmail.com
https://orcid.org/0009-0000-7997-2811

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Abstract

Pseudomonas aeruginosa is a Gram-negative aerobic bacterium that has become one of the most nosocomial pathogens. The main goal of this research project was to determine genotypes associated with LPS transport and antibiotic-resistance genes (*lptD* and *lptE*) of clinical isolates of *P. aeruginosa*. Characterization of the strains of *P. aeruginosa* that are widespread in Iraqi patients was done by collecting 140 clinical samples from wounds, burns, ear swaps, sputum, and urinary tract infections taken from seven general hospitals in Baghdad. Isolation methods for identifying *P. aeruginosa* relied on culture methods; 102 was positive growth while the remaining 38 had negative growth; biochemical tests and conventional culture method on selective media showed that only 50 from 102 isolates were *P. aeruginosa* VITEK-2 system was used to confirm the diagnosis, and also antibiotic sensitivity test, The results showed that 100% of these 50 isolates were *P. aeruginosa*. In this study, molecular techniques to identify *P. aeruginosa*, including DNA extraction and PCR, were used. To determine the presence of the virulence genes *lptD* and *lptE*, which play a role in lipopolysaccharide (LPS) transport across the outer membrane, polymerase chain reaction (PCR) was used to amplify specific regions of DNA. In conclusion, the prevalence of antibiotic-resistant *P. aeruginosa* strains among hospitalized patients presents a significant challenge in managing infections. These genes were found to be closely associated with LPS transport and, hence, the ability of these bacteria to resist antibiotics.

Keywords: *P. aeruginosa*, *lptD*, *lptE*, Burn Wound Infection, LPS

Introduction

Pseudomonas aeruginosa, a versatile gram-negative bacterium with rod-shaped, thrives in various environments such as water, soil, plants, and even humans [1] [2] [3]. It's remarkably resilient, enduring temperatures between 4 to 42°C and flourishing in hard conditions. Notably, it can persist for up to six months on dry surfaces within hospital environments, showcasing its adaptability and survival skills [4] [5].

In both community and hospital settings, *P. aeruginosa* stands out as a prominent opportunistic pathogen, frequently associated with otitis media, nosocomial infections, respiratory tract infections and

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burns [6] [7]. The emergence of multidrug-resistant strains further complicates treatment, leading to an increasing incidence of *P. aeruginosa* infections, which pose significant challenges in healthcare settings [8].

The bacterium's ability to infect various tissues contributes to its morbidity and mortality rates, particularly evident in burn wound infections where it finds an ideal niche [9] [10]. Burn patients are particularly susceptible due to factors such as compromised immunity, extensive hospital stays, and exposure to large body surface areas[11] [12].

P. aeruginosa harbors numerous virulence factors, including flagella, pili, and LPS, aiding in host adhesion and colonization. Additionally, it produces causative tissue damage, proteases and toxins, to deliver effectors and toxins into the host secretion systems utilizes and employs biofilm formation and quorum sensing to communicate and resist antimicrobial therapies[13] [14] [15].

In a new study on *P. aeruginosa*, scientists investigated a protein transport system (Lpt) crucial for building the bacterial outer membrane. This shell relies on LPS. While previous research showed seven proteins called Lpt proteins were essential for LPS transport via the outer membrane [16] [17], they found some surprise with *LptE*. mutations in the *LptE* gene didn't prevent bacterial growth, but the bacteria were weaker [18] [19].

They were easier to kill with antibiotics and less infectious in insects. Interestingly, the LPS transport system appeared to function adequately even in the absence of *LptE*. However, the levels of another key protein, *LptD*, were much lower. This suggests *LptE* might not directly move LPS, but instead helps *LptD* function properly [20] [21].

Even though the bacteria can survive with a flawed *LptD*, it makes them more vulnerable. Understanding *LptE*' role could be a new way to fight this pathogen [22] [23]. By targeting *LptE*, scientists might be able to make these bacteria easier to treat with antibiotics or less able to cause infections [24] [23].

Materials and Methods

Bacterial Isolates Collection

For four months from October 2023 to January 2024, one hundred and forty clinical specimens were grabbed from hospitalized patients at general hospital in Baghdad City, Iraq. These isolates were obtained from both genders and varying ages of patients. Then, the samples were transferred to the lab. As part of the sampling method, from clinically deep burn wound sites that showed clinical symptoms of wound infection after changing the wound bandage, swaps were taken, and other sources such as wound, sputum, ear swap and urinary tract infection. By bacterial cultures (on nutrient agar and MacConkey agar) subculture on Cetrimide agar which is selective media the isolates were determined to be *P. aeruginosa* according to previous studies, these pure colonies were identified based on their cultural characteristics. In addition, VITEK-2 system was used to identify *P. aeruginosa* isolates.

Bacterial DNA Extraction from *P. aeruginosa*

Genomic bacterial DNA extraction from isolates was conducted using a high-yield DNA Purification Kit following the instructions of the manufacturer (presto mini gDNA Bacterial kit/Taiwan). The purity of the bacterial DNA was evaluated using a Nanodrop on UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and stored at –20°C for subsequent PCR amplification. Table 1 outlines the primer sequences and expected amplification band sizes for *lptD* and *lptE*. Each PCR mixture (20 µl) comprised 10 µl of green master mix (Promega, USA), 5 µl of nuclease-free water, 1 µl of each primer (forward and reverse primers in this study were designed by NCBI and are suitable for the working conditions.) and 3 µl of DNA template. Table 2 shows the reaction conditions for all primers in detail. The resulting amplicons were visualized via agarose gel electrophoresis and exposed to UV light (Cleaver Scientific Ltd., Rugby, UK). Furthermore, by comparison with 100 to a 1500–bp DNA ladder (Promega, USA) which was used to determine the size of the amplicons (fig 1).

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Table 1: Forward and Reverse primers sequences used in this study.

Genes	Primers sequences (5′→3′)	size (bp)	References
LptD	**F: AACTCGCCTTCCAGCATCAT**	333 bp	This study
	R: GCTCAAGGATGGCACCTACA		
LptE	**F: GGAGTTCGAGCTGACCAACA**	349 bp	
	R: TCACGGGGTGGGGAACTC		

Table 2: A mixture of working solution PCR reaction.

Components	Reaction Volume (µl)
Forward Primer	1
Reverse Primer	1
Templet DNA	3
Nuclease-free water	5
Master Mix	10
Total volume	**20**

Results and Discussion

Isolation and identification of P. aeruginosa

One hundred and forty different clinical specimens were collected from various clinical sources and patients in Baghdad General Hospitals, from October 2023 to January 2024. The results showed that from a total of 140 specimens, only 102 (72.8%) were clinical positive growth samples, while the rest 38 (27.14 %) were negative growth samples. Negative growth results may suggest absence of infection at the time of sample collection or successful treatment of infection, some organisms may not grow easily in standard lab conditions.

Depending on the conventional culture method, and morphological trait, biochemical and microscopic examination found that 50 isolate was *P. aeruginosa* while the rest 38 (27.14%) samples were other bacterial isolates. The high isolation rate was from burn infections 48 (47.05%) isolates, wounds 30 (29.41%) isolates, sputum 11 (10.78%), urine sample 8 (7.84%) and 5 (4.9%) from ear swap sample, as explained in Table (3).

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Table 3: Number and percentage of isolates according to the source of samples.

No.	Source of isolates	No. of isolate	%
1	Burns	48	47.05%
2	Wounds	30	29.41%
3	Sputum	11	10.78%
4	Urine	8	7.84%
5	Aer swap	5	4.9%
6	**Total**	**102**	**100%**

Figure 1: This is a figure of *p. aeruginosa* on MacConkey agar

VITEC-2 Test

Fifty isolate (35.71%) from positive growth were identified as *P. aeruginosa* by culturing on selective medium such as MacConkey agar, cetrimide agar and some biochemical tests as well as VITEK-2 system as a confirmatory test. The isolates were diagnosed as *P. aeruginosa*. The VITEK-2 System test identified the presence of *P. aeruginosa* in all 50 (100%) samples with 95-99% identity. Additionally, the result of VITEK-2 system test mentioned in Fig. 2 showed the antibiotic sensitivity of *P. aeruginosa* isolates which was resist to more than 8 antibiotics.

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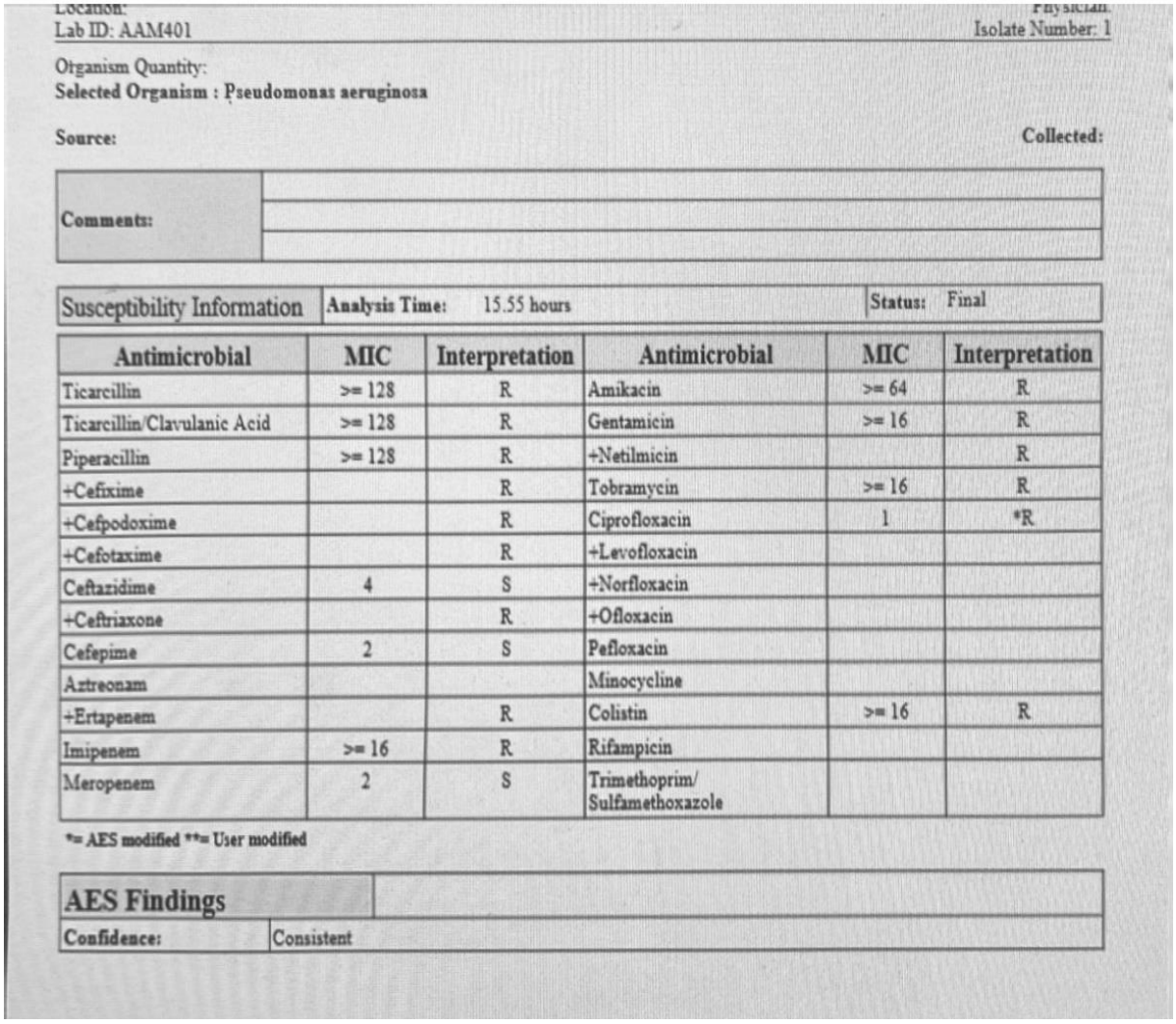

Location:
Physician:
Lab ID: AAM401
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source:
Collected:

Comments:	

Susceptibility Information	**Analysis Time:** 15.55 hours	**Status:** Final

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ticarcillin	>= 128	R	Amikacin	>= 64	R
Ticarcillin/Clavulanic Acid	>= 128	R	Gentamicin	>= 16	R
Piperacillin	>= 128	R	+Netilmicin		R
+Cefixime		R	Tobramycin	>= 16	R
+Cefpodoxime		R	Ciprofloxacin	1	*R
+Cefotaxime		R	+Levofloxacin		
Ceftazidime	4	S	+Norfloxacin		
+Ceftriaxone		R	+Ofloxacin		
Cefepime	2	S	Pefloxacin		
Aztreonam			Minocycline		
+Ertapenem		R	Colistin	>= 16	R
Imipenem	>= 16	R	Rifampicin		
Meropenem	2	S	Trimethoprim/ Sulfamethoxazole		

*= AES modified **= User modified

AES Findings	
Confidence:	Consistent

Figure 2: Results of Vitek2 test for *P. aeruginosa*.

PCR Analysis

A total of 50 isolates of *P. aeruginosa* which identified by VITEK-2 system has been used for molecular detection of both *lptD* and *lptE* genes. The presence of genes was detected using PCR, which amplifies specific regions of DNA. The result of PCR products were then separated using agarose gel electrophoresis based on their molecular weight which showed that 48 of 50 (96%) *P. aeruginosa* isolates gave a positive result for the *lptD* gene represented by observation of the *lptD* gene band of 333 bp molecular weight as shown in Fig. 3 while only 8 of 50 (16%) gave a positive result for the *lptE* gene band with molecular weight 349 bp as shown in Fig. 4 in the bottom line, only 7 of 50 (14%) isolate carried both virulence gene *lptD* and *lptE* ,table 4 referred to the seven isolate that already mentioned and their sensitivity.

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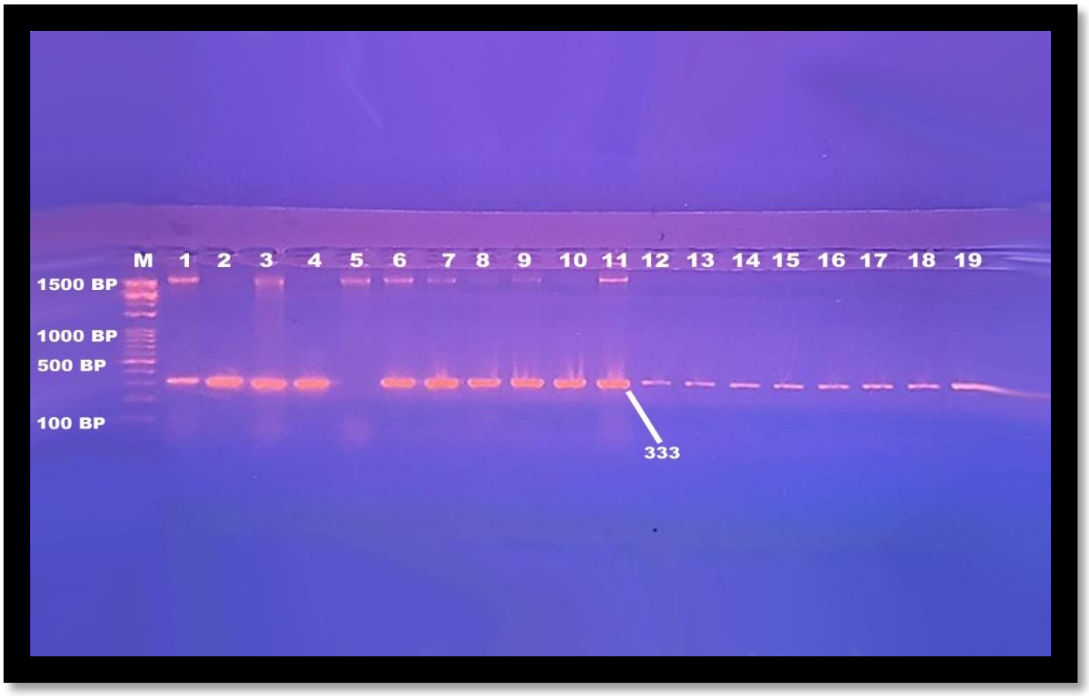

Figure 3: PCR profile for the amplified *lptD* gene bands on 333bp of *P. aeruginosa* isolates were fractionated on agarose 1.5% at 100v/m Amp for 60 minutes, Lane M: DNA ladder marker (100 bp-1500 bp).

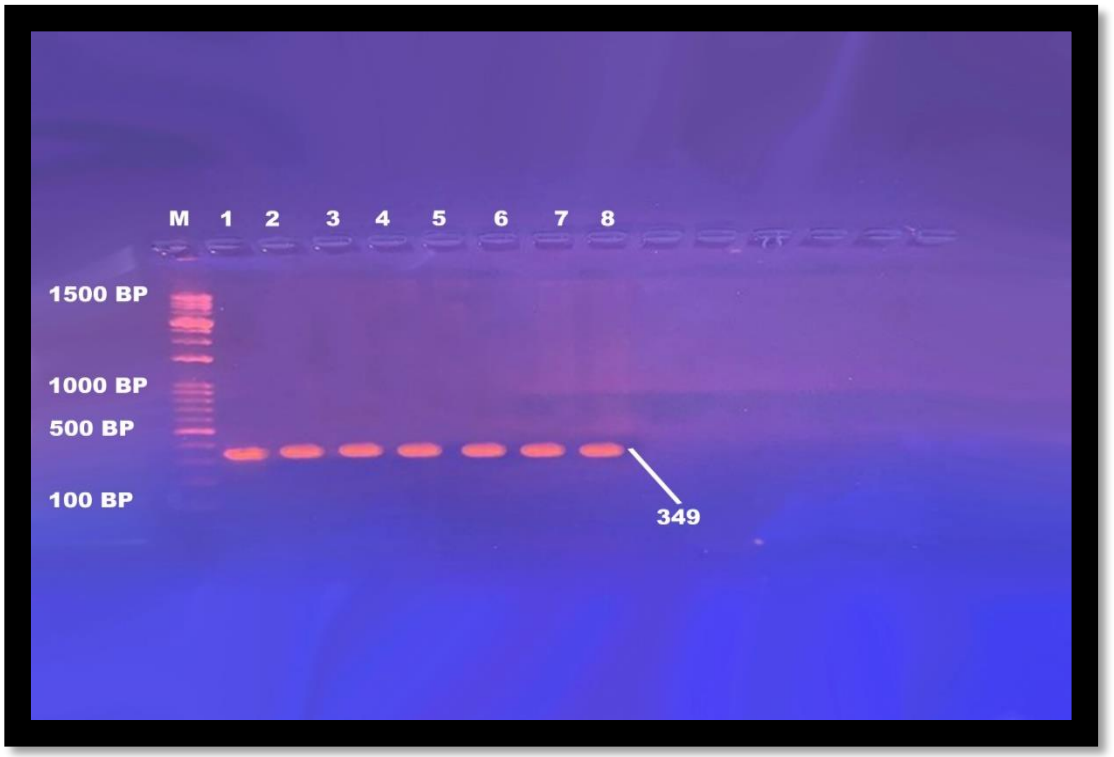

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Figure 4: PCR profile for the amplified *lptE* gene bands on 349 bp of *Pseudomonas aeruginosa* isolates were fractionated on agarose 1.5% at 100v/m Amp for 60 minutes, Lane M: DNA ladder marker (100 bp-1500 bp).

Table 4: Antibiotic sensitivity test on *P. aeruginosa* isolates

Isolates	Antibiotic															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
C-3	R	R	R	S	S	S	R	R	S	S	S	S	S	S	S	S
C-4	R	R	R	S	R	R	R	R	S	S	S	S	S	S	S	S
C-5	R	R	R	S	R	S	R	R	S	S	S	S	S	S	S	S
C-7	S	S	R	S	R	S	R	R	S	S	S	S	S	S	S	R
Y-6	R	R	R	S	S	S	R	R	S	S	S	S	S	S	S	S
K-1	R	R	R	S	S	S	R	R	S	S	S	S	S	S	S	S
K-2	R	R	R	S	S	R	R	R	S	S	S	S	S	S	R	R

C: Imam Ali Hospital, Y: Al-Yarmouk Teaching Hospital, K: Al-Kindy Teaching Hospital, S: Sensitive, R: Resistance, 1: Ampicillin /Sulbactam, 2: Piperacillin /Tazobactam, 3: Cefazolin, 4: Ceftazidime, 5: Ceftriaxone, 6: Cefepime, 7: Aztreonam, 8: Ertapenem, 9: Imipenem, 10: Meropenem, 11: Amikacin, 12: Gentamicin, 13: Tobramycin, 14: Ciprofloxacin, 15: Levofloxacin, 16: Tigecycline, 17: Trimethoprim /Sulfamethoxazole

Discussion

P. aeruginosa is one of the most common microbes causing hospital-acquired infections, after completing the process of culturing bacteria on dishes in order to diagnose the bacteria and confirm their diagnosis using the VITEK-2 system device, the confirmatory results of the device showed that 102 isolates (72.85%) out of 140 isolates (100%) were infected with *Pseudomonas* bacteria. The reason for not finding this bacterium in all the isolates is due to sample collection conditions where isolates were collected from more than one place. In the current study, the highest rate of infection with this bacterium was in samples isolated from burn patients, which was about 62 isolates (44.29%). The reason for this percentage is because the number of samples that were isolated from burns was numerous and immediate infections, and this percentage is considered close to a study conducted by the researcher [25]. where the rate of infection with this bacterium among burn patients was about 71 isolates out of 110 samples, or about (64.55%), which is an intuitive percentage. While in previous studies in Morocco and Egypt, it was about 15.1% and 19.8%, respectively, and these percentages are not consistent with the results of the current study [26].

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The results of the current study showed that the number of infected women was about 82 samples (58.58%), while among males there were 58 isolates (41.5%). These differences in percentage can be attributed to biological and physiological factors in women, this percentage is similar to a previous study conducted by researcher [25], where the infection rate for males was about (26%) and for females was (38%).

Based on the PCR results, the current study found that 48 out of 50 isolates of *P. aeruginosa* bacteria contained the *lptD* gene, while only 8 isolates contained the *lptE* gene. Based on the results of gel electrophoresis, only 7 out of 50 isolates carried both genes. The reason for this result is that the presence of these genes *lptD* and *lptE* in different isolates of these bacteria may be affected by many factors such as natural genetic differences, environmental pressure, horizontal genetic transfer, as well as the use of antibiotics, as the pressure that results from the use of antibiotics can lead to this. To an increase in the spread of resistance genes, these results may also indicate that the *lptD* gene provides more benefit to the bacteria in the environments from which it was isolated, or that the environmental conditions in which the isolates were collected may have a significant impact on this.

The name *LptD* comes from the LPS protein transport because of its intrusion in OM biogenesis [27] [28]. *LptD* in *P. aeruginosa* is also involved in LPS transport, controls membrane permeability, and confers resistance to many known antibiotics [29] [30]. The findings of Sundar Pandey and co-workers certainly suggest that *LptD*, an amino acid transporter protein, may have a specific role in regulating alginate production Since the *P. aeruginosa LptD* importance has been experimentally demonstrated [31], this evidence suggests that very low amounts of *LptD* and even *LptE* may be sufficient to support LPS transport and growth in *P. aeruginosa*, as previously observed in *Neisseria meningitidis* [32]. It is noteworthy that *LptE*-deficient cells were competent to grow under laboratory conditions but were severely resistant to detergents and antibiotics. This result was confirmed in our study, where all isolates showed significant growth, while the PCR results confirmed the absence of these genes in a number of isolates, meaning that depletion of these genes does not negatively affect growth.

According to a study that proved that *lptE* is directly involved in LPS transport and is essential for it in *E. coli* [33] [23], while it can be dispensed with for LPS transport to the OM in *Neisseria meningitidis* [34] *lptE*, in both bacteria was found to play an important role in the maturation of the integral OM component of the LPS transport mechanism *LptD*, where it acts as a companion to stabilize *LptD* and/or assist *LptD* in folding and inserting into the OM [34] [24].

This infection assay shows that depletion of *LptE* severely weakens the ability of *P. aeruginosa* to cause disease in *Galleria mellonella* larvae, indicating that this protein can play an important role during the *P. aeruginosa* infection process[24]. As a result of our study, it was found that the most resistant isolates are those carrying the *LptE* gene.

Conclusions

The study points to the importance of multiple virulence factors that contribute to the severity and delayed recovery of infections caused by *P. aeruginosa*. The study shows that this bacterium possesses a wide range of virulence traits that enable it to adapt to diverse conditions and cause different types of infections, making it difficult to treat. Multi-drug resistance among *P. aeruginosa* isolates poses a major challenge, which may be attributed to the overuse or inappropriate use of antibiotics. This resistance increases the complexity of infection management and limits effective therapeutic options. The *lptD* and *lptE* genes are associated with the transport of LPS which contribute to bacterial resistance to antibiotics. The technique used to detect these genes revealed a great diversity among different *P. aeruginosa* isolates, reflecting the complexity of this infection.

Overall, the study highlights the importance of:

- Understanding the mechanisms of virulence and resistance in *P. aeruginosa* to develop effective therapeutic strategies.
- Judgmental use of antibiotics to avoid the development of drug resistance.
- Developing accurate tests to identify genes responsible for virulence and resistance to improve diagnosis and treatment of infections.

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Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

[First Author]: Contributed to the conception and design of the study, conducted some experiments, data rearrangement and drafted the initial manuscript.

[Second Author]: conducted some experiments, collected a part of literature review and conducted some characteristics of the products.

References

1. That, B. R. & Environment, H. Biomedicines-11-01221.Pdf. (2023).
2. Al-yasiri, S. & Risan, M. H. Identification , characterization and antibiotic susceptibility testing of Pseudomonas aeruginosa isolated from clinical sources. (2024).
3. Ahmad, R., Mohammed, R. & Alagely, H. Study the Phenotypic and Antimicrobial Susceptibility of Pseudomonas aeruginosa Isolated Clinically from Baghdad Hospitals. *J. Biotechnol. Res. Cent.* **18**, (2024).
4. Sathe, N. *et al.* Pseudomonas aeruginosa: Infections and novel approaches to treatment "Knowing the enemy" the threat of Pseudomonas aeruginosa and exploring novel approaches to treatment. *Infect. Med.* **2**, 178–194 (2023).
5. Morley, S. A. *et al.* Testing the Resilience, Physiological Plasticity and Mechanisms Underlying Upper Temperature Limits of Antarctic Marine Ectotherms. *Biology (Basel).* **13**, (2024).

6. Young, L. S. & Amstrong, D. Pseudomonas Aeruginosa Infections. *CRC Crit. Rev. Clin. Lab. Sci.* **3**, 291–347 (1972).

7. Wood, S. J., Kuzel, T. M. & Shafikhani, S. H. Pseudomonas aeruginosa: Infections, Animal Modeling, and Therapeutics. *Cells* **12**, 1–37 (2023).

8. Elfadadny, A. *et al.* Antimicrobial resistance of Pseudomonas aeruginosa: navigating clinical impacts, current resistance trends, and innovations in breaking therapies. *Front. Microbiol.* **15**, 1374466 (2024).

9. Roy, S. *et al.* Microbial infections in burn patients. *Acute Crit. Care* **39**, 214–225 (2024).

10. Maslova, E., Eisaiankhongi, L., Sjöberg, F. & McCarthy, R. R. Burns and biofilms: priority pathogens and in vivo models. *npj Biofilms Microbiomes* **7**, 1–9 (2021).

11. Maitz, J., Merlino, J., Rizzo, S., McKew, G. & Maitz, P. Burn wound infections microbiome and novel approaches using therapeutic microorganisms in burn wound infection control. *Adv. Drug Deliv. Rev.* **196**, 114769 (2023).

12. Ahmed, A. S. Production of silver nanoparticles from the aerobic and anaerobic cultures supernatants of Pseudomonas aeruginosa. 1–10 (2024).

13. Jurado-Martín, I., Sainz-Mejías, M. & McClean, S. Pseudomonas aeruginosa: An Audacious Pathogen with an Adaptable Arsenal of Virulence Factors. *Int. J. Mol. Sci.* **22**, (2021).

14. Holban, A. M., Gregoire, C. M. & Gestal, M. C. Conquering the host: Bordetella spp. and Pseudomonas aeruginosa molecular regulators in lung infection. *Front. Microbiol.* **13**, 1–18 (2022).

15. Liao, C., Huang, X., Wang, Q., Yao, D. & Lu, W. Virulence Factors of Pseudomonas Aeruginosa and Antivirulence Strategies to Combat Its Drug Resistance. *Front. Cell. Infect. Microbiol.* **12**, 926758 (2022).

16. Bertani, B. & Ruiz, N. Function and Biogenesis of Lipopolysaccharides. *EcoSal Plus* **8**, (2018).

17. Okuda, S., Sherman, D. J., Silhavy, T. J., Ruiz, N. & Kahne, D. Lipopolysaccharide transport and assembly at the outer membrane: the PEZ model. *Nat. Rev. Microbiol.* **14**, 337–345 (2016).

18. Kaito, C. *et al.* Non-pathogenic Escherichia coli acquires virulence by mutating a growth-essential LPS transporter. *PLoS Pathog.* **16**, e1008469 (2020).

19. Stocks, N. 済無No Title No Title No Title. 1–23 (2016).

20. Chng, S.-S., Ruiz, N., Chimalakonda, G., Silhavy, T. J. & Kahne, D. Characterization of the two-protein complex in Escherichia coli responsible for lipopolysaccharide assembly at the outer membrane. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 5363–5368 (2010).

21. Romano, K. P. & Hung, D. T. Targeting LPS biosynthesis and transport in gram-negative bacteria in the era of multi-drug resistance. *Biochim. Biophys. acta. Mol. cell Res.* **1870**, 119407 (2023).

22. Romano, K. P. & Hung, D. T. Targeting LPS biosynthesis and transport in gram-negative bacteria in the era of multi-drug resistance. *Biochim. Biophys. Acta - Mol. Cell Res.* **1870**, 119407 (2023).

23. Lo Sciuto, A. *et al.* Pseudomonas aeruginosa LptE is crucial for LptD assembly, cell envelope integrity, antibiotic resistance and virulence. *Virulence* **9**, 1718–1733 (2018).

24. Chimalakonda, G. *et al.* Lipoprotein LptE is required for the assembly of LptD by the β-barrel assembly machine in the outer membrane of Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 2492–2497 (2011).

25. Reem, A. *et al.* Pseudomonas aeruginosa and related antibiotic resistance genes as indicators for wastewater treatment. *Heliyon* **10**, e29798 (2024).

26. Mahmoud, A., Zahran, W., Hindawi, G., Labib, A. & Galal, R. Prevalence of Multidrug-Resistant Pseudomonas aeruginosa in Patients with Nosocomial Infections at a University Hospital in Egypt, with Special Reference to Typing Methods. *J. Virol. Microbiol.* **2013**, 1–13 (2013).

27. Braun, M. & Silhavy, T. J. Imp/OstA is required for cell envelope biogenesis in Escherichia coli. *Mol. Microbiol.* **45**, 1289–1302 (2002).

28. Sperandeo, P. *et al.* Characterization of lptA and lptB, two essential genes implicated in lipopolysaccharide transport to the outer membrane of Escherichia coli. *J. Bacteriol.* **189**, 244–253 (2007).

29. Werneburg, M. *et al.* Inhibition of Lipopolysaccharide Transport to the Outer Membrane in Pseudomonas aeruginosa by Peptidomimetic Antibiotics. *ChemBioChem* **13**, 1767–1775 (2012).

30. Balibar, C. J. & Grabowicz, M. Mutant alleles of lptD increase the permeability of Pseudomonas aeruginosa and define determinants of intrinsic resistance to antibiotics. *Antimicrob. Agents Chemother.* **60**, 845–854 (2016).

31. Hassett, D. J. Anaerobic production of alginate by Pseudomonas aeruginosa: Alginate restricts diffusion of oxygen. *J. Bacteriol.* **178**, 7322–7325 (1996).

32. Bragonzi, A. *et al.* Nonmucoid Pseudomonas aeruginosa expresses alginate in the lungs of patients with cystic fibrosis and in a mouse model. *J. Infect. Dis.* **192**, 410–419 (2005).

33. Wu, T. *et al.* Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 11754–11759 (2006).

34. Malojčić, G. *et al.* LptE binds to and alters the physical state of LPS to catalyze its assembly at the cell surface. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 9467–9472 (2014).

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Serum level of IgM and IgG in Responses to COVID-19 Vaccination in Iraq

Ayat S. Saif[1*]; *Shahlaa M. Salih*[2]

[1,2] **Department of Molecular and Medical Biotechnology, College of Biotechnology, Al-Nahrian University, Baghdad, Iraq**
*Corresponding Author
E. mail: ayat.mbm23@ced.nahrainuniv.edu.iq
https://orcid.org/0000-0002-1679-9262

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Abstract

The COVID-19 is pandemic disease, caused by the novel coronavirus SARS-CoV-2. It has enhanced extensive research about the immune responses of infection and vaccination. This study aimed to measurement of serum antibodies (IgM and IgG) levels in vaccinated individuals in Iraqi patients, and compares the responses between unvaccinated, Pfizer-vaccinated, Sinopharm-vaccinated, and COVID-19-infected groups. A total of 300 participants were categorized into four subgroups: 30 non-vaccinated and uninfected, 90 Pfizer-vaccinated, 90 Sinopharm-vaccinated after the second dose, and 90 COVID-19 infected, which assessed immune responses to Pfizer and Sinopharm COVID-19 vaccines over the first three months post-vaccination. IgM levels were significantly elevated in both the Sinopharm and Pfizer vaccine groups compared to the control group. Specifically, Sinopharm recipients had IgM levels of 5.33 ± 2.1 ng/ml, Pfizer recipients had 3.61 ± 1.14 ng/ml, and the control group had 0.6 ± 1.14 ng/ml. Notably, there were no significant differences in IgM levels between the Sinopharm and Pfizer groups. IgM levels in both Sinopharm and Pfizer groups slightly increased after the third month of vaccination. Sinopharm recipients exhibited a slight rise to 2.1 ± 0.7 ng/ml, while Pfizer recipients had a slight increase to 1.0 ± 0.3 ng/ml. IgG levels were significantly higher in the Pfizer vaccine group compared to the Sinopharm group and the control group. Pfizer recipients had a serum IgG level of 78.4 ± 12.3 ng/ml, followed by Sinopharm recipients at 68.4 ± 9.10 ng/ml, and the control group at 0.71 ± 0.02 ng/ml. After the third month of vaccination, both Sinopharm and Pfizer groups showed a decline in IgG levels. However, the Pfizer group maintained a higher level, with 50.2 ± 9.8 ng/ml compared to the Sinopharm group's 32.1 ± 5.4 ng/ml. These findings underscore the distinct immune responses elicited by different vaccines, providing valuable information for optimizing COVID-19 vaccination programs.

Keywords: COVID-19, IgM, IgG, Pfizer, Sinopharm.

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Introduction

A modified virus called SARS-CoV2 is the source of the Coronavirus Disease (COVID-19). The virus was discovered for the first time in December 2019 in Wuhan, China[1]. Three vaccines have been globally administered in Iraq: the Pfizer vaccine (is made from messenger RNA (mRNA) that has been engineered to encode the SARS-CoV-2 spike protein (S)), the Oxford AstraZeneca vaccine (employs a modified chimpanzee DNA adenovirus), and the Sinopharm vaccine (contains the inactivated SARS-CoV-2 virus)[2]. Despite the different modes of action of the COVID-19 vaccines, all of them target the spike protein because of its vital function[3].SARS-CoV-2's spike (S) protein is essential to infection by attaching itself to the ACE-2 receptor on host cells, it functions as a key, allowing the virus to fuse and enter[4]. Immunological markers, particularly immunoglobulin G (IgG) levels, are useful tools for evaluating the efficacy of virus vaccinations, because IgG represents the body's long-term humoral immune response[5]. IgG antibodies take longer to develop but provide more persistent protection than IgM antibodies, which are first produced by the immune system in response to a novel infection, including viruses. Consequently, after vaccination, higher IgG than IgM antibody levels suggest a stronger immunological response and possibly higher vaccine efficacy[6]. A SARS-CoV-2 infection can cause IgM antibodies to appear as early as 4 days after infection and to peak at roughly 20 days following, but IgG antibodies rise approximately 7 days after infection and reach their peak at about 25 days[7,8]. Conversely, serum may already have significant levels of IgG against SARS-CoV-2, which may be found alongside or ahead of IgM. Another study found that the levels of IgG and IgM specific to the SARS-CoV-2 virus peaked 17–19 days and 20–22 days after the onset of symptoms, respectively. Several types of seroconversions have been reported: IgG and IgM seroconversion concurrently, IgM seroconversion prior to IgG, and IgM seroconversion subsequent to IgG[9].

Studies on the immune response to SARS-CoV-2 after spontaneous infection have revealed that convalescent COVID-19 patients have IgG antibodies for several months after the onset of symptoms, though these antibodies capacity to neutralize the virus gradually deteriorates[10]. For the adaptive immune response to be effective against the SARS-CoV-2 immunoglobulins IgG and IgM are indispensable. IgG mainly works by neutralizing the virus, blocking its entrance into cells, and stimulating immune cells to destroy infected cells. The first immune response, IgM, on the other hand, helps to activate B cells so they can produce more IgG[11].

Assessing IgG and IgM levels after vaccination or infection is crucial for evaluating vaccine efficacy, determining the duration of protective immunity, and advancing the development of novel therapeutic approaches[12].

Materials and Methods

Subject:

There were Three hundred Iraqis that participated in this study. The participants were divided into four subgroups: thirty people were ranged from 24 to 35 (male equal to female), who were not vaccinated and uninfected, ninety people who received the Pfizer vaccine, ninety people who received the Sinopharm vaccine following the second dose of vaccination, and ninety people who were infected with COVID-19 but had not received the vaccine. The observation period was for the first three months after vaccination or

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infection. The ages of the vaccinated and infected groups varied from (25 to 40) years. Each of these groupings was then divided into three categories based on the length of vaccination or infection:

one month, two months, and three months. Between October 2021 and January 2022, medical professionals oversaw the collection of contaminated samples from Baghdad Teaching Hospital and Ibn Al-Kateeb Hospital in Baghdad, Iraq

Estimation Serum Level of IgM and IgG

Serum samples were obtained from all participants. Anti-SARS-CoV-2 IgM and IgG levels were quantified using Sunglong, China ELISA kits.

Statistical analysis:

The data analysis tools used were GraphPad Prism 9 and SPSS version 23. The mean, standard deviation, ANOVA, and ROC curves were among the statistical tests used.

Results and Discussion

Evaluation of Anti-S covid-19 immunoglobulin M (IgM) in the infected and vaccinated group:

Compared to the controls (0.6±0.01 ng/ml) and vaccinated groups, the follow-up data analysis revealed a significantly higher serum level of IgM in hospitalized patients after one month of infection (14.3±2.8 ng/ml). Following the first month of vaccination, the IgM levels were substantially higher in the Sinopharm vaccinate (5.33±2.1 ng/ml) and Pfizer vaccinate (3.61±1.14 ng/ml) groups than in the control group. However, no significant differences were observed within the vaccinated groups.

In comparison to the control group, the infected group exhibited a significantly higher IgM level (7.3±2.8 ng/ml) and Sinopharm vaccinate (3.9±1.3 ng/ml) in the second month following infection and vaccination, respectively. However, no significant differences were observed within the vaccinated groups. Pfizer vaccinate did not exhibit any significant differences in comparison to controls (1.4±0.6 ng/ml), respectively.

Compared to controls (0.6±0.01 ng/ml) and vaccinated groups, the IgM levels in patient (3.2±1.1ng/ml) and Sinopharm (2.1±0.7 ng/ml) and Pfizer (1.0±0.3 ng/ml) were slightly higher after the third month of infection and vaccination, as demonstrated in (figure 1).

Anti-S COVID-19 Immunoglobulin G (IgG) in the infected and vaccinated group: The data analysis showed that the infected group had a greater level of IgG identified in the first three months after infection and vaccination, and that after one month, the amount of serum IgG was higher (114.3±13.6 ng/ml). Compared to the controls (0.71±0.02 ng/ml), the Pfizer vaccinates had a higher serum IgG level (78.4±12.3 ng/ml), followed by the Sinopharm vaccinates (68.4±9.10 ng/ml).

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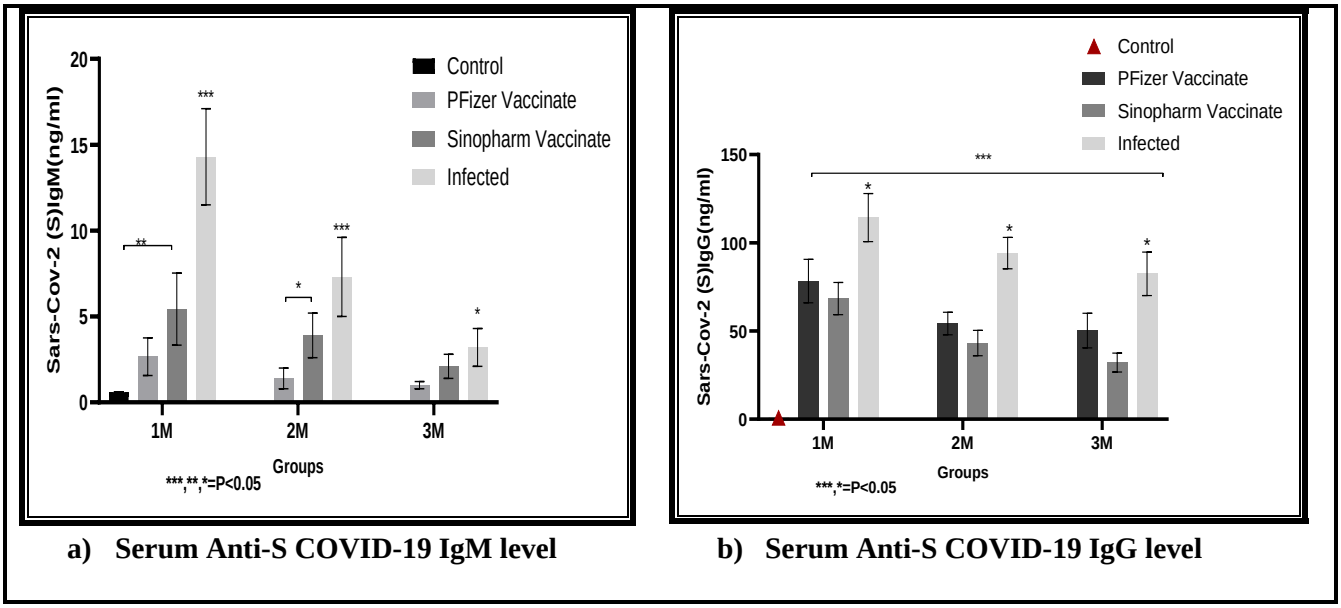
The second month after infection and vaccination showed notable variations as well. The IgG levels in the infected groups were substantially higher (94.2±8.9 ng/ml) than in the Sinopharm group (43.2±7.2 ng/ml), Pfizer vaccinated group (54.2±6.4 ng/ml), and control groups ($p<0.05$). The immunization group did not differ significantly from one another, nevertheless ($p>0.05$).

In comparison to the Pfizer (50.2±9.8 ng/ml) and Sinopharm (32.1±5.4 ng/ml) groups, as well as the control group (0.71±0.02 ng/ml), the infected groups also showed a considerable increase in the third month (82.4±12.3 ng/ml). However, as shown in (figure 1), no notable variations were found within the previously indicated categories.



a) Serum Anti-S COVID-19 IgM level **b) Serum Anti-S COVID-19 IgG level**

Figure 1. a) IgM and b) IgG serum levels in the infected and vaccinated groups.

One month after infection, higher serum levels of IgM were found in hospitalized patients compared to both the control and vaccinated groups. This finding emphasizes the immediate immunological response to SARS-CoV-2 pneumonia. The study's findings which support the findings of[13,14], showed that IgM levels are rising quickly. They observed that IgM antibodies against SARS-CoV-2 could be found within days of infection, peaking approximately three weeks later. Interestingly, the differential IgM response among vaccinated individuals, with Sinopharm recipients showing the highest levels followed by Pfizer, suggests vaccine-specific immune activation profiles. This phenomenon can be attributed to varying vaccine platforms and adjuvants used, affecting the immunogenicity and efficiency of the immune response[15].

Results showed a significant rise in IgG levels following vaccination and infection, indicating the beginning of a more robust immune response. In the first month after vaccination and infection, the infected group had the greatest IgG levels; thereafter, the Pfizer and Sinopharm vaccines showed significant increases in IgG levels. These outcomes are consistent with[16], who reported the presence of immunoglobulin G in high quantities in the post-infection period.

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Results are also in line with the study by[17], who demonstrated that IgG antibody levels in fully vaccinated individuals with the Pfizer-BioNTech vaccine exhibited higher quantitative efficiency compared to those who received the Sinopharm vaccine. Both immunizations produce S protein IgG and NAbs over a period of several months. They also show a modest TH2 response and a robust T helper (TH) 1 response.

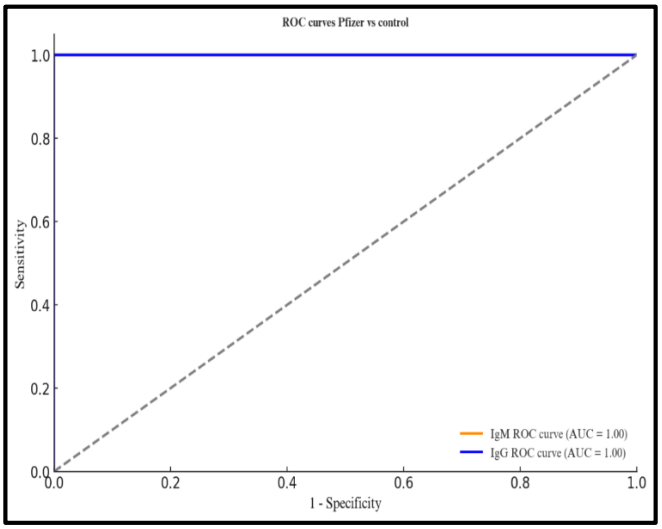
The weeks following vaccination, the anti-spike IgG concentration for the Pfizer vaccination varied greatly; as the weeks pass, the concentration rises, reaching its maximum between the sixth and seventh weeks and its lowest between the tenth and subsequent weeks, as demonstrated in[18].

ROC test Analysis:

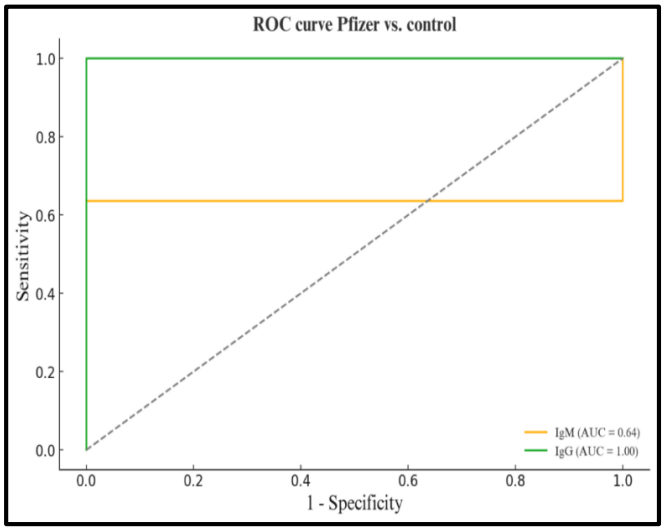
The Roc curve analysis of the Pfizer vaccine for IgM and IgG during the first month demonstrated (Sensitivity 100%, Specificity 100%, AUC: 1), while the analysis in third month demonstrated that IgM (Sensitivity 63.6 %, Specificity 63.6 %, AUC: 0.64 ± 0.10), and the IgG (Sensitivity 100%, Specificity 100%, AUC: 1.00 ± 0.00). This is shown in fig 2.

The Sinopharm vaccine, during the first month the ROC curve analysis for IgM and IgG showed higher sensitivity and specificity (Sensitivity 100%, Specificity 100%, AUC: 1), while in the third month the result for IgM yielded (Sensitivity 100%, Specificity 36.364%, AUC: 1.00 ± 0.10), and the IgG yielded (Sensitivity %: 100, Specificity %: 18.182, AUC: 1.00 ± 0.00) as shown in fig 3.

ROC analysis of the infected individual recorded in the first and the third months that IgM (Sensitivity 100%, Specificity 100%, AUC: 1.00±0.00) ROC analysis recorded that IgG (Sensitivity 100%, Specificity 100%, AUC 1.00±0.00) as shown in fig 4



a) ROC test of IgM and IgG after the first month



b) ROC test of IgM and IgG after the third month

Figure 2. Roc test of IgM and IgG after Pfizer vaccination

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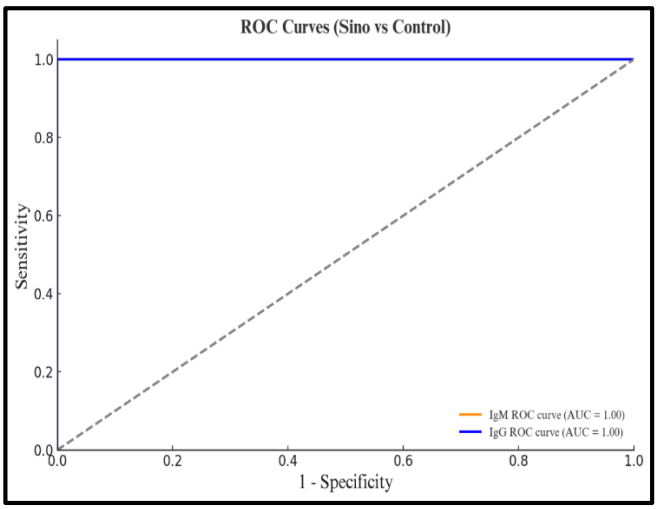
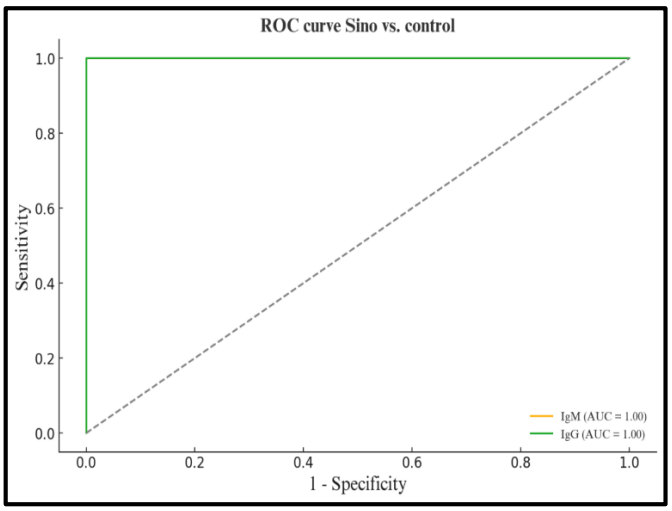
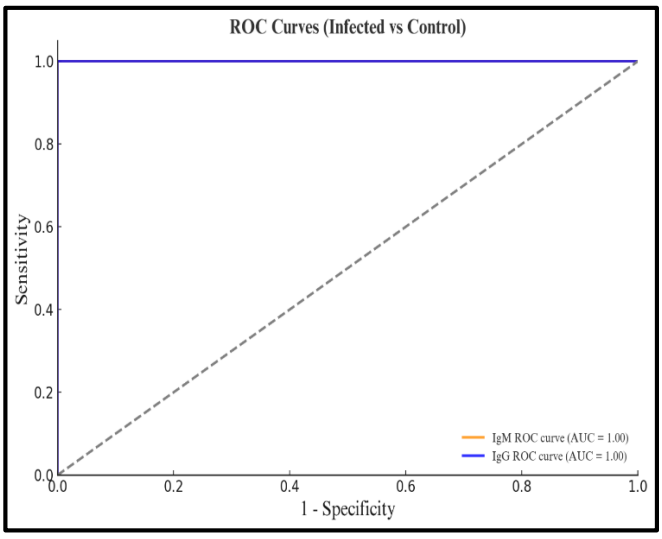


a) ROC test of IgM and IgG after the first month

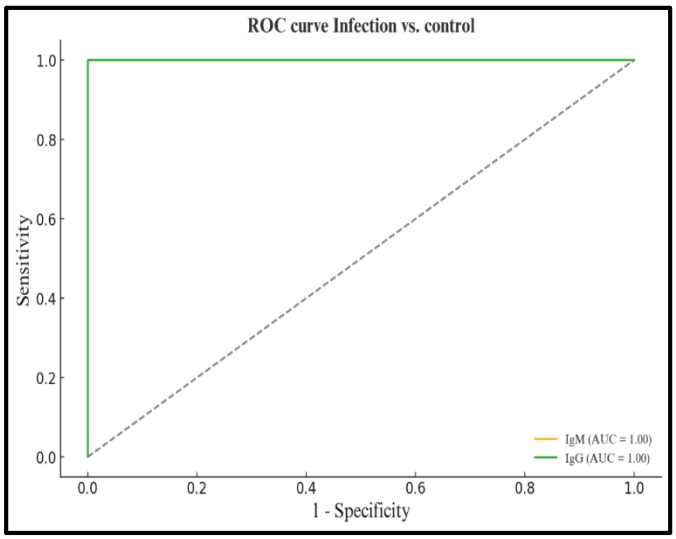


b) ROC test of IgM and IgG after the third month

Figure 3. Roc test of IgM and IgG after Sinopharm vaccination



a) ROC test of IgM and IgG after the first month



b) ROC test of IgM and IgG after the third month

Figure 4. Roc test of IgM and IgG after infection

A ROC analysis revealed that IgM and IgG levels are highly effective diagnostic markers for COVID-19 infection and the immune response to Pfizer and Sinopharm vaccines. IgM and IgG antibodies were identified as the most reliable indicators for assessing both infection status and vaccine efficacy.

These markers demonstrated exceptional sensitivity and specificity of 100%, as well as high area under the curve (AUC) values, particularly within the first month after vaccination.

IgM levels exhibited a decline in sensitivity over time, especially in the third month following vaccination. In contrast, IgG levels maintained consistently high sensitivity and specificity throughout the study period. The superior diagnostic accuracy of IgG compared to IgM was further evidenced by the AUC (IgG) > AUC (IgM) result[19]. Additional research has demonstrated that SARS-CoV-2 serological testing can serve as a valuable adjunct to the current RT-PCR assay, enabling more accurate and timely identification of COVID-19 cases[20].

Overall, this investigation highlights the potential of utilizing various immunological markers to diagnose COVID-19 infection and assess vaccine response. The Pfizer vaccine group demonstrated superior diagnostic potential for both IgM and IgG compared to the Sinopharm group, which exhibited lower sensitivity and specificity values [21].

Conclusions

According to the study, the Pfizer vaccine induces high levels of IgM and IgG antibodies. IgG levels often reach their peak several weeks following the second dosage and stay there for three months, whereas the Sinopharm immunization tends to cause a quick reduction in antibody response.

Acknowledgments

The authors would like to express our deepest gratitude to all those who contributed to the completion of this study. Our sincere thanks go to the healthcare providers and staff at Baghdad Teaching Hospital and Ibn Al-Kateeb Hospital for their invaluable assistance in sample collection and patient care. We are also grateful to the participants for their cooperation and willingness to be part of this study. extend our heartfelt appreciation to the faculty and staff of the College of Biotechnology, Al-Nahrain University, for their support and guidance throughout this research.

Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

[First Author's Name]: Played a critical role in the statistical analysis of the data and interpretation of the results.

[Second Author's Name]: Played a critical role in supervising the research, providing guidance, and designing the study

References

1. Zhou, P., Yang, X.L., Wang, X.G., Hu, B., Zhang, L., Zhang, W., Si, H.R., Zhu, Y., Li, B., Huang, C.L. and Chen, H.D., 2020. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *nature*, *579*(7798), pp.270-273.

2. Wilder-Smith, A. and Mulholland, K., 2021. Effectiveness of an inactivated SARS-CoV-2 vaccine. *New England Journal of Medicine*, *385*(10), pp.946-948.

3. Tran, B.X., Ha, G.H., Nguyen, L.H., Vu, G.T., Hoang, M.T., Le, H.T., Latkin, C.A., Ho, C.S. and Ho, R.C., 2020. Studies of novel coronavirus disease 19 (COVID-19) pandemic: a global analysis of literature. *International journal of environmental research and public health*, *17*(11), p.4095.

4. Mascellino, M.T., Di Timoteo, F., De Angelis, M. and Oliva, A., 2021. Overview of the main anti-SARS-CoV-2 vaccines: mechanism of action, efficacy and safety. *Infection and drug resistance*, pp.3459-3476.

5. Inchingolo, A.D., Malcangi, G., Ceci, S., Patano, A., Corriero, A., Vimercati, L., Azzollini, D., Marinelli, G., Coloccia, G., Piras, F. and Barile, G., 2022. Effectiveness of SARS-CoV-2 vaccines for short-and long-term immunity: a general overview for the pandemic contrast. *International Journal of Molecular Sciences*, *23*(15), p.8485.

6. Hou, H., Wang, T., Zhang, B., Luo, Y., Mao, L., Wang, F., Wu, S. and Sun, Z., 2020. Detection of IgM and IgG antibodies in patients with coronavirus disease 2019. *Clinical & translational immunology*, *9*(5), p.e1136.

7. Long, Q.X., Liu, B.Z., Deng, H.J., Wu, G.C., Deng, K., Chen, Y.K., Liao, P., Qiu, J.F., Lin, Y., Cai, X.F. and Wang, D.Q., 2020. Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nature medicine*, *26*(6), pp.845-848.

8. Jin, Y., Wang, M., Zuo, Z., Fan, C., Ye, F., Cai, Z., Wang, Y., Cui, H., Pan, K. and Xu, A., 2020. Diagnostic value and dynamic variance of serum antibody in coronavirus disease 2019. *International journal of infectious diseases*, *94*, pp.49-52.

9. Lee, Y.L., Liao, C.H., Liu, P.Y., Cheng, C.Y., Chung, M.Y., Liu, C.E., Chang, S.Y. and Hsueh, P.R., 2020. Dynamics of anti-SARS-Cov-2 IgM and IgG antibodies among COVID-19 patients. *The Journal of infection*, *81*(2), p.e55.

10. Xia, S., Zhang, Y., Wang, Y., Wang, H., Yang, Y., Gao, G.F., Tan, W., Wu, G., Xu, M., Lou, Z. and Huang, W., 2021. Safety and immunogenicity of an inactivated SARS-CoV-2 vaccine, BBIBP-CorV: a randomised, double-blind, placebo-controlled, phase 1/2 trial. *The Lancet infectious diseases*, *21*(1), pp.39-51.

11. Schroeder Jr, H.W. and Cavacini, L., 2010. Structure and function of immunoglobulins. *Journal of allergy and clinical immunology*, *125*(2), pp.S41-S52.

12. Chang, S.E., Feng, A., Meng, W., Apostolidis, S.A., Mack, E., Artandi, M., Barman, L., Bennett, K., Chakraborty, S., Chang, I. and Cheung, P., 2021. New-onset IgG autoantibodies in hospitalized patients with COVID-19. *Nature communications*, *12*(1), p.5417.

13. Narasimhan, M., Mahimainathan, L., Araj, E., Clark, A.E., Markantonis, J., Green, A., Xu, J., SoRelle, J.A., Alexis, C., Fankhauser, K. and Parikh, H., 2021. Clinical evaluation of the Abbott Alinity SARS-CoV-2 spike-specific quantitative IgG and IgM assays among infected, recovered, and vaccinated groups. *Journal of clinical microbiology*, *59*(7), pp.10-1128.

14. Ruggiero, A., Piubelli, C., Calciano, L., Accordini, S., Valenti, M.T., Dalle Carbonare, L., Siracusano, G., Temperton, N., Tiberti, N., Longoni, S.S. and Pizzato, M., 2022. SARS-CoV-2 vaccination elicits unconventional IgM specific responses in naïve and previously COVID-19-infected individuals. *EBioMedicine*, *77*.

15. Bettini, E. and Locci, M., 2021. SARS-CoV-2 mRNA vaccines: immunological mechanism and beyond. *Vaccines*, *9*(2), p.147.

16. Zhou, C., Bu, G., Sun, Y., Ren, C., Qu, M., Gao, Y., Zhu, Y., Wang, L., Sun, L. and Liu, Y., 2021. Evaluation of serum IgM and IgG antibodies in COVID-19 patients by enzyme linked immunosorbent assay. *Journal of medical virology*, *93*(5), pp.2857-2866.

17. Alqassieh, R., Suleiman, A., Abu-Halaweh, S., Santarisi, A., Shatnawi, O., Shdaifat, L., Tarifi, A., Al-Tamimi, M., Al-Shudifat, A.E., Alsmadi, H. and Al Sharqawi, A., 2021. Pfizer-BioNTech and Sinopharm: a comparative study on post-vaccination antibody titers. *Vaccines*, *9*(11), p.1223.

18. Hassan, R.T. and Mohammed, S.H., 2022. Evaluation of immunoglobulin G level among subjects vaccinated with different types of COVID-19 vaccines in the karbala population, Iraq. *Biomedical and Biotechnology Research Journal (BBRJ)*, *6*(3), pp.466-471.

19. Bayraktar, N., Turan, H., Bayraktar, M., Ozturk, A. and Erdoğdu, H., 2022. Analysis of serum cytokine and protective vitamin D levels in severe cases of COVID-19. *Journal of medical virology*, *94*(1), pp.154-160.

20. Khan, W.H., Hashmi, Z., Goel, A., Ahmad, R., Gupta, K., Khan, N., Alam, I., Ahmed, F. and Ansari, M.A., 2021. COVID-19 pandemic and vaccines update on challenges and resolutions. *Frontiers in cellular and infection microbiology*, *11*, p.690621.

21. Adjobimey, T., Meyer, J., Sollberg, L., Bawolt, M., Berens, C., Kovačević, P., Trudić, A., Parcina, M. and Hoerauf, A., 2022. Comparison of IgA, IgG, and neutralizing antibody responses following immunization with Moderna, BioNTech, AstraZeneca, Sputnik-V, Johnson and Johnson, and Sinopharm's COVID-19 vaccines. *Frontiers in Immunology*, *13*, p.917905.

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Streptomyces actinomycinicus CMU-RKDM30 Bacterial Active Compounds Revealed by High-Performance Liquid Chromatography

***Bassam Qasim Mohammed*[1*]; *Mohsen Hashim Risan*[2]**

[1]**Ibn Sina university of Medical and Pharmaceutical Science**
[2]**Al-Nahrain University, College of Biotechnology, Baghdad, Iraq**

*Corresponding Author: E. mail: Bassam.qasim@ibnsina.edu.iq

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Abstract

Streptomyces actinomycinicus is a bacterium species from the genus of *Streptomyces* which has been isolated from soil. *Streptomyces actinomycinicus* has the ability to degrade pol(3-hydroxyalkanoate). This species produces Actinomycin, exfoliamycin and many secondary metabolite. The 52 isolated obtained from the isolation processes were sub-cultured on yeast extract-malt extract agar. The results obtained of our isolation *Streptomyces actinomycinicus* CMU-RKDM30 which detected by PCR based on the study of the 16S rRNA gene sequence and phylogenetic relationship. The results of biochemical tests revealed the amylase, urease, catalase, gelatinase, protease, cellulose, phosphatase tests were positive, while the indole production and soluble pigment tests were negative. The HPLC of extracellular crude extract of our isolation *Streptomyces actinomycinicus* showed three different antibiotics (Azithromycin, Amoxicillin, and actinomycin).

Keywords: *Streptomyces*, actinomycin, *actinomycinicus*,16S rRNA, HPLC.

Introduction

Actinomycetes are part of the phylum Actinobacteria, subclass Actinobacteridae, and order Actinomycetes. It is estimated that over 70% of naturally derived antibiotics currently in clinical use are sourced from soil actinomycetes. Some well-known examples of antibiotics produced by actinomycetes include streptomycin, erythromycin, vancomycin and tetracycline. This order is further divided into 14 suborders, 49 families, and over 140 genera. Actinomycetes are widely distributed in various environments, including soil, water, and even in some extreme conditions. They play a crucial role in decomposing organic matter and contributing to the nutrient cycling in ecosystems [1].These antibiotics have been crucial in treating bacterial infections and have played a significant role in advancing medical practices[2]. The ability of actinomycetes to produce such a wide array of secondary metabolites is thought to be linked to their complex life cycle and their role in competing with other microorganisms in their ecological niche.

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Research on actinomycetes and their secondary metabolites is focused on discovering therapeutic compounds and addressing antibiotic resistance in pathogenic bacteria[3]. *Streptomyces actinomycinicus* is a bacterium species from the genus of *Streptomyces* which has been isolated from soil. *Streptomyces actinomycinicus* has the ability to degrade poly (3-hydroxyalkanoate). This species produces Actinomycin, exfoliamycin and many secondary metabolite [4]. *Streptomyces* are widely distributed bacteria found on land, known for their strong metabolic powers and adaptability. These bacteria can use new growth and dispersion characteristics as they compete for environmental niches. In addition, they utilize their varied metabolic abilities for a range of purposes, such as optimizing food absorption, impeding phage replication, and suppressing bacterial and fungal proliferation [5]. They are increasingly discovered to exist in symbiosis with plants and insects, frequently providing protective advantages to their host by producing antimicrobial chemicals that inhibit pathogens [6]. One form of liquid chromatography that has proven useful in the separation and quantification of dissolved compounds is high performance liquid chromatography, or HPLC. One way to find out how much of a certain chemical is in a solution is to utilize HPCL. The various solutes in the sample solution interact with the stationary phase in high-performance liquid chromatography (HPLC) and other methods that involve contacting the sample solution with a second solid or liquid phase. As a result of their unique interactions with the column, various components of the sample can be effectively separated into [6]. Various compounds have various retention durations, Rt, because their motilities cause them to escape the column at different times. The objective of this study is to isolate, identify, and analyze the active compounds produced by Streptomyces actinomycinicus CMU-RKDM30, a bacterium isolated from soil. Through a combination of molecular techniques, including PCR-based identification of the 16S rRNA gene, and biochemical testing, the study aims to confirm the strain's identity and its ability to produce bioactive metabolites. Using High-Performance Liquid Chromatography (HPLC), the extracellular crude extract from this strain was analyzed, with the goal of detecting and quantifying the presence of important antibiotics. By comparing the chromatographic data to known standards, the study seeks to reveal the specific antibiotic compounds produced by the isolate, thereby contributing to the growing body of research on the therapeutic potential of Streptomyces species. This investigation has the broader goal of exploring new sources of bioactive compounds, particularly antibiotics, that could address the rising issue of antibiotic resistance.

Materials and Methods

One gram of dried and treated soil samples was used to make suspension by adding it in 99 ml of sterile distilled water (stock suspension) and they were shacked in a shaker at 160 rpm for 30 minutes at room temperature [7]. Serial dilutions from 10^1 to 10^5 were made from the stock suspension and left for 10 minutes. After shaking, 0.1 ml of each dilution were culture on Yeast Extract and Malt Extract (YEME) with Streptomycin 50 ug/ml, then spread by sterile swab for making uniform distribution of the suspension on the surface of the media. The inoculated plates were incubated at 28°C for 7 to 10 days. For the purpose of making a suspension, utilized one gram of treated and dried soil samples [8]. This was mixed with 99 ml of sterile distilled water to create a stock suspension. The mixture was then shaken at 160 rpm for 30 minutes at room temperature. The stock suspension was diluted in a series of steps from 10^1 to 10^5 and then left to sit for 10 minutes. Following shaking, 0.1 ml of each dilution was cultured on Yeast Extract and Malt Extract (YEME) with 50 ug/ml of streptomycin. The suspension was then evenly distributed over the surface of the media using a sterile swab. For 7 to 10 days, the inoculation plates were kept at 37°C for incubation. Based on cultural characteristics, suspected colonies of *Streptomyces* were selected which are characterized as small, white, pin-point, rough, chalky and a clear zone of inhibition around them, these colonies was confirmed their identification by (types of Gram's stain, aerial and substrate mycelium color, pigment production and pigment color).These colonies were transferred from the mixed culture into separate agar plates and incubated at 28±1°C for 7 days. Streptomyces were re-streaked on the International

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Streptomyces Project (ISP2) to obtain pure colonies for identification. Cultural characteristics confirmed the identification of small, white, pinpoint, rough, chalky Streptomyces colonies. These colonies were cultured at 28±1°C for 7 days[10].

Standard Preparation

To get a concentration of 200 parts per million (ppm), 10 milligrams of standards were dissolved in 50 milliliters of high-performance liquid chromatography (HPLC) grade methanol. This concentration was then further diluted by dissolving 1 milliliter of the solution in 50 milliliters of methanol [11].

Sample Preparation

Twenty ml of the sample were diluted in 50 ml of HPLC-grade methanol (99%). A 1ml of the solution was added to 50ml of 99% HPLC grade methanol to further dilute it.[12]. Analyzing the extracellular extract of Streptomyces using High-Performance Liquid Chromatography (HPLC). 20 ml of the standard and 20ml of the sample were injected into the HPLC machine to get a chromatogram. The content of the sample was then estimated by comparing it to the standard. The concentrations were determined using the following equation: The formula for concentration is given by multiplying the ratio of the area of the sample to the area of the standard by the sample concentration and the dilution factor.

The equation for determining the concentration of the sample based on the described method can be written as follows:

Concentration of sample = (Area of sample / Area of standard) x Concentration of standard x Dilution factor

Where:

- Area of sample / Area of standard: This is the ratio of the chromatogram peak areas.
- Concentration of standard: This is the known concentration of the standard used in the experiment.
- Dilution factor: The factor accounting for the sample dilution, calculated as:
  Dilution factor = (50/20) x (50/1)

Thus, the final concentration of the sample is:

Concentration of sample = (Area of sample / Area of standard) x Concentration of standard x (50/20 x 50/1). The table 1 shows format to present the circumstances of the deportation and the device used.

Table 1: The circumstances of the deportation and the device used in this study

Item	Description
Device Used	PrimeQ Real-Time PCR System
Deportation Circumstances	Sample preparation and analysis of extracellular extracts from *Streptomyces actinomycinicus* CMU-RKDM30
Purpose of Deportation	Identification and quantification of antibiotic compounds (e.g., Azithromycin, Amoxicillin, Actinomycin) in extracellular extract
Process	1. Sample isolation and preparation using ISP2 medium. 2. Dilution and injection into HPLC machine.
Analysis Method	High-Performance Liquid Chromatography (HPLC)
Results Measurement	Comparison of chromatogram peak areas between the sample and standard
Outcome	Identification of active antibiotic compounds based on retention times and peak areas

Primers Used in this Study

The following primers were used for detecting *Streptomyces actinomycinicus* 16S ribosomal RNA gene for strain CMU-RKDM30:

- **Forward primer (5'---3')**: AGCGTTGTCCGGAATTATTG
- **Reverse primer (5'---3')**: TCCAGACGTTTCCGGTGTAT

Electrophoresis

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Electrophoresis was used to confirm the isolation of genomic DNA and validate PCR results. After PCR amplification, the DNA bands were visualized under UV light and photographed following staining with ethidium bromide. The amplified DNA bands confirmed the presence of the target gene for the detection of *Streptomyces actinomycinicus* gene for 16S ribosomal RNA. in the isolated samples.

Results and Discussion

The results of our isolation of *Streptomyces actinomycinicus* CMU-RKDM30 which detected by PCR based on the study of the 16S rRNA gene sequence and phylogenetic relationship were consistent with other studies findings [13,14], regarding the similar habitats in which *Streptomyces* diversity was observed. The results observed a few different colony types, and the isolation process typically yielded plates with one or a few colony types, ranging from 2-4 colonies as shown in the (figure 1).

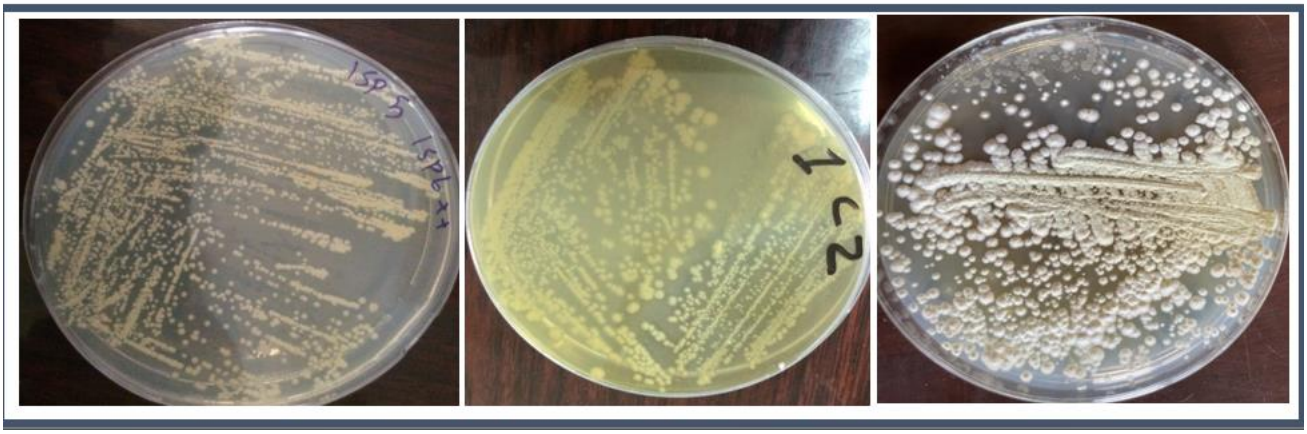

Figure 1. Isolation of *Streptomyces actinomycinicus,* ISP2 (International Streptomyces Project Medium 2) or Starch Casein Agar. The culture was incubated at 28°C for 7-14 days.

Biochemical and physiological characteristics

The *Streptomyces* spp. biochemical and physiological tests are listed in table (2) Amylase, urease, catalase, protease, cellulase, and phosphatase were among the extracellular enzymes produced by the *Streptomyces*. The utilization of citrate was positive, there was no formation of HCN and volatile organic compounds toxicity. Several researchers found consistent results when they studied the *Streptomyces* strains for nutritional uptake, physiological and biochemical characteristics [15]. *Streptomyces actinomycinicus* identification is a laborious procedure. Spore shape, mycelium service, carbon utilization and culture on international *Streptomyces* project (ISP) were the primary criteria for the *Streptomyces* taxonomy. When it comes to classifying members of the Streptomycetaceae family, morphological and biochemical traits are crucial [16].

Table2: Results of biochemical analysis of *Streptomyces* spp.

Reaction	Response	Result
1- Urease	Red to deep pink	Positive
2- Catalase	Bubbles	Positive
3- Amylase	Clear zone	Positive
4- Protease	Clear zone	Positive
5- Gelatinase	Narrow zone	Positive
6- Cellulase	Clear zone	Positive
7- Phosphatase	Clear zone	Positive
8- Indole production	No color zone	Negative

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9- Citrate Utilization	Deep blue color	Positive
10- Soluble pigment	No brown	Negative

The results in the table above show that the amylase, urease, catalase, gelatinase, protease, cellulose, phosphatase tests were positive, while the indole production and soluble pigment tests were negative these results are consistent when they studied the *Streptomyces* strains for nutritional uptake, physiological and biochemical characteristics. Isolation and purification of the
extracellular crude extract from the isolated strain the extracellular crude extract was partially purified using High Performance Liquid Chromatography (HPLC) to isolate and identify the bioactive components. This was done by performing bioautographic in the presence of a standard antibiotic[19].

Molecular identification of *Streptomyces*

The study confirmed the isolation of genomic DNAs from Streptomyces isolates through electrophoresis and PCR. 12 isolates were positive, and a single 480 bp band was observed in all tested isolates. The 16S rRNA gene sequence of the T2 strain was identified. The DNA extraction was effective and confirmed through electrophoresis analysis.

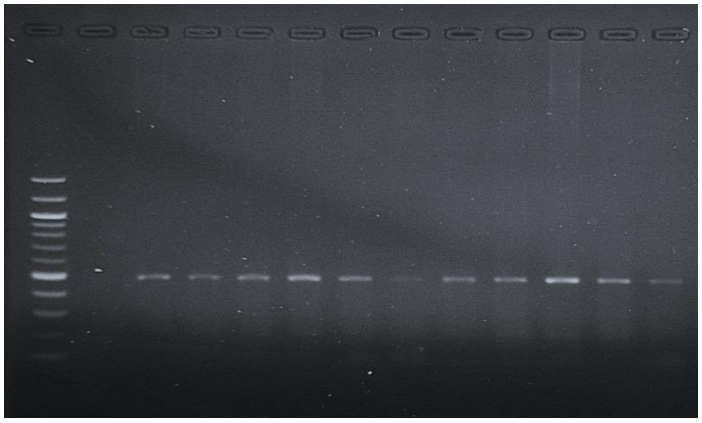

Figure 2: Agarose gel electrophoresis of PCR products detection and isolation *Streptomyces actinomycinicus* gene for 16S ribosomal RNA

Purification by High Performance Liquid Chromatography (HPLC)

The HPLC analysis was done to detect the concentration of important active compounds present in *Streptomyces*. HPLC analysis of extracellular extract of *Streptomyces* indicated the presence of three active compounds. The figure (2) revealed different peaks of antibiotic present in extracellular extracts of *Streptomyces* in same retention time in compare with a stander but with different area.

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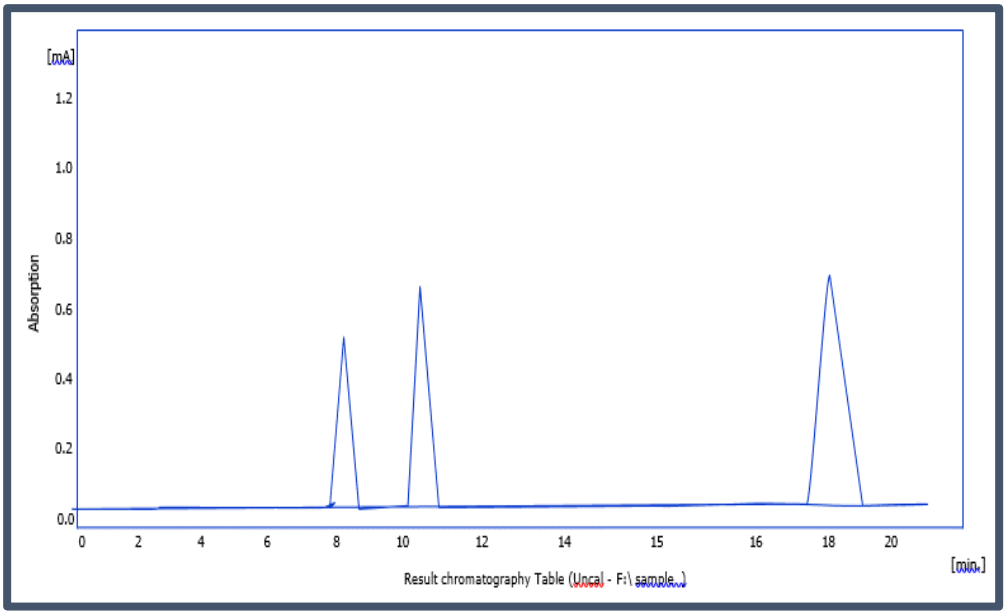

Figure 2: High Performance Liquid Chromatography of standard antibiotic

The HPLC of extracellular crude extract showed three different antibiotics (Azithromycin, Amoxicillin, actinomycin). As presented in figure (3) revealed that the same retention time of the sample in compare with a stander but with different area. The results in table (2) presents the retention times (Rf values), areas, and concentrations of various standard antibiotics identified in *Streptomyces* spp[18]. The table lists three antibiotics: Azithromycin, Amoxicillin, and Actinomycin. Azithromycin, detected at 7.80 minutes, accounts for 30% of the area and height. Amoxicillin, with a longer retention time of 10.75 minutes, contributes to 45% of the total area and height, indicating its significant presence in the sample. Additionally, Actinomycin, detected at 17.21 minutes, shows the highest individual contribution with 65.12% of the total area and 51% of the height, highlighting its dominant concentration within the *Streptomyces* spp. The cumulative area and height percentages across these compounds amount to 100%, reflecting the total analytical output of the chromatographic analysis [17].

Table (2) show the R_f values for each standard antibiotic and area also the concentration of each antibiotic found in *Streptomyces spp*.

N	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	7.80	526.07	600.98	30.00	30.00	0.15	Azithromycin
2	10.75	380.90	800.11	45.00	45.00	0.20	Amoxicillin
3	17.21	509.81	854.12	65.12	51.00	0.26	Actinomycin

The chromatogram in figure (3) shows four distinct peaks at retention times of 7.80, 10.75, and 17.21 minutes. Each peak corresponds to a different compound separated by the chromatographic method. The first peak at 7.80 minutes is slightly larger, corresponding to Azithromycin. The second peak at 10.75 minutes is prominent and sharp, indicating a significant presence of Amoxicillin. Finally, the largest peak occurs at 17.21 minutes, which likely corresponds to Actinomycin, the most abundant compound in this mixture.

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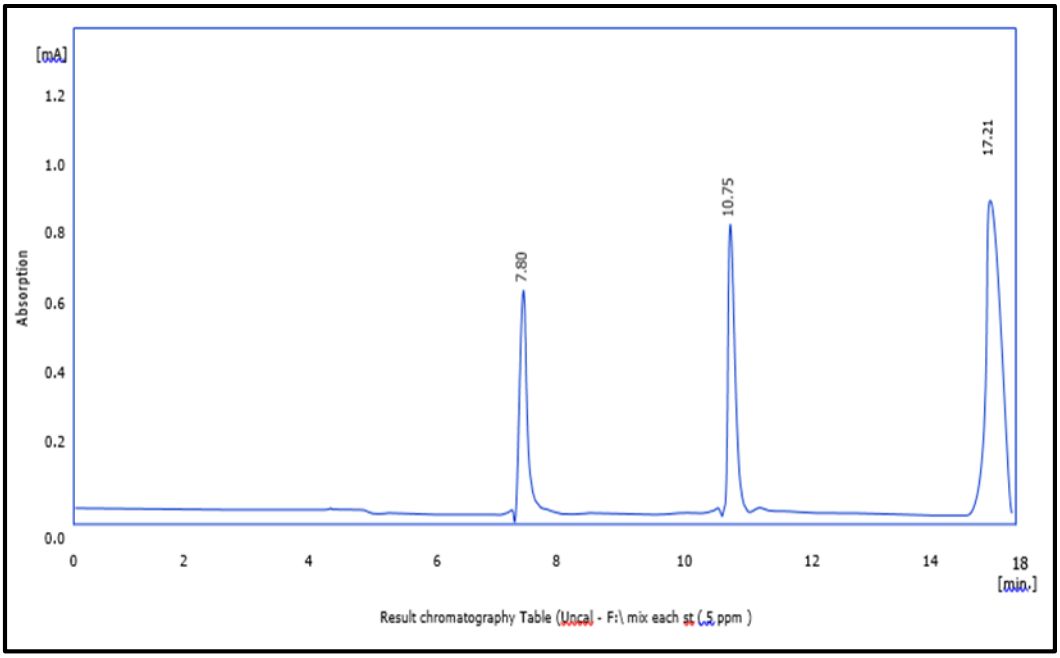

Figure (3) High Performance Liquid Chromatography for extracellular extract of *Streptomyces*.

A study described that thin high performance liquid chromatography are regularly used for analysis and characterization of antimicrobial compounds from producing microorganisms [17].

Conclusions

Actinomycetes were observed in culturing the diluted soil sample (10^{-5}) for 7-10 days on ISP2 agar. The results of our isolation of *Streptomyces actinomycinicus* HPLC analysis of extracellular extract of *Streptomyces* indicated the presence of three active compounds

Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

Bassam Qasim Mohammed: Contributed to the conception and design of the study, conducted some experiments, data rearrangement and drafted the initial manuscript.

Mohsen Hashim Risan: conducted some experiments, collection a part of literature review and conducted some characteristics of the products.

References

1. Abd-Elhalim, B.T., Hemdan, B.A., El-Sayed, S.M. Enhancing durability and sustainable preservation of Egyptian stone monuments using metabolites produced by *Streptomyces exfoliatus*. *Sci Rep* 13, 9458 (2023)

2. Al-Rubaye, T. S., Risan, M. H., Al-Rubaye, D., & Radi, R. O. (2018b). Identification and In vitro antimicrobial activities of Marine Streptomyces spp. Bacteria from Tigris River Sediments in Baghdad City. World Journal of Pharmaceutical and Life Sciences, 4(10), 120-134.
3. Awais, M.; Shah, A.; Hammed, A. and Hasan, F. (2007). Isolation, identification and optimization of bacitracin produced by *Bacillus* sp. *Pak J Bot.* 39:1303-1312.
4. Shepherdson EM, Baglio CR, Elliot MA. Streptomyces behavior and competition in the natural environment. *Curr Opin Microbiol*. 2023;71:102257. doi:10.1016/j.mib.2022.102257
5. Jin, Z., Jiang, F., Wang, L. Arbuscular mycorrhizal fungi and *Streptomyces*: brothers in arms to shape the structure and function of the hyphosphere microbiome in the early stage of interaction. *Microbiome* 12, 83 (2024).
6. Maitland, P. D,. (2010). A Comprehensive Working, Principles and Applications of Thin Layer Chromatography: Are we getting it right. *J Biolog Edu.* 37(1): 6-8.
7. Naine, S. J.; Devi, C. S.; Mohanasrinivasan, V. and Vaishnavi. B. (2015). Antimicrobial, Antioxidant and Cytotoxic Activity of Marine Streptomyces parvulus VITJS11 Crude Extract. *Braz Arch Biol Technol.* 58(2): 198-207.
8. Nonoh, J.; Lwande, W.; Masiga, D.; Herrmann, R.; Presnail, J.; Schepers, E.; Okech, MA.; Bagine, R.; Mungai, P.; Bernard Nyende, A. and Boga H.I. (2010). Isolation and characterization of *Streptomyces* species with antifungal activity from selected national parks in Kenya. *Afri J Microb Res.* 4(9): 856-864.
9. Oskay, M.; Tamer, U.A. andAzeri, C. (2004). Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. *Afr. J. Biotechnol.*, 3: 441-446.
10. Portillo, M. C.; Saiz-Jimenez, C. and Gonzalez, J. M. (2009). Molecular characterization of total and metabolically active bacterial communities of "white colonizations" in the Altamira Cave, *Spain Res Microbiol.* 160: 41-Qasim B, Risan M H. (2017). Antitumor and Antimicrobial Activity of Antibiotic Produced by Streptomyces spp. World Journal of Pharmaceutical Research, 6(4): 116-128.
11. Rajaseka, S. and Elango, R. (2011). Estimate of alkaloid content of Ashwagandle *(Withania Somnifera)* with HPLC Methods. *J Experi science*, 2(5):39-41.
12. Rajeb ES, Risan MH, Hassan ZY. (2021). Effect of purified bioactive compounds for Streptomyces griseus on proliferation lymphocytes in vitro. Indian J Ecol; 48:1858-62.
13. Risan MH, Rusul J, Subhi SA. (2019). Isolation, characterization and antibacterial activity of a rare actinomycete: Saccharopolyspora sp. In Iraq. East Afr Scholars J Biotechnol Genet;1:60-49.
14. Risan M. H., Qasim B., Abdel-jabbar B., & Muhsin, A. H. (2017). Identification Active Compounds of Bacteria *Streptomyces* Using High-Performance Liquid Chromatography, World Journal of Pharmaceutical and Life Sciences, 3(6), 91-97. 44.
15. Cuozzo S, de Moreno de LeBlanc A, LeBlanc JG, Hoffmann N, Tortella GR. Streptomyces genus as a source of probiotics and its potential for its use in health. *Microbiol Res*. 2023;266:127248. doi:10.1016/j.micres.2022.127248
16. Bhandari, S.; Bhattarai, B.R.; Adhikari, A.; Aryal, B.; Shrestha, A.; Aryal, N.; Lamichhane, U.; Thapa, R.; Thapa, B.B.; Yadav, R.P.; et al. Characterization of *Streptomyces* Species and Validation of Antimicrobial Activity of Their Metabolites through Molecular Docking. *Processes* **2022**, *10*, 2149.
17. Zhou, J.; Gu, Y.; Zou, C. and Mo, M. (2007). Phylogenetic diversity of bacteria in an earth-cave in Guizhou province, southwest of China. *J. Microbiol.* 45: 105-112.
18. AL-Yasiri. S., Risan M. H., Urker.O., and Yousif. E. "Identification, characterization and antibiotic susceptibility testing of Pseudomonas aeruginosa isolated from clinical sources." *Iraqi Journal of Bioscience and Biomedical* 1.1 (2024): 27-37
19. Mahmood M. H., and Ahmed A. S "Heavy Metals Removal from Drinking Water by Nanocomposite Tannin Resin- TiO2". *Iraqi Journal of Bioscience and Biomedical*, vol. 1, no. 1, May 2024, pp. 38-47,

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Prevalence of quorum sensing genes in *Pseudomonas aeruginosa* isolated from clinical specimens

Noor Mustafa Ghazi [1*]; ***Hameed M. Jasim*** [2]

[1,2] **Molecular and Medical Biotechnology, College of Biotechnology, Al-Nahrian University, Baghdad, Iraq**
*Corresponding Author
E. mail: noormu071@gmail.com

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Abstract

Pseudomonas aeruginosa, similar to several bacteria, use chemical signals for intercellular communication through the quorum sensing (QS) mechanism. QS enables bacterial groups to detect population density and, in reaction to variations in cell density, to synchronize their behaviors. The current work intended to identify quorum sensing virulence factor genes in *P. aeruginosa* isolated from clinical tissues. A total of 155 clinical samples were obtained from burns, wounds, urine, and ears for isolation of *P. aeruginosa*. Bacterial isolates were identified according to their biochemical reactions, then antibiotic susceptibility and Biofilm formation by isolates of *P. aeruginosa* was investigated, and the genomic DNA was extracted from each bacterial isolate for detection of quorum sensing genes (*lasR, lasI, rhlR, and rhlI*). From different clinical specimens, twenty-five *P. aeruginosa* isolates were identified. these isolates were resistant to most antibiotics, and it was found that only 10(40%) of these isolates were biofilm producers. Biofilm virulence genes *lasR, lasI, rhlR, and rhlI* were detected in all *P. aeruginosa* isolates examined. *lasR, lasI, rhlR, and rhlI* genes were common in *P. aeruginosa* isolates that have high rate of resistance to all antibiotics, and strong ability in biofilm formation.

Keywords: Quorum sensing, *Pseudomonas aeruginosa,* Biofilm virulence genes

Introduction

Pseudomonas aeruginosa is among the most opportunistic microorganisms, bacterial infections obtained in hospitals continue to result in antibiotic resistance, which is a major healthcare issue [1]. Due to the regulation of bacterial activity and the formation of biofilms by quorum sensing (QS), *Pseudomonas* infections are frequently challenging to treat[2]. When a bacterial population reaches a certain concentration, or quorum, a bacterium known as QS is able to sense information from other cells in the population and using autoinducers, which are tiny signalling molecules, for communication purposes with them in order to regulate the synthesis of several virulence factors[3]. *Rhl* and *Las* have been recognized as *P. aeruginosa's* two main quorum sense systems. The autoinducer synthases lasI and *rhlI*, together with the related transcriptional regulators *lasR* and *rhlR*, comprise each system. Moreover, *P. aeruginosa* has the integrated

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QS (IQS) system, a newly discovered fourth system, and pseudomonas quinolone signal (PQS), a third QS system[4]. In order to orchestrate the production of various virulence factors in response to a variety of environmental stimuli at different infection sites, *P. aeruginosa*, which is an important nosocomial pathogen, utilises a complex network of hierarchical quorum-sensing (QS) systems. These QS systems include the *las*, *rhl*, and 2-alkyl-4-quinolone-related QS systems. Biofilm production is crucial to pathogenicity and antibiotic resistance[5]. This study was designed to gather information on the existence, distribution, and occurrence rate of quorum sensing in *P. aeruginosa* clinical isolates regarding their capacity to form biofilms.

Materials and Methods

Clinical Specimens

A total of 155 clinical samples were collected from patients ranging in age from one to sixty years old who were receiving medical care at Al-Imamain Teaching Hospital, Baghdad Teaching Hospital, Al-Karkh General Hospital, and Al-Yarmouk Teaching Hospital throughout the months of September and November in the year 2023. Otitis from the ears, burns, and wounds were among the samples that were collected. Urine from urinary tract infections was also included. After being rapidly cultured on MacConkey agar, the swap samples were next incubated at 37 degrees Celsius for a period of twenty-four hours [6].

Identification of *Pseudomonas aeruginosa*

Identification of *P. aeruginosa* was initially performed according to their morphological and cultural characteristics, and biochemical reactions. Identification of isolates was confirmed by using automated VITEK2 system (BioMérieux, France).

Antibiotic Susceptibility Testing

Using growth-based technologies, the VITEK 2 system is computerized microbiology software. The arrangement of the bacterial suspension followed the guidelines provided by the manufacturers. A suitable number of colonies were taken out of the overnight pure culture and placed in a 12 x 75 mm transparent polystyrene test tube with 3.0 ml of sterile saline. DensiChek turbidity meter was used to set the turbidity to 0.5 McFarland. Subsequently, the VITEK 2 compact system with GN-ID employed the same suspension. Lastly, the specimen suspension tubes and the GN-ID cassette were placed into the VITEK 2 chamber[7].

Biofilm formation and quantification

Utilizing Luria Bertani (LB) broth medium, 96-well microtiter plates (MTP) were used to evaluate the in vitro biofilm growth utilizing the following protocols[8]: Once the identified strains of bacteria were diluted in LB broth at a ratio of 1:100, they were then seeded onto a microtiter plate with an initial turbidity of 0.05 at 600 nm. Finally, after being cultivated in the broth for the night, the microtiter plate was seeded with 200 µl of each well. The mean was calculated after each strain was examined three times in three different studies. Control wells numbered eight. The exhausted wells held sterile LB broth. The plates were then kept in an aerobic environment at 37 °C for a whole day. After the wells were incubated, their contents were decanted. Each was thoroughly rinsed three times with 200 µl of 0.9 percent sterile saline. Once the biofilm layer had adhered to each MTP well, 150 µl of 0.1% crystal violet was stained and allowed to stand at room temperature for a duration of 15 minutes. Following staining, excess dye was removed from the MTP by immersing it in a large container of water using a pipette[9]. The strains may be categorised as non-biofilm producers (0), weak biofilm producers (+ or 1), moderate biofilm producers (++ or 2), and strong biofilm producers (+++ or 3). This classification is based on the OD values that were calculated before. For

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the purpose of this computation, it is essential to keep in mind that the ODc value should not be removed from the average OD value of the strain. The following will make the interpretation of the results easier to understand: OD ≤ODc=no biofilm producer; ODc<OD≤2*ODc=weak biofilm producer; 2*ODc<OD≤4*ODc=moderate biofilm producer; 4*ODc<OD=strong biofilm producer.

DNA extraction

The Wizard Genomic DNA Purification Kit, manufactured by Promega in the United States, was used to extract genomic DNA from every single bacterial isolate. The efficiency of the chromosomal DNA extraction was validated by the process of electrophoresis of the DNA extracts on a 1% agarose gel that had been stained with ethidium bromide. Within the scope of this study, the NanoDrop 2000 spectrophotometer was used to assess the levels of purity and concentration.

Amplification of biofilm genes

The genes *lasI*, *lasR*, *rhlI*, and *rhlR* were shown to be involved in the creation of biofilms in *P. aeruginosa*. These genes were found to contribute to the formation of biofilms. Amplification by polymerase chain reaction (PCR) was performed using the primers that are specified in table (1).

Table (1): Oligonucleotide primers used for amplification of biofilm genes

Gene	Sequences (5' → 3')	T_m (℃)	Product size (bp)	Reference
lasI	F:GTGTTCAAGGAGCGCAAAG R:GAAACGGCTGAGTTCCCAGA	62	240	10
lasR	F: AGATCCTGTTCGGCCTGTTG R:CTGCTTTCGCGTCTGGTAGA	62.5	194	
rhlI	F:GCTACCGGCATCAGGTCTTC R:GTTTGCGGATGGTCGAACTG	63.5	100	
rhlR	F:ACCAGCAGAACATCTCCAGC R:CATTGCAGGATCTCGCGTTC	64	157	

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Primer's preparation: In order to achieve a stock solution with a final concentration of 100 pmol/µl, the lyophilised primers that were indicated in Table 2 were reconstituted in water that was free of nuclease. For the purpose of achieving a working primer concentration of 10 pmol/µl, a working primer solution was created by combining 10 µl of primer stock solution with 90 µl of nuclease-free water.

Optimization of PCR conditions: Amplification of biofilm genes was carried out using thermal cycler (Fisher Scientific, USA). Optimum conditions for amplification of these genes described in Table (2).

Table (2): PCR amplification program for *P. aeruginosa* biofilm genes

Step	Temperature (°C)	Time	No. of cycles
Initial denaturation	95	2 minutes	1
Denaturation	95	30 seconds	35
Annealing	57		
Extension	72		
Final extension	72	5 minutes	1

Results and Discussion

Isolation and identification of *P. aeruginosa*

For isolation of *P. aeruginosa*, clinical samples were cultured on enrichment and differential media. From these samples, a total of 102 bacterial isolates were obtained. Different bacterial colonies were examined according their cultural and microscopic characteristics. Colonies suspected to be *P. aeruginosa* appeared as slightly curved, short to medium size, grouped as single or pairs, non-spore forming, non-motile, non-lactose fermenter, negative for Gram staining. They produce blue-green pyocyanin pigments that gave them the appearance of pale-yellow colonies. These bacterial isolates were further subjected to biochemical identification. Results showed that among the total isolates, only 25 isolates were identified as *P. aeruginosa* , as they were positive for Catalase, Oxidase, Gelatinase, and Hemolysine production tests. While they were negative for Indole and Lactose fermentation tests as indicated in table (3). There was a confirmation of the positive identification of *P. aeruginosa* isolates by the use of the Vitek 2 compact system.

Table (3): Biochemical test results for identification of *P. aeruginosa* isolates

Biochemical test	Result
Catalase	+
Oxidase	+
Indole	-
Hemolysine production	+
Lactose fermintation	-

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Motility test	+
Gelatinase production	+
Grow at 42℃	+
Grow at 4℃	-
Gram staining	-

(+): Positive result; (-): Negative result

The high isolation rate was from burn infections (50%), wound infections (24.5%), urinary tract infections (14.7%), and otitis media (10.8%). Whereas the rest 77 isolates were related to other pathogenic bacteria from different genera (*Klebsiella pneumonia*, *Proteus mirabilis*, *Staphylococcus aureus* and *Escherichia coli*).

The study focused on isolating *Pseudomonas aeruginosa* from clinical samples using enrichment and differential media, resulting in 102 bacterial isolates. Twenty-five of them were identified as *P. aeruginosa* by means of the Vitek 2 compact system, biochemical testing, microscopy, and culture.

Antibiotic Susceptibility of *P. aeruginosa* isolates

The Kirby-Bauer disc diffusion technique was used in order to ascertain the antibiotic susceptibility of the *P. aeruginosa* isolates. Results depicted in figure (1) demonstrated that multidrug resistance was prevailing in these clinical isolates, with 100% of the bacterial isolates exhibiting resistance to Amoxcillin-clavululanate, 96% were resistant to Amoxcillin and Cefazolin, 88% to Tetracycline, 64% to Colistin, 48% to Levofloxacin ,44% to Aztreonam , 40% to Ceftazidim and Cefepime, 36 % to Piperacillin-sulbactum, 28% to Ceftazidime-avibactam,, 24% to Tobramycin and Ciprofloxacin, 20 % to Ceftolozane-tazobactam and Meropenem-vaborbactam 16% to was resistance Doripenem, and 4% to Meropenem. Results also showed that (100%) of the *P. aeruginosa* isolates from burn, wound, UTI, and otitis infections had multi drug resistance to more than three antibiotic groups as shown in figure (1).

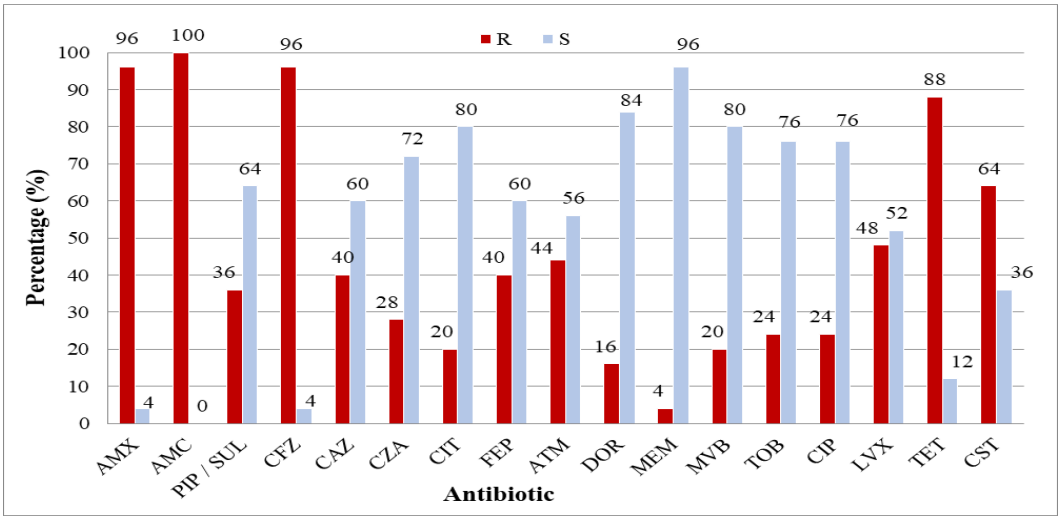

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Figure (1): Antibiogram of *P. aeruginosa* isolates and the susceptibility percentage against different antibiotics. Amx: Amoxcillin; Amc: Amoxcillin-clavululanate; Pip/sulb:Piperacillin sulbactum; CFZ: Cefazolin; CAZ:Ceftazidim; CZA:Ceftazidime-avibactam; C/T:Ceftolozane-tazobactam; FEP:Cefepime; ATM: Aztreonam; DOR: Doripenem; MEM: Meropenem; MVB:Meropenem-vaborbactam; TOB: Tobramycin; CIP: Ciprofloxacin; LVX: Levofloxacin; TET: Tetracycline; CST: Colistin.

The antibiotic susceptibility results and resistance pattern of the current study that showed in Figure (1), were in agreement with Chika *et al*. [11], who found that 100% of *P. aeruginosa* isolates were multi-drug-resistant *P. aeruginosa*. The high rate of multidrug resistance may be caused by haphazard antibiotic use. Additionally, There are many ways that MDR *P. aeruginosa* might develop resistance, including the formation of β-lactamases, enzymes that change aminoglycosides, efflux pumps that are resistant to several drugs, and a decrease in the permeability of the outer membrane. *P. aeruginosa* strains isolated from burn patients admitted to hospitals in Tehran demonstrated a rise in the rate of multiple drug resistance due to the improper use of antibiotics[12]. Additionally, changes in bacterial enzymes and the overuse or improper administration of antibiotics by doctors and their patients may be linked to resistance.

Ability of *P. aeruginosa* isolates in biofilm formation

The microtiter plate method was used in order to evaluate the capacity of *P. aeruginosa* isolates to generate biofilm. Results showed as in figure (2) that all bacterial isolates (100%) were biofilm formants with variable degrees, as there is 21(84%) of the isolates were weak biofilm formats, 3(12%) were moderate biofilm formats, and only 1 isolate (4%) was strong biofilm format.

The study evaluated that all isolates were confirmed to produce biofilm, demonstrating varying strengths as showed in figure 2. Biofilms have remarkable capabilities in terms of both their physical and physiological adaptability to antimicrobial drugs. As a result of their method for improving antimicrobial tolerance, biofilms are able to withstand longer exposure to typical antimicrobial treatments, which allows them to keep their cell viability[13].

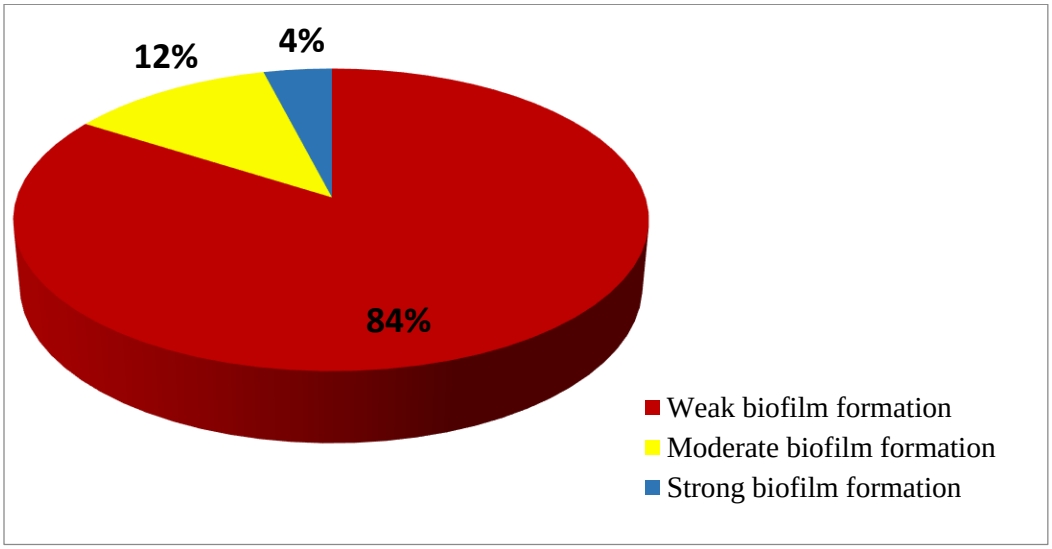

Figure (2): Ability of *P. aeruginosa* isolates in biofilm formation DNA Extraction and Purification

DNA Extraction and Purification

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Twenty-five *P. aeruginosa* samples were used to extract genomic DNA using the Wizard genomic DNA purification kit (Promega, USA). When the extracted DNA was stained with EB dye, it was verified by gel electrophoresis on a 1% agarose gel and seen under UV light[20], as shown in Figure 3.

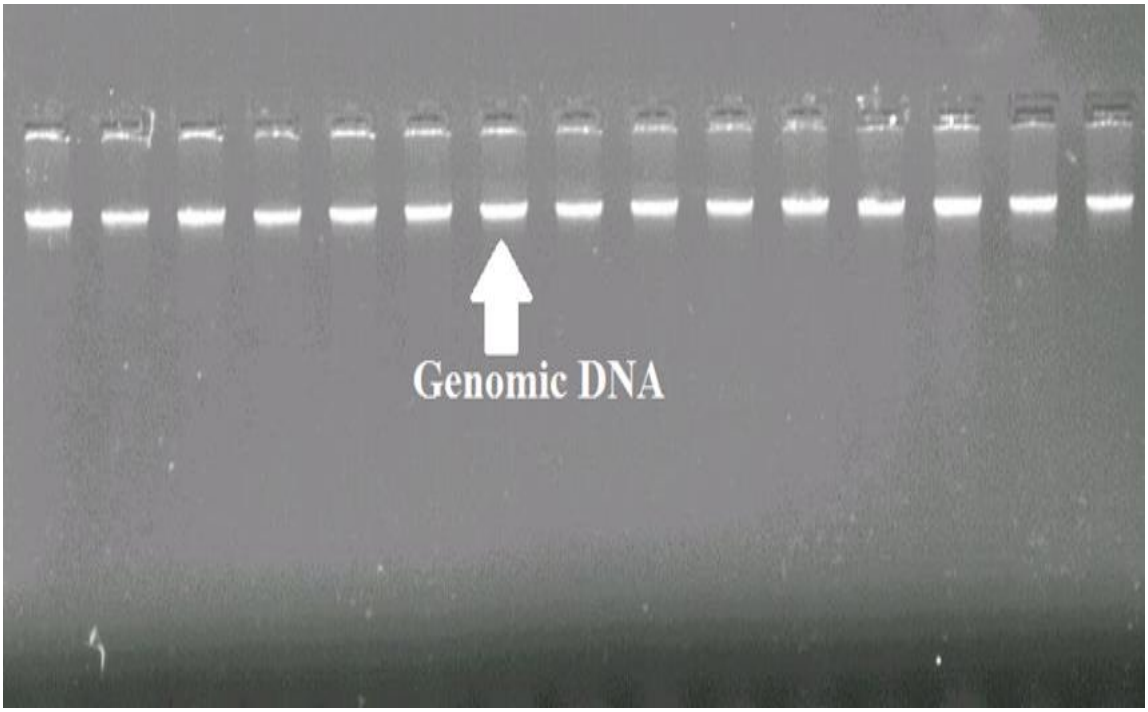

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Figure (3): Electrophoresis of genomic DNA on 1% agarose gel after extraction from *P. aeruginosa* isolates

Molecular Detection of Biofilm Genes

Biofilm genes were detected in *P. aeruginosa* isolates. These genes are quorum sensing genes belong to two types, the first is auto inducers genes (*lasI* and *rhlI*), the second is transcription regulatory genes (*lasR* and *rhlR*) [14] .The virulence genes were amplified with particular primers, and the resultant products were visualised on agarose gel.

Molecular Detection of biofilm auto inducer gene *lasI*

LasI was amplified using certain primers to determine its frequency among the 25 *P. aeruginosa* isolates. The amplified product was then subjected to 1.5% agarose gel analysis. Results illustrated in

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figure(4) showed an amplified product of 240 bp was visualized on agarose gel represents *lasI* gene prevalent in 24(96%) isolates of *P. aeruginosa*.

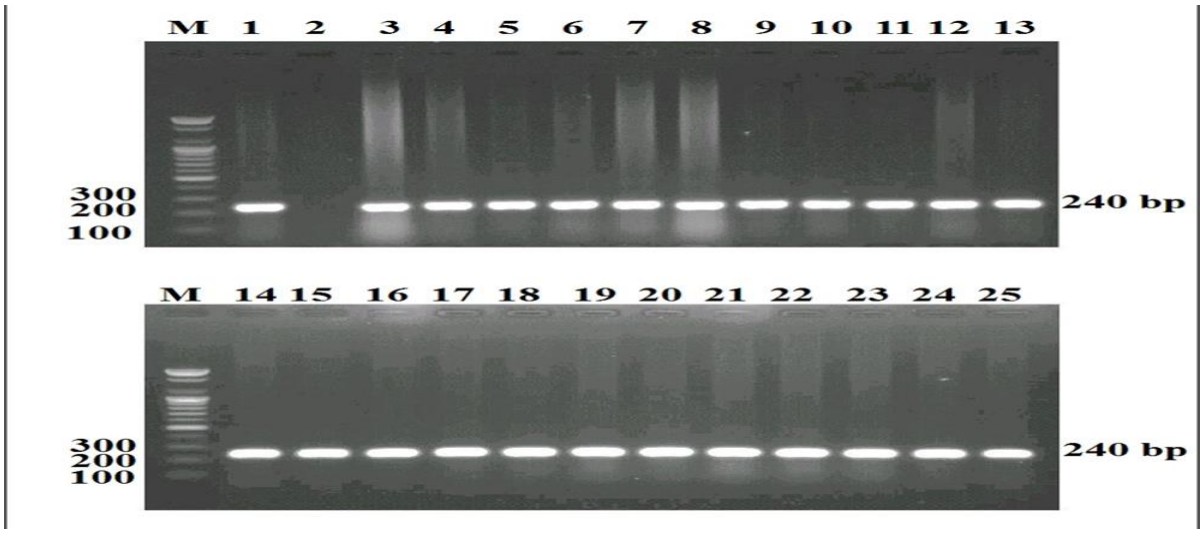

Figure (4): *lasI* gene detection after amplification of genomic DNA of *P. aeruginosa* isolates and electrophoresis on agarose gel (1.5%) for one hour. Lane (M): DNA Ladder marker; Lanes (1-25): *P. aeruginosa* isolates.

The results of the current study regarding biofilm auto inducer gene *lasI* referred that *lasI* gene may be differently distributed in clinical isolates and was compatible with those obtained by Limaa who found that percentage *lasI* occurrence was found to be 97.5% in *P. aeruginosa* isolates. As though Sabharwal *et al*. revealed that *lasI* was detected in 75% of *P. aeruginosa* isolated from urinary tract infections [15].

Molecular Detection of biofilm auto inducer gene *lasR*

As shown in figure **(**5), PCR was employed to amplify the *lasR* gene in 25 *P. aeruginosa* isolates using specific primers. Results of amplification and electrophoresis on 1.5% agarose gel showed an amplified product of 194 bp was visualized on agarose gel represents *lasR* prevalent in all 25 (100%) isolates of *P. aeruginosa***.**

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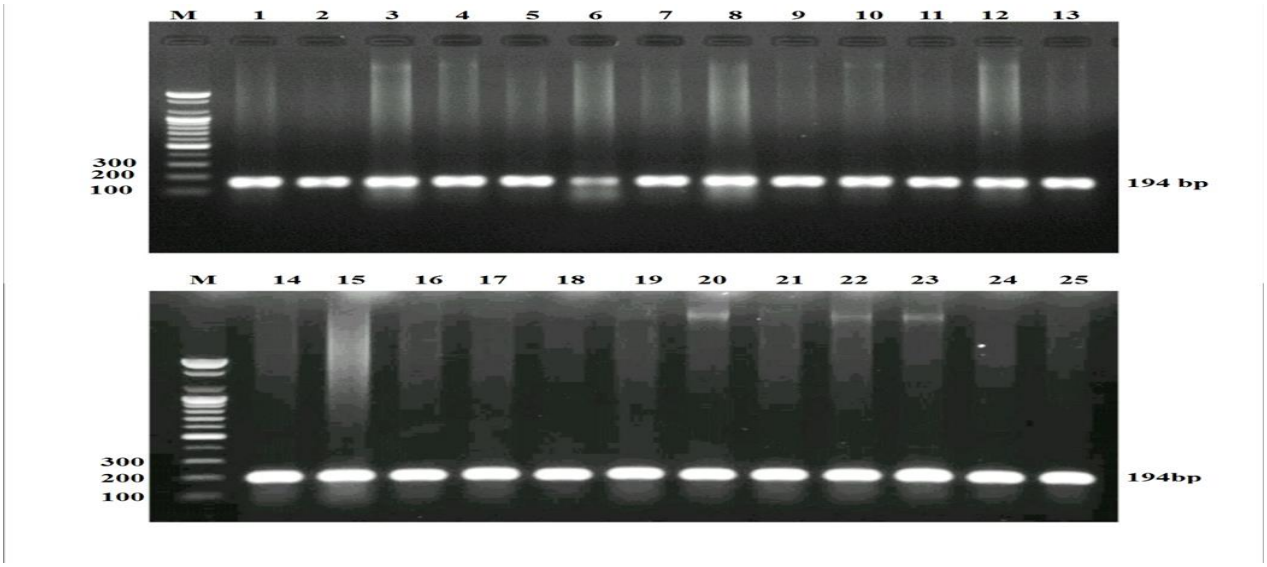

Figure (5): *lasR* gene detection after amplification of genomic DNA of *P. aeruginosa* isolates and electrophoresis on agarose gel (1.5%) for one hour. Lane (M): DNA Ladder marker; Lanes (1-25): *P. aeruginosa* isolates.

The *lasR* gene encodes the transcription factor that activates many target genes, primarily associated with quorum sensing in *P. aeruginosa* and findings are similar to those obtained by Lima[10] who found, *P. aeruginosa* isolates with the *lasR* were all 100% present. In contrast, Hemmati's [16] findings shown that the identification of QS genes was lower than that of the present research (78.3% for the *lasR* gene).

Molecular Detection of biofilm auto inducer gene *RhlI*

The present investigation employed polymerase chain reaction to identify the *rhlR* gene, which encodes rhamnolipid, by amplification with particular primers to ascertain gene frequency among *P. aeruginosa* isolates. Results illustrated in figure (6) showed that *RhlI* gene was also present in all 25 (100%) bacterial isolates.

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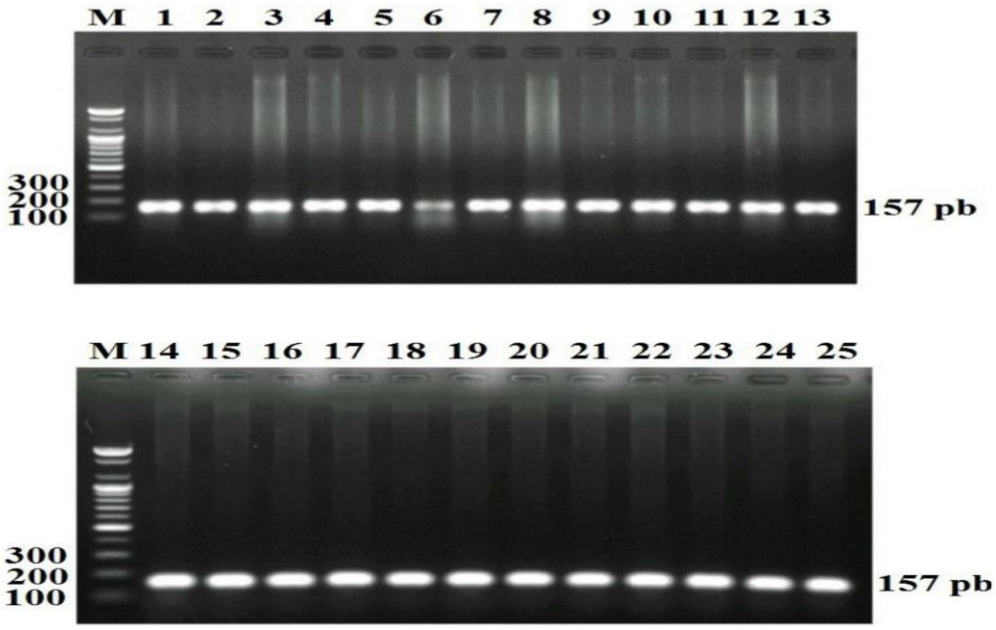
Figure (6): *RhlI* gene detection after amplification of genomic DNA of *P. aeruginosa* isolates and



electrophoresis on agarose gel (1.5%) for one hour. Lane (M): DNA Ladder marker; Lanes (1-25): *P. aeruginosa* isolates.

According to the findings of the present investigation on the biofilm auto inducer gene *RhlI*, in local research carried out by Al-Kilabi [17], 31 isolates of *P. aeruginosa* were tested for the presence of the *RhlI* gene; the results showed that 25 isolates (or 80.6%) had a positive gene frequency. Nevertheless, when the *RhlI* gene was detected molecularly in *P. aeruginosa* isolates, 56 of the isolates (93.33%) tested positive for this virulence gene [18].

Molecular Detection of biofilm auto inducer gene (*RhlR*)

RhlR gene was amplified using specific primers to determine its frequency in MDR and high pathogenicity *P. aeruginosa* isolates. Figure (7) illustrates the amplification and electrophoresis results on a 1% agarose gel, which showed that the *RhlR* gene was present in only 24(96%) isolates of *P. aeruginosa*. the genomic DNA of isolate.

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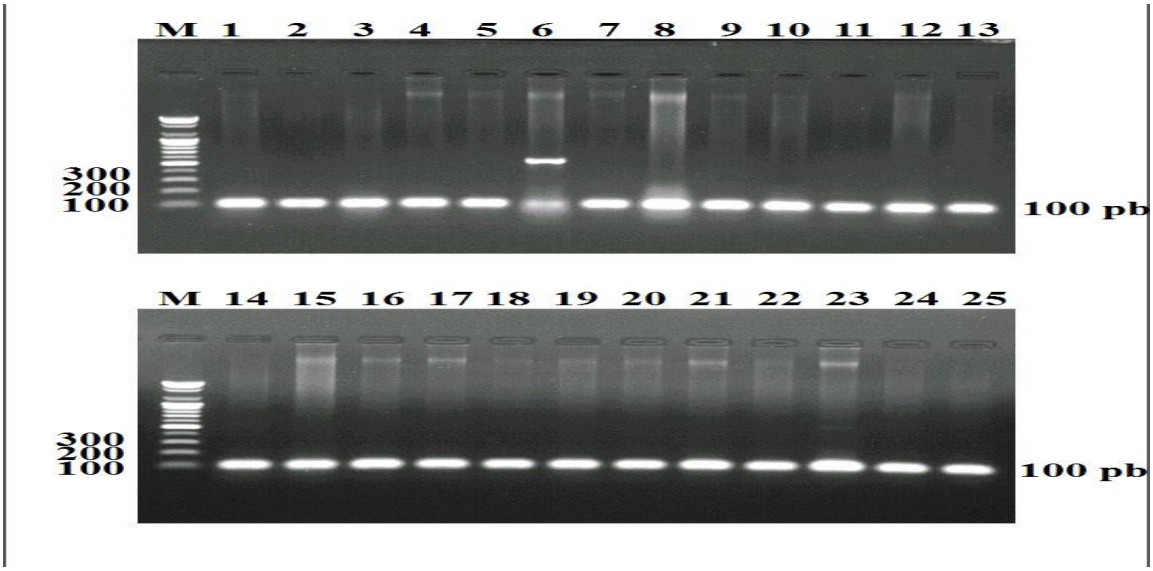

Figure (7): *RhlR* gene detection after amplification of genomic DNA of *P. aeruginosa* isolates and electrophoresis on agarose gel (1.5%) for one hour. Lane (M): DNA Ladder marker; Lanes (1-25): *P. aeruginosa* isolates.

The findings of biofilm auto inducer gene *RhlR* are similar to those obtained by Lima[17] that 100% of *P. aeruginosa* carrying *RhlI* gene. In another study conducted in Egypt, it was found *RhlI* gene was prevalent in 45(90%) of *P. aeruginosa* isolates[19].

Conclusions

lasR, lasI, rhlR, and rhlI genes were common in *P. aeruginosa* isolates that have high rate of resistance to all antibiotics, and strong ability in biofilm formation.

Acknowledgments

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Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.

- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.

- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

[First Author]: participated in the collection of clinical specimens, and did experiments.

[Second Author]: Design of the study, and conceived the manuscript.

Both authors reviewed the manuscript.

References

1. Sumaya, A. Y., Risan, M. H., Ürker, O., & Yousif, E. (2024). Identification, characterization and antibiotic susceptibility testing of Pseudomonas aeruginosa isolated from clinical sources. *Iraqi Journal of Bioscience and Biomedical*, *1*(1), 27-37.
2. Almuttairi, A. A. H., & Abdulla, A. A. (2023). Biofilm Formation and Virulence Factors among Multidrug Resistant *Pseudomonas Aeruginosa* Isolated from Patients in Babylon Province. *Medical Journal of Babylon*, *20*(2), 368-374.
3. Rutherford, S. T., & Bassler, B. L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harbor perspectives in medicine*, *2*(11), a012427.
4. Sarabhai, S., Kaur, A., Capalash, N., & Sharma, P. (2016). Quorum sensing in Pseudomonas aeruginosa: mechanism and regulation of virulence. In *Pseudomonas: Molecular and Applied Biology* (pp. 231-256). Springer, Cham.
5. Kruczek, C., Qaisar, U., Colmer-Hamood, J. A., & Hamood, A. N. (2014). Serum influences the expression of *P. seudomonas* aeruginosa quorum-sensing genes and QS-controlled virulence genes during early and late stages of growth. *MicrobiologyOpen*, *3*(1), 64-79.
6. Roberts, L., & Zeibig, E. A. (2007). Specimen collection and processing. *Textbook of diagnostic microbiology, 4th edition. Saunders Elsevier*, 111-125.
7. Angaali, N., Vemu, L., Padmasri, C., Mamidi, N., & Teja, V. D. (2018). Direct identification and susceptibility testing of Gram-negative bacilli from turbid urine samples using VITEK2. *Journal of laboratory physicians*, *10*(03), 299-303.
8. Stepanović, S., Vuković, D., Hola, V., Bonaventura, G. D., Djukić, S., Ćirković, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis*, *115*(8), 891-899.
9. Stepanović, S., Vuković, D., Hola, V., Bonaventura, G. D., Djukić, S., Ćirković, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis*, *115*(8), 891-899.
10. Lima, J. L. D. C., Alves, L. R., Jacomé, P. R. L. D. A., Bezerra, J. P., Maciel, M. A. V., & Morais, M. M. C. D. (2018). Biofilm production by clinical isolates of *Pseudomonas aeruginosa* and structural changes in LasR protein of isolates non-biofilm-producing. *Brazilian Journal of Infectious Diseases*, *22*(2), 129-136.
11. Chika, E. O., Nneka, A. R., Dorothy, O. N., & Chika, E. (2017). Multi-Drug-Resistant Pseudomonas aeruginosa Isolated from Hospitals in Onitsha, South-Eastern Nigeria. *Int Arch BioMed Clin Res*, *3*(3), 17-21.

12. Ranjbar, R., Owlia, P., Saderi, H., Mansouri, S., Jonaidi-Jafari, N., Izadi, M., ... & Arjomandzadegan, M. (2011). Characterization of *Pseudomonas aeruginosa* strains isolated from burned patients hospitalized in a major burn center in Tehran, Iran. *Acta Med Iran*, *49*(10), 675-9.
13. Cadavid, E., & Echeverri, F. (2019). The search for natural inhibitors of biofilm formation and the activity of the autoinductor C6-AHL in *Klebsiella pneumoniae* ATCC 13884. *Biomolecules*, *9*(2), 49.
14. EC, P. (1999). Quorum sensing in Pseudomonas aeruginosa. *Cell to cell signaling in bacteria*, 147-155.
15. Sabharwal, N., Dhall, S., Chhibber, S., & Harjai, K. (2014). Molecular detection of virulence genes as markers in *Pseudomonas aeruginosa* isolated from urinary tract infections. *International journal of molecular epidemiology and genetics*, *5*(3), 125.
16. Hemmati, J., Nazari, M., Abolhasani, F. S., Ahmadi, A., & Asghari, B. (2024). In vitro investigation of relationship between quorum-sensing system genes, biofilm forming ability, and drug resistance in clinical isolates of *Pseudomonas aeruginosa*. *BMC microbiology*, *24*(1), 99.
17. Al-Kilabi, A. A. K., Al-Turaihi, T. S., & Al Mohammed, H. S. (2020). Molecular detection of Quorum sensing genes in *Pseudomonas aeruginosa* isolated from CSOM patients and their relationship to biofilm ability. *Eurasia J Biosci*, *14*, 4929-4934.
18. Al-Azawi, I. H., & Al-Shabbani, H. A. (2022). Molecular study of quorum sensing and biofilm formation genes in Pseudomonas aeruginosa isolated from UTIs patients. *International Journal of Health Sciences*, *6*(S4), 12121-12134.
19. Elnegery, A. A., Mowafy, W. K., Zahra, T. A., & Abou El-Khier, N. T. (2021). Study of quorum-sensing LasR and RhlR genes and their dependent virulence factors in Pseudomonas aeruginosa isolates from infected burn wounds. *Access Microbiology*, 000211. Elnegery, A. A., Mowafy, W. K., Zahra, T. A., & Abou El-Khier, N. T. (2021). Study of quorum-sensing LasR and RhlR genes and their dependent virulence factors in *Pseudomonas aeruginosa* isolates from infected burn wounds. *Access Microbiology*, 000211.
20. Detection of Tn1545 conferring erythromycin resistance in clinical isolates of Streptococcus pneumonoiae. (2024). *Iraqi Journal of Bioscience and Biomedical*, *1*(1), 11-19.

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Detection of Clindamycin-Resistant *Staphylococcus aureus* Isolated from clinical specimens in Baghdad

***Samah A. Thamer*[1*]; *Dhafar N. Al-Ugaili*[2]**

[1,2] **Department of Molecular and Medical Biotechnology, College of Biotechnology, Al-Nahrian University, Baghdad, Iraq**
*Corresponding Author
E. mail: semah.mbm23@ced.nahrainuniv.edu.iq
https://orcid.org/0000-0002-1679-9262

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Abstract

A total of 150 samples were collected from clinical sources in Baghdad. All samples were subjected to analyses and confirmed by VITEK2 system. As a result, 80 isolates were recognized as *S. aureus*. Disc diffusion method was used to test antibiotic susceptibility profile for the isolates towards 8 antibiotics. The results indicated that 12 isolates were multi-drug resistant (MDR), 11 isolates were extensively drug-resistant (XDR) and 8 were pandrug-resistant (PDR). Twenty-eight (35%) of the isolates were clindamycin resistant and 21 (26%) of these were constitutive clindamycin resistant as they show resistance towards erythromycin too; while 52 (65%) of the isolates were sensitive to both clindamycin and erythromycin and 7 isolates were reported to be erythromycin-sensitive but clindamycin-resistant. It is essential that all healthcare facilities should continuously monitor *S. aureus* to avoid treatment failures associated with induced lincosamide antibiotics resistance.

Keywords: Antibiotics, *Staphylococcus aureus*, VITEK 2 system, wounds, burns.

Introduction

Staphylococcus aureus is a ubiquitous Gram-positive bacterium, which may resident in various parts of body, especially skin and mucous membrane, as a human normal flora. In some cases, this bacterium can cause both community and nosocomial infections, including fatal pneumonia, bacteremia, osteomyelitis, infective endocarditis, mild skin and soft tissue infections [1–3].

Staphylococcus aureus has several virulence factors, including enzymes, proteins, toxins and others. In order to survive in various environmental conditions [4]. This bacterium is rapidly acquires resistance to antimicrobial drugs, making it a major contributor to the spread of diseases in healthcare settings [5].

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When compared to illnesses caused by susceptible strains of bacteria, those caused by antibiotic-resistant bacteria carry a higher risk of death and higher treatment costs [6]. Genomic mutations that change the target DNA-gyrase or decrease the number of proteins in the outer membranes can reduce drug absorption and so impart drug resistance [7,8].

Most antibiotics cannot kill Methicillin-resistant *S. aureus* bacteria, and some strains have even evolved resistance to drugs used as a last resort [9] .Resistance strains have regularly emerged shortly after new antibiotic were introduced to combat this infection. The most notable development is the rise in frequency of *S. aureus* isolates resistant to β-lactams. Therefore, efforts were focused on the use of clindamycin which is a lincosamide antibiotics that belongs to the macrolide-lincosamide-streptogramin B (MLS_B) antibiotic group [10]. The clindamycin was characterized by its pharmacokinetic properties as it inhibits toxin production through its ability to penetrate to the soft tissue, in addition to its availability in oral as well as parenteral formulation.However, the frequent and extensive use of this antibiotic has led to the development of resistance to it [11].

The resistance mechanism of clindamycin depends upon the ribosomal target site modification by the *23SrRNA* methylases that is mediated by one or more *erm* genes (*ermA, ermB, ermC, and ermF*) among which *ermA* and *ermC* are predominant genes [12]. This resistance mechanism could be either constitutive or induced in such a way that when the rRNA methylase is usually produced, the resistance phenotype is constitutive (cMLSB) but when an inducing agent such as erythromycin induced the production of methylase it said to be induced clindamycin resistance (iMLSB) [13]. The aims of this study was to determine the prevalence of constitutive and induced clindamycin resistance among multidrug resistant S. aureus isolates from different clinical samples.

Materials and Methods

Collection of clinical specimens

From November 2023 to January 2024, a total of 150 clinical samples were collected from individuals suffering from a range of infections. These samples included ear, nose, wound, burn swabs, urine, and blood samples. The samples were collected from Mahmoudiya Hospital and Al-Imamain AL-Kadhimain Medical City.

Bacterial isolation and identification

Blood agar and mannitol salt agar (MSA) were used as differential and selective media for cultivation of *Staph. aureus*. the samples were incubated at 37°C for 24 hours. For further identification of *Staphylococcus* to the species level, morphological, biochemical (coagulase, catalase, oxidase) and confirmed using VITEK2.

Antimicrobial susceptibility test

The Kirby-Bauer method, as specified in Clinical and Laboratory Standards Institute (CLSI) guideline [14] was utilized to test susceptibility profile for the isolates against the applied antibiotic using

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Muller Hinton agar as a culture media. The antimicrobial discs listed in (table1) were supplied by Himedia/India.

Table (1): The antimicrobial agents of this study.

Antimicrobial	Symbol	Disk content	Antimicrobial Class
Erythromycin	ERY	15 µg	Macrolides
Azithromycin	AZM	15 µg	Macrolides
Clindamycin	CL	2 µg	Lincosamide
Cefoxitin	CXN	30 µg	Cephamycin (2nd-generation)
Vancomycin	VA	30 µg	Glycopeptide
Gentamicin	GEN	10 µg	Aminoglycoside
Tetracycline	TE	30 µg	Tetracyclines
Ceftazidime	CAZ	30 µg	Cephalosporins (3rd-generation)

Results and Discussion

Based on used examination procedures; 80 samples (53%) were positively identified as *Staph. aureus* as they appear grape-like clusters under microscope forming golden yellow colonies on MSA and their mode of ß-hemolysis on blood agar in addition to their ability to produce catalase and coagulase enzyme while they were unable to produce oxidase enzyme and this was confirmed by the results of the VITEK 2 system. According to the isolation source; 47 (58.75%) the isolates were from skin samples that include epidermal (30%; n=24/80), wounds (20%; n=16/80) and burns (9%; n=7/80) followed urine (n=12; 15%), blood (n=10; 12%), nasal (n= 4; 5%), sputum (n= 3; 4%), ear (n=2; 2%), knee joint (n=2; 3%) as represented in figure (1).

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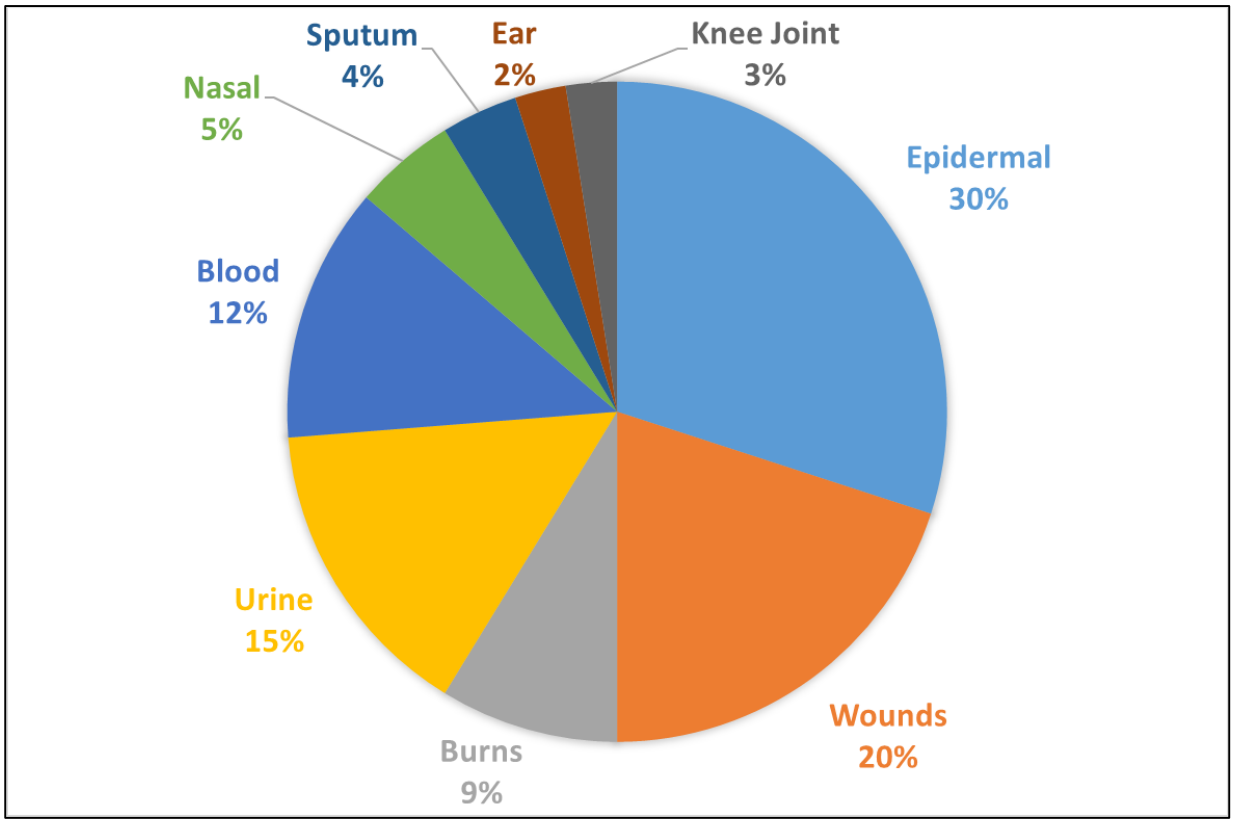

Figure (1): The distribution of *S. aureus* isolates among samples.

From these 80 isolates, resistance to both CL and CXN was (35%, n=28/80), for AZM (26%, n=21/80), for GEN (28%, n=22), for ERY (26%, n=21/80), for TE (50%; n=40/80), for CAZ (24%; n=19/80) and the lowest resistant ratio was recorded for VA (14% n=11/80), as represented in figure (2).

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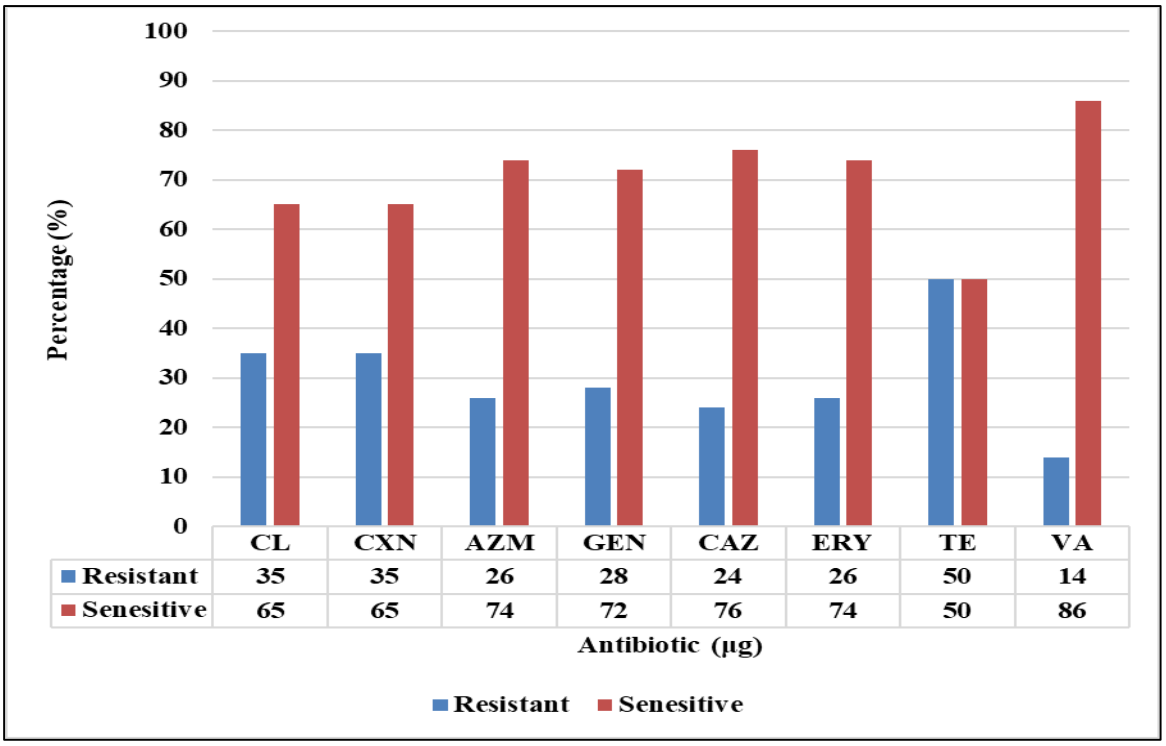

Figure (2): Antibiotic resistance patterns of *S. aureus* isolates.

ERY=Erythromycin
CL=Clindamycin
CXN=Cefoxitin
AZM=Azithromycin
GEN=Gentamicin
CAZ=Ceftazidime
TE=Tetracycline
VA=Vancomycin

The antibiotic resistance categories were determined as MDR, XDR and PDR. Among 80 isolates, n=12/80 (15%) were MDR, n=11/80 (13.75%) were XDR and n=8/80 (10%) were PDR, while the rest of isolates (62.5%); n=50 being out these categories.

Table (2): The percentages of distribution for isolates among the categories of antibiotic resistance.

Category	Isolates	
	Number	Percentage (%)
MDR	12	15
XDR	11	13.75
PDR	8	10
Non-MDR, -XDR and -PDR	50	62.5
Total	80	100

* MDR: non-susceptible to ≥1 agent in ≥3 antimicrobial categories, XDR: non-susceptible to ≥1 agent in all but ≤2 categories and PDR: non-susceptible to all antimicrobial agents listed [15].

For phenotypic detection of clindamycin and erythromycin-resistant *S. aureus*, the results of this study are represented in table (3). Among these, 28 (35%) of the isolates were clindamycin resistant and 21 (26%) of these were recorded as constitutive clindamycin resistance as they show resistance towards erythromycin too; while 52 (65%) of the isolates were sensitive to both clindamycin and erythromycin and

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7 isolates were reported to be erythromycin sensitive but clindamycin resistant. None of the examined isolates were resistant to ERY and sensitive to CL together, whereas they not considered asinduced macrolide-lincosamide- streptogramin B ($iMLS_B$).

Table (3): The distribution for clindamycin-resistant isolates among the categories of antibiotic resistance.

Susceptibility pattern (phenotype)	Total	
	No.	%
ERY=R; CL=R ($cMLS_B$)	21	26
ERY=S; CL=S	52	65
ERY= R; CL=S ($iMLS_B$: D test positive)	0	0
ERY=S; CL=R	7	8.75
Total	80	100

R=resistant
S=sensitive
ERY=Erythromycin
CL=Clindamycin
iMLSB= induced macrolide-lincosamide- streptogramin B
cMLSB= Constitutive macrolide-lincosamide- streptogramin B

In this study, 53% of *S. aureus* isolates were obtained form 150 clinical samples with a ratio compared with that reported by Shakir *et al.* [16] as they used different sources of the isolate *S. aureus* with a ratio of 25.5% and that obtained by Onanuga *et al.* [17] who isolated only 20.8% of *S. aureus* from various sources, while the results of this study were closely related with both studies reported by Uwaezuoke *et al.* [18] and Yassein *et al.* [19], whereas they obtained 36% and 48% of *S. aureus* isolates from different samples, respectively.

This study reported that 12, 15 and 20% of isolates of *S. aureus* from blood, urine and wound samples, which were different from findings of the study reported by Nsofor *et al.* [20], who obtained 6.7, 25.4 and 29.4% of isolates from blood, urine and wound samples, respectively.

One possible explanation for this patrtern of prevalence. Because of its adaptability to various bacterial environments, *Staphylococcus aureus* is the most common pathogele explanation for this pattern of n found in clinical specimens, and this fact is supported by the high prevalence of the organism.

Among the different clinical specimens studied, the one with the highest prevalence of *Staphylococcus aureus* was found in epidermal swab samples (30%), followed by wound swab samples (20%). This finding is in line with previous reports from Obiazi *et al.* [21] and Nwoire *et al.* [22] which also found that wound swabs contained 48% and 42.53% of *S. aureus* isolates, respectively. While Chigbu *et al.* [23] found the greatest occurrence rate in urine specimens (76.4%), current data show the opposite.

Inadequate personal hygiene and the wounds' exposure may have increased their susceptibility to contamination and infection, leading to the high prevalence of *S. aureus* in wound swabs found in this investigation. Another possible explanation for the high prevalence of *S. aureus* colonisation in this research is that some residents of the region self-medicate their wounds or seek out the services of unlicensed or poorly educated quacks before seeking professional medical help.

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The high resistance of *Staph. aureus* to a wide range of routinely used antibiotics results in a subsequent treatment failure and progress of uncomplicated infection to a major problem in the healthcare and community systems. Eight types of antibiotics belong to seven different classes were applied in this study, including CL, CXN, AZM, GEN, VA, TE, CAZ and ERY. According to the results, the tested isolates showed different degrees of resistance to the applied antibiotics such that 12 isolates were recorded as multi-drug resistant (MDR); 11 isolates were extensively drug-resistant (XDR) and 8 were pandrug-resistant (PDR). Among these, 28 (35%) of the isolates were recorded as resistant to CLI and among them 21 (26%) were also resistant to ERY . so, they are considered to have constitutive resistance as they show resistance to both clindamycin and erythromycin. while 52 (65%) of the isolates were sensitive to both clindamycin and erythromycin. furthermore, 7 isolates were reported to be erythromycin sensitive but clindamycin resistant, an indication for expected genetic exchange at the sequence of the gene coding for the erythromycin ribosomal methylase and this result was closely related to that obtained by Moroi and his colleagues in 2019 in Korea [24].

The same proportion of the examined isolates (26%) was found to be lower than findings of Gurung *et al.*[25], who found that (65%) of these isolates were resistant to AZM.

This study found that 26% of isolates were resistant to ERY, which was lower than the findings of Mokta *et al.* [26] who showed that 39% of isolates in their specimens were resistant to ERY. In studies by Jarajreh et al., [27], Thapa *et al.* [28] and Gurung *et al.* [25], 78, 47 and 46.2% of isolates were resistant to ERY, respectively. Another study reported that resistance rates to ERY were (58%) [29], which was higher than the findings of this study. Also, Kishk *et al.* [13], indicated that (54%) of their isolates showed resistance to ERY.

In study conducted by Gurung *et al.* [25], 75% of isolates were resistant to FOX, which is higher than that we recorded. According to this study, 35% of isolates were resistant to CLI, which was higher than finding of study reported by Gurung *et al.* [25], who found that 25% of isolates were resistant to this antibiotic. This variability against antibiotics among different years may due to the variability in the number of isolates and mechanisms of antimicrobial resistance in bacteria are due to several mechanisms included degradations of antibacterial drugs by enzyme, alterations of bacterial proteins that are antimicrobials targets, and alteration in permeability of membranes to antibiotics. Antibiotic resistance can be by plasmids mediated and transposons [30]. So, the resistance to antibiotic is considered an international problem that is associated with serious infections that are difficult or cannot be treated. Bacteria that important in people infection have increasing resistance that's include *S. aureus* and *Escherichia coli* that considered very common in this field [1]. Suffering, increased complications and higher death rates are seen with people that infected in resistant bacteria. Wherever antibiotics are used, antibiotic resistance is developed in both medicine and community.

Poor hygiene, poor water sanitation and poor infection control supply good environments for spreading the resistant bacteria. Most the antibiotic utilization in the world involves in food animals. This utilization result in the growing of resistant bacteria that transmit to people by water and food chain [31].

One of the most popular drugs treating Staphylococcal infections, especially those of the skin and soft tissues, is clindamycin [32]. Isolates with an inducible MLSB (iMLSB) phenotype must be distinguished from those with an MS phenotype to avoid treatment failures caused by clindamycin. According to the results of this study, majority of isolates were $cMLS_B$, which disagreed with Tiwari *et al.* [33], who indicated that 64% of isolates were $cMLS_B$ and 38 of isolates were $iMLS_B$. Erythromycin resistance was detected in 61.4% of the isolates in investigation of Gupta *et al.* [34], which is higher than that found in the results of this study

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(26%). They reported that a total of 47.20 percent of the erythromycin-resistant *Staphylococcus aureus* isolates had the MS phenotype, 31.67% had cMLSB resistance, and 21.1% had iMLSB resistance.

The highest frequency of the iMLSB phenotype was recorded by Steward et al. [35] at 16.4%, with cMLSB at 12.5% and MS at 7.8%. The results of this study disagree with both Regha *et al*. [36] and Deotale *et al*. [37] who found that iMLSB was the most common phenotype, with cMLSB coming in second and MS phenotypic third. In contrast, Dubey et al. [38] found that iMLSB was the highest, followed by MS phenotypic and cMLSB, demonstrating that researchers found varying rates in their investigations. In addition, Prabhu *et al*. [39] 9.47 and 10.52% of isolates were $cMSL_B$ and $iMSL_B$, respectively, which was lower than that found in this study.

Generally, reasons for the discrepancy in results of different studies, in addition to results of this study, include differences in antibiotic exposure between populations or regions, which in turn cause different patterns of resistance. However, resistance rates may be influenced by the kinds and frequency of antibiotics administered in a certain community or healthcare system. The development of resistance to antibiotics might be accelerated by their misuse or excessive usage [40,41]. The reliability of resistance rate estimations is affected by the sample population's size and representativeness. Adaptation by microbes, changes in antibiotic prescription practices, and new infection control methods may all cause resistance patterns to develop over time [42,43].

Conclusions

Vancomycin (VA.) not recommended as effective antibiotic against *S. aureus.* There is a correlation between regional differences in infection patterns and medication use and the prevalence of MLS_B resistance. Thus, in order to prevent treatment failures, it is imperative that all healthcare facilities continuously monitor *S. aureus* for MLS_B resistance by testing erythromycin and clindamycin-resistant isolates with the D-test Inducible clindamycin resistance *in vitro* is another important concept for physicians to be aware of. According to the results obtained, possible medication to explore for treatment in such circumstances include vancomycin and Cefoxitin.

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Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

[Samah Anwer Thamer]: Played a critical role in the statistical analysis of the data and interpretation of the results.

[Dhafar N. Al-Ugaili2]: Played a critical role in supervising the research, providing guidance, and designing the study **.**

References

22. Guo, Y., Song, G., Sun, M., Wang, J., and Wang, Y. (2020). Prevalence and therapies of antibiotic-resistance in *Staphylococcus aureus*. *Frontiers in Cellular and Infection Microbiology*, *10*, 107.

23. Abd, F. B., Al-Ugaili, D. N., and Risan, M. H. (2022). Biofilm Formation and Molecular Analysis of Icaabcd Genes Among *Staphylococcus Aureus* Strains Isolated from Different Clinical Sources. HIV Nursing, 22(2), 369–372.

24. Najim, R. S., Al-ugaili, D. N., and Risan, M. H. (2022). Biofilm forming ability in multidrug-resistant Staphylococcus aureus strains isolated from local clinical sources. Journal of Pharmaceutical Negative Results, 13(4), 418–422.

25. Cheung, G. Y. C., Bae, J. S., and Otto, M. (2021). Pathogenicity and virulence of *Staphylococcus aureus*. *Virulence*, *12*(1), 547–569.

26. Fair, R. J., and Tor, Y. (2014). Antibiotics and bacterial resistance in the 21st century. *Perspectives in Medicinal Chemistry*, *6*, PMC-S14459.

27. Moloney, M. G. (2016). Natural products as a source for novel antibiotics. *Trends in Pharmacological Sciences*, *37*(8), 689–701.

28. Yang, J.-J., Cheng, A., Tai, H.-M., Chang, L.-W., Hsu, M.-C., and Sheng, W.-H. (2020). Selected mutations by nemonoxacin and fluoroquinolone exposure among relevant gram-positive bacterial strains in Taiwan. *Microbial Drug Resistance*, *26*(2), 110–117.

29. Kime, L., Randall, C. P., Banda, F. I., Coll, F., Wright, J., Richardson, J., Empel, J., Parkhill, J., and O'Neill, A. J. (2019). Transient silencing of antibiotic resistance by mutation represents a significant potential source of unanticipated therapeutic failure. *MBio*, *10*(5), 10–1128.

30. Welte, T., Kantecki, M., Stone, G. G., and Hammond, J. (2019). Ceftaroline fosamil as a potential treatment option for *Staphylococcus aureus* community-acquired pneumonia in adults. *International Journal of Antimicrobial Agents*, *54*(4), 410–422.

31. Andreadis, T. (2020). Characterisation of phenotypic and genotypic antibiotic resistance with special consideration of macrolide, lincosamide and streptogramin B (MLS) antibiotics in methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from European hares (Lepus europaeus) and companion animals originating from the German North Frisian Island Pellworm (European hares) as well as Austria (companion animals, European hares).

32. Anandabaskar, N. (2021). Protein synthesis inhibitors. Introduction to Basics of Pharmacology and Toxicology: Volume 2: Essentials of Systemic Pharmacology: From Principles to Practice, 835–868.

33. Abu Lila, A. S., Alharby, T. N., Alanazi, J., Alanazi, M., Abdallah, M. H., Rizvi, S. M. D., Moin, A., Khafagy, E.-S., Tabrez, S., and Al Balushi, A. A. (2023). Clinical resistant strains of Enterococci and their correlation to reduced susceptibility to biocides: phenotypic and genotypic analysis of macrolides, lincosamides, and streptogramins. *Antibiotics*, *12*(3), 461.

34. Kishk, R. M., Anani, M. M., Nemr, N. A., Soliman, N. M., and Fouad, M. M. (2020). Inducible clindamycin resistance in clinical isolates of *Staphylococcus aureus* in Suez Canal University Hospital, Ismailia, Egypt. *The Journal of Infection in Developing Countries*, *14*(11), 1281–1287.

35. Sahu, C., Jain, V., Mishra, P., and Prasad, K. N. (2018). Clinical and laboratory standards institute versus European committee for antimicrobial susceptibility testing guidelines for interpretation of carbapenem antimicrobial susceptibility results for *Escherichia coli* in urinary tract infection (UTI). *Journal of Laboratory Physicians*, *10*(03), 289–293.

36. Magiorakos, A.-P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., and Olsson-Liljequist, B. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, *18*(3), 268–281.

37. Kandel, S. N., Adhikari, N., Dhungel, B., Shrestha, U. T., Angbuhang, K. B., Karki, G., Adhikari, B., Banjara, M. R., Rijal, K. R., and Ghimire, P. (2020). Characteristics of *Staphylococcus aureus* isolated from clinical specimens in a tertiary care hospital, Kathmandu, Nepal. *Microbiology Insights*, *13*, 1178636120972695.

38. Onanuga, A., and Awhowho, G. O. (2012). Antimicrobial resistance of Staphylococcus aureus strains from patients with urinary tract infections in Yenagoa, Nigeria. *Journal of Pharmacy and Bioallied Sciences*, *4*(3), 226–230.

39. Uwaezuoke, J. C., and Aririatu, L. E. (2004). A survey of antibiotic resistant *Staphylococcus aureus* strains from clinical sources in Owerri. *Journal of Applied Sciences and Environmental Management*, *8*(1), 67–69.

40. Yassin, N. A., Mohammed, H. H., and Ahmad, A. M. (2013). Antibiograming profiles of *Staphylococcus aureus* isolated from various clinical specimens in Duhok City, Iraq.

41. Nsofor, C. A., Nwokenkwo, V. N., and Ohale, C. U. (2016). Prevalence and antibiotic susceptibility pattern of *Staphylococcus aureus* isolated from various clinical specimens in south-East Nigeria. *MOJ Cell Sci Rep*, *3*(2), 1–5.

42. Obiazi, H. A. K., Nmorsi, O. P. G., Ekundayo, A. O., and Ukwandu, N. C. D. (2007). Prevalence and antibiotic susceptibility pattern of *Staphylococcus aureus* from clinical isolates grown at 37 and 44oC from Irrua, Nigeria. *Afr J Microbiol Res*, *1*(5), 57–60.

43. Nwoire, A., Madubuko, E., Eze, U., Oti-Wilberforce, R., Azi, S., Ibiam, G., Egwu, I. H., Okereke, E. C., and Obi, I. A. (2013). Incidence of *Staphylococcus aureus* in clinical specimens in Federal Teaching Hospital, Abakaliki, Ebonyi State. *Merit Research Journal of Medicine and Medical Sciences*, *1*(3), 43–46.

44. Chigbu, C. O., and Ezeronye, O. U. (2003). Antibiotic resistant *Staphylococcus aureus* in Abia state of Nigeria. *African Journal of Biotechnology*, *2*(10), 374–378.

45. Moroi, H., Kimura, K., Ido, A., Banno, H., Jin, W., Wachino, J., Yamada, K., Kikkawa, F., Park, Y.-J., and Arakawa, Y. (2019). Erythromycin-susceptible but clindamycin-resistant phenotype of clinical ermB-PCR-positive Group B. streptococci isolates with IS1216E-inserted ermB. *Japanese Journal of Infectious Diseases*, *72*(6), 420–422.

46. Gurung, R. R., Maharjan, P., and Chhetri, G. G. (2020). Antibiotic resistance pattern of Staphylococcus aureus with reference to MRSA isolates from pediatric patients. *Future Science OA*, *6*(4), FSO464.

47. Mokta, K. K., Verma, S., Chauhan, D., Ganju, S. A., Singh, D., Kanga, A., Kumari, A., and Mehta, V. (2015). Inducible clindamycin resistance among clinical isolates of *Staphylococcus aureus* from Sub Himalayan Region of India. Journal of Clinical and Diagnostic Research: JCDR, 9(8), DC20.

48. Jarajreh, D., Aqel, A., Alzoubi, H., and Al-Zereini, W. (2017). Prevalence of inducible clindamycin resistance in methicillin-resistant *Staphylococcus aureus*: the first study in Jordan. *The Journal of Infection in Developing Countries*, *11*(04), 350–354.

49. Thapa, D., Pyakurel, S., Thapa, S., Lamsal, S., Chaudhari, M., Adhikari, N., and Shrestha, D. (2021). Staphylococcus aureus with inducible clindamycin resistance and methicillin resistance in a tertiary hospital in Nepal. *Tropical Medicine and Health*, *49*, 1–7.

50. Timsina, R., Shrestha, U., Singh, A., and Timalsina, B. (2020). Inducible clindamycin resistance and erm genes in *Staphylococcus aureus* in school children in Kathmandu, Nepal. *Future Science OA*, *7*(1), FSO361.

51. Vrancianu, C. O., Popa, L. I., Bleotu, C., and Chifiriuc, M. C. (2020). Targeting plasmids to limit acquisition and transmission of antimicrobial resistance. Frontiers in Microbiology, *11*, 492455.

52. Collignon, P., Athukorala, P., Senanayake, S., and Khan, F. (2015). Antimicrobial resistance: the major contribution of poor governance and corruption to this growing problem. *PloS One*, *10*(3), e0116746.

53. Heath, D. M., Boyer, B. J., Ghali, A. N., Momtaz, D. A., Nagel, S. C., and Brady, C. I. (2022). Use of clindamycin for necrotizing soft tissue infection decreases amputation rate. *Journal of Orthopaedic Trauma*, *36*(7), 327–331.

54. Tiwari, S., Rani, E., and Kumar, A. (2020). Inducible and constitutive clindamycin resistance in Staphylococcus aureus, isolated from clinical samples. *IP International Journal of Medical Microbiology and Tropical Diseases*, *6*(3), 157–160.

55. Gupta, D. K., Pandey, A., Thakuria, B., Chauhan, K., and Jindal, S. (2019). Occurrence of inducible clindamycin resistance in clinical isolates of *Staphylococcus aureus* in a tertiary care hospital. *Int J Health Sci Res*, *9*(8), 71–77.

56. Steward, C. D., Raney, P. M., Morrell, A. K., Williams, P. P., McDougal, L. K., Jevitt, L., McGowan Jr, J. E., and Tenover, F. C. (2005). Testing for induction of clindamycin resistance in erythromycin-resistant isolates of Staphylococcus aureus. *Journal of Clinical Microbiology*, *43*(4), 1716–1721.

57. Regha, I. R., Harichandran, D., and Sulekha, B. (2016). Inducible clindamycin resistance among clinical isolates of *Staphylococcus aureus* in a tertiary care centre, Kerala, India. *Int J Curr Microbiol App Sci*, *5*, 929–934.

58. Deotale, V., Mendiratta, D. K., Raut, U., and Narang, P. (2010). Inducible clindamycin resistance in *Staphylococcus aureus* isolated from clinical samples. *Indian Journal of Medical Microbiology*, *28*(2), 124–126.

59. Dubey, D., Rath, S., Sahu, M. C., Rout, S., Debata, N. K., and Padhy, R. N. (2013). A report on infection dynamics of inducible clindamycin resistance of *Staphylococcus aureus* isolated from a teaching hospital in India. *Asian Pacific Journal of Tropical Biomedicine*, *3*(2), 148–153.

60. Prabhu, K., Rao, S., and Rao, V. (2011). Inducible clindamycin resistance in *Staphylococcus aureus* isolated from clinical samples. *Journal of Laboratory Physicians*, *3*(01), 25–27.

61. Zhuang, M., Achmon, Y., Cao, Y., Liang, X., Chen, L., Wang, H., Siame, B. A., and Leung, K. Y. (2021). Distribution of antibiotic resistance genes in the environment. *Environmental Pollution*, *285*, 117402.

62. Larsson, D. G., and Flach, C.-F. (2022). Antibiotic resistance in the environment. *Nature Reviews Microbiology*, *20*(5), 257–269.

63. Li, W., and Zhang, G. (2022). Detection and various environmental factors of antibiotic resistance gene horizontal transfer. *Environmental Research*, *212*, 113267.

64. Andersson, D. I., Balaban, N. Q., Baquero, F., Courvalin, P., Glaser, P., Gophna, U., Kishony, R., Molin, S., and Tønjum, T. (2020). Antibiotic resistance: turning evolutionary principles into clinical reality. *FEMS Microbiology Reviews*, *44*(2), 171–188.

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Estimation the Total Flavonoid, Reductive Ability, Free Radical Scavenging Potentials and Reactive Oxygen Species Reduction of *Ginkgo biloba* Ethanolic Extract

Zeina Tariq Salih [1*]

[1] **Molecular and Medical Biotechnology, College of Biotechnology, Al-Nahrian University, Baghdad, Iraq**

*Corresponding Author
E. mail: zeinatariqalfalahi@gmail.com

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Abstract

Oxidative stress is a pathological condition characterized by an imbalance between the production of reactive oxygen species and the body's antioxidant defenses. This imbalance can lead to cellular damage and has been implicated in various disease states. *Ginkgo biloba*, a widely studied medicinal plant, is known to possess potent antioxidant properties due to its phytochemical composition. This study aimed to comprehensively evaluate the antioxidant activity and protective effects of *G. biloba* ethanolic extract (GB-EE). The total flavonoid content of the extract was determined, and its free radical scavenging ability and reducing power were assessed. GB-EE exhibited a concentration-dependent DPPH radical scavenging activity, with an IC_{50} of 19.45 µg/mL, comparable to the positive control, ascorbic acid (26.35 µg/mL). Additionally, the extract demonstrated moderate reduction ability, as evidenced by its ferric-reducing antioxidant power. Furthermore, *in vivo* studies on mice revealed that GB-EE treatment protected against hydrogen peroxide-induced oxidative stress, significantly reducing malondialdehyde levels and restoring glutathione concentrations in the kidney and liver tissues. These findings suggest that GB-EE possesses potent antioxidant properties and can exert a protective effect against oxidative damage, highlighting its potential therapeutic applications.

Keywords: *Ginkgo biloba*, Antioxidants, DPPH assay, FRAP assay, Oxidative Stress.

Introduction

For centuries, medicinal plants have been an essential resource and remain a mainstay in traditional medicine for treating local ailments of the indigenous people [1]. The World Health Organization (WHO) estimates that 88% of member countries use herbal plants, acupuncture, yoga, and local remedies in traditional medicine to treat some local diseases [2]. Medicinal plants represent major resources of natural products that have the ability to fight many serious diseases [3]. *Ginkgo biloba* is the oldest living tree with a long history of use as a therapeutic plant in traditional Chinese medicine [4]. Several studies showed that leaf

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extracts of *G. biloba* have significant contents of phytochemicals products like flavonoids (24%), terpenoids (6%), and natural antioxidants ginkgolic acids (less than 5 ppm) [5].

Flavonoids are secondary metabolites in plants and can be divided into flavones, flavonols, dihydroflavonols, dihydroflavones, flavanones and isoflavones [6]. More than 5000 flavonoids have been found in edible plants. Dietary flavonoids possess multiple bioactivities, including antioxidative, anti-obesity and antiadipogenic effects. However, flavonoids as natural antioxidants have the ability for reducing (ROS) in human bodies [7].

Reactive oxygen species (ROS) are highly reactive due to the presence of unpaired electrons [8]. In case of oxidative stress, ROS that occurred during aerobic metabolic activities such as superoxide, hydroxyl, nitric acid, hypochloric acid, proxynitrite and peroxyl radicals play an essential role in the pathogenesis of various serious diseases [9]. Oxidative damage by free radicals has been considered as a mechanism involved in the pathogenesis of many chronic diseases such as atherogenesis and other heart diseases [10], in cancer [11], neural disorders [12], and it also play an adverse effect on the female reproductive functions [13]. ROS significantly impacts reproductive function by inducing oxidative stress, damaging cellular components like DNA, proteins, and lipids [14]. Therefore, reducing the rates of ROS in the bodies to the lowest levels became extremely required.

Since dietary plants contain most antioxidant compounds that are consumed by human bodies, it is essential to find methods that can measure the total flavonoids of plant extracts directly [15]. In the current study, total flavonoids and antioxidant activities were evaluated in *G. biloba* ethanolic extract through DPPH Radical - Scavenging Activity (1,1-diphenyl-2-picrylhydrazyl) and Ferric-reducing antioxidant power (FRAP) assays and examine its alleviating effect *in vivo*.

Materials and Methods

Plant Collection and Identification

Dried *G. biloba* leaves used in this study were obtained from local markets of Baghdad-Iraq which previously identified by National Herbarium of Iraq. The plant's leaves were thoroughly washed in tap water to remove impurities, dried and grinded into fine powder using electrical grinder.

Preparation of *G. biloba* Ethanolic Extract (GB-EE)

This method was carried out according to [16]. The plant ethanolic extract was prepared by adding 50 g of leaves powder in 250 mL of ethanol (99%). Then the mixture was incubated in shaker-incubator at 37°C for 24 hours. At first, the mixture was filtered by multiple layers of gauze followed by Whatmans filter paper No.1. In addition, the filtrate was concentrated well by rotary evaporator. Stock solution was perpared, then different concentrations of the extract were prepared 25, 50,100, and 200 µg/mL.

Determination of Total Flavonoid Content

The aluminum chloride colorimetric method which was described by [17] was used to evaluate the total flavonoid content Spectro-photosynthetically. Briefly, 3.5 mg of *G. biloba* ethanolic extract was dissolved in 5 mL of half ethanol, followed by addition of 1 ml of 5% (w/v) NaNO2 solution. After 6 min, 1 mL of a 10% (w/v) $AlCl_3.6H_2O$ solution was added and the mixture was left for a further 5 min before adding 10 mL of a 10% (w/v) NaOH and the mixture was made up with 50 mL of distilled water and mixed well. Furthermore, the absorbance was evaluated using a spectrophotometer and it was 460 nm. A linear standard curve of catechin was prepared. Total flavonoid was calculated based on standard curve equation

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y =3.25x + 0.019, where y = absorbance at 460 nm and x = flavonoid concentration. The obtained result was expressed as means (SD) mg of (+)-catechin equivalents per gramme gram for the *G. biloba* ethanolic extract.

DPPH Radical - Scavenging Activity

The measurement of the antioxidant activity of *G. biloba* ethanolic extract and the standard were done according to the radical–scavenging effect of the stable 1,1 diphenyl-2-pecrylhdrazyl (DPPH) free radical method [18]. An aliquot of 0.1 mL of *G. biloba* ethanolic extract or standard (Ascorbic Acid) (25, 50, 100 and 200 µg/mL) was added to 3.9 mL of DPPH solution in a test tube. And the mixture was incubated in dark at 37°C for 30 min. In addition, spectrophotometer apparatus was used to determine the absorbance of each mixture at 517 nm. All measurements were performed in triplicates. The ability to scavenge DPPH free radical was calculated by the equation given below:

$$(DPPH)\ Radical\ Scavenging\ Activity\ (\%) = \left(1 - \frac{Absorbance\ of\ Sample}{Absorbance\ of\ Standard}\right) \times 100$$

Reductive Ability

To determine the reductive ability of *G. biloba* ethanolic extract, the method that described by [19] was adopted. One mL of each concentration of *G. biloba* ethanolic extracts (25, 50, 100 and 200 µg/mL) or standard (Trolox) was added to 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1.5 mL of potassium ferricyanide (1%), and the mixture was incubated at 50°C in dark for 20 min. One mL trichloroacetic acid (10%) was added to the obtained solution to stop the interaction. After that, the mixture was centrifuged at 3000 rpm for 10 min. Then 2 mL of distilled water and 0.5 mL of freshly prepared ferric chloride (0.1%) was mixed with 2.5 mL of the obtained supernatant. Furthermore, the absorbance was measured at 700 nm with a spectrophotometer after 15 min and all tests were carried out in triplicate.

Experimental Animals

BALB/c male mice, approximately 30 g (12 to 14 weeks old) with total number of 40, were used in this study and kindly provided from Biotechnology Research Center – Al-Nahrain University. Mice were housed in stainless steel cages, under a 12-hour dark/12-hour light cycle at a temperature of 22±3.0°C. The investigation was conducted at a relative humidity of 55±5.0%. Mice fed water and conventional pellet meal throughout the study period.

Mice were separated into 4 groups, each group contained 10 mice as follows:

Group 1 (Untreated mice): mice were administrated with 0.1 mL PBS intraperitoneally for 14 days.

Group 2 (GB-EE Treatment): mice were intraperitoneally injected with 0.1 mL of 100 mg/kg GB-EE for 14 days.

Group 3 (H_2O_2 Treatment): mice were intraperitoneally injected with 0.1 mL of 100 mg/kg H_2O_2 for 14 days.

Group 4 (GB-EE + H_2O_2 Treatment): mice were injected with 0.1 mL (100 mg/kg) GB-EE for 5 days prior to dual H2O2 and GB-EE treatment (100 mg/kg for each) for 9 days.

At the end of the experiment, all mice were sacrificed, kidney and liver tissues were collected and homogenized in cold KCl 0.15 M using homogenizer (15,000 rpm). The homogenates underwent centrifugation, and the supernatants were preserved at -40°C until analysis. Tissue malondialdehyde (MDA) and glutathione (GSH) concentrations were quantified using a UV-spectrophotometer employing the colorimetric techniques as previously reported [20].

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Data Analysis

All values in this study are given as mean ± standard deviation (SD), and differences between means were determined by analysis of variance (ANOVA) followed by either LSD (Least Significant Difference) of Duncan's test. The analyses were achieved by using the statistical package SPSS version 20.

Results and Discussion

Total Flavonoids Content

Ethanolic extract of *G. biloba* was found to have total flavonoids of 832.1±15.8 µg/mL. Accordingly, the ethanolic extract of *G. biloba* can be considered as a rich source of flavonoids. Phenolic compounds with variable structures are the main components of flavonoids and can be found in fruits, vegetables, grains, bark, roots, stems and flowers which in turn belong to a wide-group of plant secondary metabolites. Moreover, Flavonoids have a wide range of therapeutic activities including their potential function as anti-cancer agents which give them a remarkable therapeutic feature [21, 22].

DPPH Radical Scavenging Activity

The radical scavenging activity of GB-EE was estimated by comparing the percentage inhibition of formation of DPPH radical by the extract with those of ascorbic acid. GB-EE exhibited a dose-dependent antioxidant capabilities with maximum scavenging of DPPH was 76.23±2.022% at 200 µg/mL. Compared to ascorbic acid (as a positive control), the pattern of GB-EE scavenging activity showed no significant differences with ascorbic acid treatment, indicating the substantial antioxidant activity of GB-EE. The calculated IC_{50} was 19.45 and 26.35 µg/mL for GB-EE and ascorbic acid, respectively (Table 1).

(Table 1): DPPH radical scavenging activity of GB-EE.

Treatment (µg/mL)	Antioxidant Activity (Mean ± SD) %		
	Ascorbic acid	GB-EE	*p* Value
25	49.04±0.8839	38.46±0.4064	0.9998 NS
50	53.86±1.381	57.56±11.27	0.7840 NS
100	69.48±4.291	60.88±1.252	0.1123 NS
200	76.23±2.022	73.15±1.140	0.8733 NS

NS: Non-significant.

GB-EE demonstrates notable antioxidant properties, attributed to its abundant bioactive compounds, especially flavonoids, terpene lactones, and polyphenolic compounds. The antioxidant activity of the extract is mainly due to its capacity to neutralize free radicals, inhibit lipid peroxidation, and safeguard cells from damage caused by oxidative stress [23]. The principal flavonoid constituents play a crucial role in enhancing antioxidant capacity via mechanisms including hydrogen atom donation and electron transfer [24].

Reductive Ability Assay

In this experiment, the assessment of the reductive ability showed that the GB-EE was moderate in such activity, in which the obtained results were concentration-related. GB-EE exhibited efficient reduction ability for all concentrations used with significant differences ($p < 0.01$) compared to trolox (positive

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control). At the concentration 0.64 mg/mL of GB-EE the highest absorbance was observed in comparison with that of trolox and the absorbance values were 0.4±0.01 and 0.278±0.01 respectively (Table 2).
The moderate reduction activity of the extract is evidenced by its ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions, as measured by the FRAP (Ferric Reducing Antioxidant Power) assay. This reducing power, while not as potent as its antioxidant capacity, plays a crucial role in maintaining cellular redox homeostasis. The synergistic interaction between the extract's reducing and antioxidant properties enhances its overall therapeutic potential, particularly in conditions characterized by oxidative stress and redox imbalance [25].

(Table 2): Reductive ability of GB-EE.

Treatment (mg/mL)	Absorbance (Mean ± SD)		
	Positive Control (Trolox)	*G. biloba*	*p Value*
0.02	0.109±0.002	0.179±0.003	<0.0001 **
0.04	0.117±0.002	0.234±0.005	<0.0001 **
0.08	0.138±0.008	0.337±0.007	<0.0001 **
0.16	0.164±0.015	0.361±0.007	<0.0001 **
0.32	0.230±0.022	0.390±0.008	<0.0001 **
0.64	0.278±0.010	0.400±0.010	<0.0001 **

****: $p < 0.01$.**

GB-EE Exhibited Protective Effect Against H_2O_2 Toxicity

Results in Fig. (1) and Table (3), show that the level of MDA and GSH in mice treated with GB-EE was close to control group, however, H_2O_2 treatment resulted in extreme changes at these levels. In H_2O_2 treated mice, the level of MDA was significantly ($p < 0.05$) increased compared to control, while the level of GSH was reduced dramatically ($p < 0.05$). Both observations were detected in kidney and liver tissues. Interestingly, GB-EE treatment along with H_2O_2 exposure significantly ($p < 0.05$) improved the abnormal changes in the level of MDA and GSH compared with mice group treated with H2O2 only. GB-EE treatment resulted in reduction of MDA level by 53.8 and 52.2% in kidney and liver tissue, respectively after 9 days exposure to H_2O_2. On the other hand, the extract resulted in 2.28- and 3.1-fold increase in the level of GSH in kidney and liver tissue, respectively.

(Table 3): Mean ± SD MDA and GSH level in mice liver and kidney tissues upon different treatments.

Groups	MDA (Mean ± SD) nmol/g	
	Kidney	Liver
I	0.31±0.02a	0.33±0.04a
II	0.36±0.04a	0.35±0.04a
III	1.19±0.12b	1.34±0.14b
IV	0.64±0.07c	0.7±0.08c
Groups	**GSH (Mean ± SD) mg/g**	
	Kidney	**Liver**
I	0.86±0.06a	0.90±0.08a
II	0.79±0.08a	0.84±0.10a
III	0.25±0.03b	0.22±0.02b
IV	0.57±0.04c	0.68±0.05c

Different letters (a, b, c) considered significantly different at $p < 0.05$.

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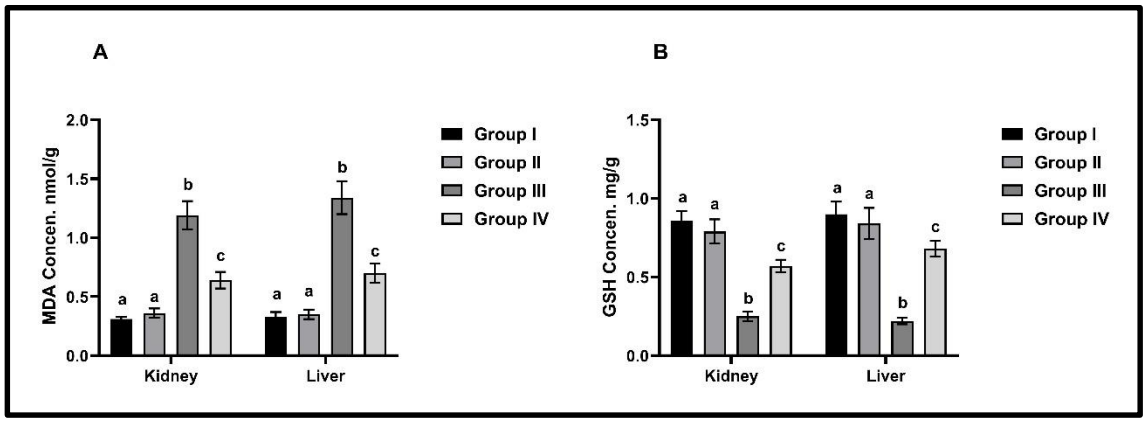

Fig. 1: Effects of GB-EE treatment on MDA and GSH levels of mice liver and kidney tissues treated with H_2O_2. Different letters (a, b, c) are significantly different at $p < 0.05$.

This study demonstrates that GB-EE treatment exhibits a protective effect against biochemical toxicity induced by H_2O_2. The protective function of GB-EE is linked to various mechanisms, notably the activation of antioxidant enzymes [26]. Ginkgolide, a component of *G. biloba* extract, has been shown to protect cells from necrosis and apoptosis by modulating glutathione peroxidase activity and reducing malondialdehyde (MDA) levels associated with lipid peroxidation [27]. Consequently, GB-EE safeguards liver and kidney tissues from damage caused by H_2O_2, decreases MDA levels, and restores the compromised antioxidant balance. A significant increase in MDA levels and a decrease in GSH levels were observed in the H_2O_2 -treated group. Elevated concentrations of H_2O_2 may induce oxidative stress, leading to cellular damage [28]. The antioxidant systems within cells protect against oxidative damage. The elevation of oxidant levels and inadequate antioxidant capacity result in irreversible cellular damage. GSH serves as a crucial antioxidant within cells, and a reduction in GSH levels signifies oxidative damage [29]. MDA results from lipid peroxidation, and elevated levels of MDA indicate oxidative damage within the cell [30]. Administration of GB-EE resulted in partial enhancement of GSH and MDA levels, demonstrating a distinct protective effect against oxidative damage.

Conclusions

The *G. biloba* ethanolic extract (GB-EE) exhibited significant antioxidant activities, including potent free radical scavenging and moderate reducing power. The extract's rich flavonoid content contributed to these antioxidant properties. Furthermore, GB-EE demonstrated a protective effect against oxidative stress-induced toxicity *in vivo*, highlighting its therapeutic potential for conditions characterized by redox imbalance

Acknowledgments

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Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.

- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

[First Author]: Contributed to the conception and design of the study, conducted all experiments, data rearrangement and drafted the initial manuscript.

References

1. Salih, S.M., A. Ahmed, and Y.W. Issa, Cytotoxic Effect of Annona muricata leaf extracts on tumor cell lines in vitro. *Iraqi Journal of Bioscience and Biomedical*, 2024. 1(1): p. 20-26.

2. World Health, O., WHO global report on traditional and complementary medicine 2019. 2019: World Health Organization.

3. Mothana, R.A., et al., Comparative evaluation of cytotoxic, antimicrobial and antioxidant activities of the crude extracts of three Plectranthus species grown in Saudi Arabia. *Saudi Pharmaceutical Journal*, 2019. 27(2): p. 162-170.

4. Abd-Allh, S.O., Precautionary Effect of Ginkgo biloba Against Mercury-Induced Acute Nephro-Hepatotoxicity in male Rats. *Journal of Babylon University/Pure and Applied Sciences*, 2014. 9: p. 2390-2405.

5. Qasim, Z.S., The Effect of Ginkgo biloba Extracts on Candida albicans Isolated from Healthy Persons. *Iraqi Journal of Pharmaceutical Sciences* (P-ISSN 1683-3597 E-ISSN 2521-3512), 2020. 29(2): p. 122-128.

6. Shen, N., et al., Plant flavonoids: Classification, distribution, biosynthesis, and antioxidant activity. *Food chemistry*, 2022. 383: p. 132531.

7. Halliwell, B., Reactive oxygen species (ROS), oxygen radicals and antioxidants: Where are we now, where is the field going and where should we go? Biochemical and biophysical research communications, 2022. 633: p. 17-19.

8. Hu, J., et al., A double-edged sword: ROS related therapies in the treatment of psoriasis. *Asian Journal of Pharmaceutical Sciences*, 2022. 17(6): p. 798-816.

9. Bhattacharyya, A., et al., Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev*, 2014. 94(2): p. 329-54.

10. Batty, M., M.R. Bennett, and E. Yu, The Role of Oxidative Stress in Atherosclerosis. Cells, 2022. 11.(23)

11. Nakamura, H. and K. Takada, Reactive oxygen species in cancer: Current findings and future directions. *Cancer Sci*, 2021. 112(10): p. 3945-3952.

12. Gogna, T., B.E. Housden, and A. Houldsworth Exploring the Role of Reactive Oxygen Species in the Pathogenesis and Pathophysiology of Alzheimer's and Parkinson's Disease and the Efficacy of Antioxidant Treatment. Antioxidants, 2024. 13, DOI: 10.3390/antiox13091138.

13. Goutami, L., et al., Pathological Role of Reactive Oxygen Species on Female Reproduction. *Adv Exp Med Biol*, 2022. 1391: p. 201-220.

14. Lu, J., et al., A novel and compact review on the role of oxidative stress in female reproduction. *Reproductive Biology and Endocrinology*, 2018. 16(1): p. 80.

15. Aparadh, V., V. Naik, and B. Karadge, Antioxidative properties (TPC, DPPH, FRAP, metal chelating ability, reducing power and TAC) within some Cleome species. ANNALI DI BOTANICA, 2012. 2: p. 49–56.

16. Maltas, E., H.C. Vural, and S. Yildiz, Antioxidant activity and fatty acid composition of Ginkgo biloba from Turkey. *Journal of Food Biochemistry*, 2011. 35(3): p. 803-818.

17. Al-Ani, N.K., et al., Evaluating the total flavonoids, Reductive ability and antibacterial potentials of Salvia officinalis aqueous extract. GPH-International *Journal of Biological & Medicine Science*, 2019. 2(09): p. 01-07.

18. Mohammed, Z.H. and R.M. Ibraheem, Anti-oxidant activity of methanol extracts of Arum maculatum L. and Physalis peruviana L. plants. Ibn AL-Haitham *Journal For Pure and Applied Science*, 2017. 28(2): p. 1-7.

19. Fu, W., et al., Antioxidant, free radical scavenging, anti-inflammatory and hepatoprotective potential of the extract from Parathelypteris nipponica (Franch. et Sav.) Ching. *Journal of ethnopharmacology*, 2010. 130(3): p. 521-528.

20. Yalçın, E., et al., In vivo protective effects of Ginkgo biloba L. leaf extract against hydrogen peroxide toxicity: cytogenetic and biochemical evaluation. *Environmental Science and Pollution Research*, 2020. 27(3): p. 3156-3164.

21. Grassi, D., G. Desideri, and C. Ferri, Flavonoids: antioxidants against atherosclerosis. Nutrients, 2010. 2(8): p. 889-902.

22. Grassi, D., et al., Flavonoids, vascular function and cardiovascular protection. Curr Pharm Des, 2009. 15(10): p. 1072-84.

23. Kobus-Cisowska, J., et al., Antioxidant properties of extracts from Ginkgo biloba leaves in meatballs. Meat Science, 2014. 97(2): p. 174-180.

24. Sati, P., et al., Ginkgo biloba flavonoid glycosides in antimicrobial perspective with reference to extraction method. *Journal of traditional and complementary medicine*, 2019. 9(1): p. 15-23.

25. Kobus, J., et al., Phenolic compounds and antioxidant activity of extracts of Ginkgo leaves. European Journal of Lipid Science and Technology, 2009. 111(11): p. 1150-1160.

26. Zhang, L., et al., Study on synergistic antioxidant effect of typical functional components of hydroethanolic leaf extract from Ginkgo Biloba in vitro. *Molecules*, 2022. 27(2): p. 439.

27. Guo, M., et al., The protective mechanism of Ginkgolides and Ginkgo flavonoids on the TNF-α induced apoptosis of rat hippocampal neurons and its mechanisms in vitro. *Heliyon*, 2015. 1.(1)

28. Wu, Z., et al., Roles of endoplasmic reticulum stress and autophagy on H2O2-induced oxidative stress injury in HepG2 cells. *Molecular medicine reports*, 2018. 18(5): p. 4163-4174.

29. Aquilano, K., S. Baldelli, and M.R. Ciriolo, Glutathione: new roles in redox signaling for an old antioxidant. *Frontiers in pharmacology*, 2014. 5: p. 196.

30. Tsikas, D., Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. *Analytical biochemistry*, 2017. 524: p. 13-30.

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Phytochemical investigation and antioxidant potential for *Vitex negundo* L. Leaves Methanolic extract

Mohamednoruldin Dh Hazim[1], Ruqaya Mohamed Al-ezzy, Saba Mahdi Al-maawi[2]

[1] Medical and molecular Biotechnology, College of Biotechnology, Al-Nahrian University, Baghdad, Iraq
[2] Oncology teaching hospital, Medical city, Baghdad, Iraq
Corresponding Author
E. mail: muhnor99@gmail.com

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Abstract

This research aimed to analyze the phytochemical constituents and antioxidant capabilities of crude methanolic extract of *Vitex negundo* L. leaves, which considered as one of medical plant. Leaves were gathered for examination followed by methanolic and essential oil extraction for biological application. Through screening processes, important compounds of therapeutic effects were investigated such flavonoids, polyphenols, and alkaloids in lower content. The quantitative assay revealed that the major content was flavonoids (513,34±23,15µg/mL), while the amount of total phenols was (350,67±27,68mg/mL). Antioxidant tests were carried out using DPPH and FRAP methods, for evaluation purposes in the current study. For DPPH, result indicated that the scavenging ability of *V. negundo* L. methanolic extract was achieved in relative to vitamin C the potential antioxidant agent at concentration (83.34±3.51µg/mL). In the FRAP experiment conducted on *V. negundo* L. plant samples showed a ferric reduction capacity of (1.92±30mg/mL) at the concentration (0.545±15mg/mL) in relative to Trolox standard. These significant findings highlight *V. negundo* L. potential as a natural source of antioxidants. These results indicate that *V. negundo* L. may be an option, for medical purposes in addressing conditions linked to oxidative stress. This supports its standing use and positions it as a remedy, in contemporary medicine.

Keywords: *Vitex negundo* L, Methanol Extraction, antioxidant activity, Phytochemical Screening.

Introduction

The Chinese chaste tree or five leaved chaste tree, with the scientific name *Vitex negundo* Linn (*V. negundo* L) is a medicinal plant belongs to the Lamiaceae family that thrives in tropical and subtropical areas like Iraq, fig 1, Southeast Asia and some parts of Africa. The plant is a fragrant shrub growing, up to 5 meters tall with compound leaves made up of five lance shaped leaflets, blue flowers and dark drupe like fruits. It flourishes in both habitats and manmade gardens is highly valued in ancient healing practices like

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Ayurvedic medicine and traditional Chinese medicine for its strong anti-inflammatory properties as well as antimicrobial and pain-relieving benefits [1].

Figure 1 Filed of *Vitex negundo* L. leaves

In Southeast Asias medicine practices *V. negundo* known as "Nirguna" in Ayurvedic medicine has played a crucial role by relieving pain and inflammation along; as recent studies indicate that *V. negundo* commonly known as the five leaved chaste tree or nirgundi in Ayurveda medicine [2], in India may have benefits in fighting cancer and has garnered interest within the field of oncology as a result of this discovery, with treating respiratory problems and female reproductive ailments. It has been utilized to address arthritis, asthma, bronchitis, menstrual disorders and enhance the healing of wounds. The diverse biological capabilities of *V. negundo* credited to a range of substances found in its leaves and bark such as flavonoids and alkaloids, among others have demonstrated effectiveness, in regulating inflammation and combating oxidative stress [3].

Studies have highlighted that extracts derived from *V. negundo* possess antioxidant properties that can regular cell growth and trigger programmed cell death in cells. Crucial components such as flavonoids and phenolic acids found in *V. negundo* are thought to be responsible for these effects which could potentially position this herb as a complementary approach, to conventional cancer treatments. In the leaves of the plant *V. negundo* various substances found; like luteolin and casticin have been found to have effects in reducing stress and inflammation—both known factors associated with the development of cancer [4]. Moreover, the essential oils from this plant contain compounds such as β caryophyllene oxide that exhibit properties like regulating the system fight against certain bacteria and reduce inflammation. These combined effects suggest that the plant may play a role, in alleviating cancer symptoms and potentially enhancing the efficacy of cancer treatments [5].

In addition, to its effects on cancer treatment *Vitex negundo* also exhibits anti-inflammatory and pain-relieving qualities that are beneficial for addressing long term inflammatory issues like arthritis and

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rheumatism. Research indicates that compounds found in the leaves of this plant have the ability to block agents such, as cytokines and enzymes that contribute to pain and inflammation. The anti-inflammatory effects of *V. negundo* plant are mostly attributed to substances such, as flavonoids and essential oils that help in decreasing inflammation by regulating pathways like COC and LOY and reducing cytokines like interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) according to Nguyen [2] study in 2024 *V. negundo* could serve as an alternative to nonsteroidal inflammatory drugs (NSAIDs) as it offers pain relief, with fewer adverse reactions as discussed by Sharma [3].

The medicinal advantages of the plant *V. negundo* also aid in maintaining health historically used in infusions or steam inhalations to alleviate respiratory problems such, as coughs and asthma symptoms while also alleviating bronchitis symptoms. The plants effects that widen the airways and reduce inflammation are attributed to substances, like α pinene and γ terpinene that assist in relaxing the muscles and decreasing mucus production. This age-old remedy shows potential as a solution, for handling term respiratory problems and improving lung wellness [6].

Aside, from its properties *V. negundo* is highly regarded for its impact on the environment as well. It is commonly used in agroforestry as a natural insect deterrent and organic fertilizer to enhance soil quality. The leaves of this plant contain substances that repel insects. When planted near crops it can naturally help control pest populations and boost crop yields. Additionally, as a shrub it plays a role, in preventing soil erosion and restoring land promoting eco agricultural methods that are sustainable [7].

Materials and Methods

Plant collection

Vitex negundo L. leaves were collected from local markets of Baghdad – Iraq and recognized by Dr. Ibrahim S. Al–Jubouri, College of Pharmacy, Al–Mustansiriyah University, during October,2023. The collected plant leaves were thoroughly washed with distilled water to remove impurities, air-dried in a shaded area to preserve phytochemicals, and then ground into a fine powder using an electric grinder. The powdered material was stored in a desiccator until further use biological experiment.

Preparation of plant methanolic extract

Approximately (50g) of the dried leaf powder was extracted with (85%) methanol (250mL) using Soxhlet apparatus at (65°C) for three hours. The obtained extract was concentrated using a rotary evaporator under reduced pressure, yielding a dry methanol extract. This extract was then stored at (-20°C) in a sealed container to prevent degradation of bioactive compounds [8].

Phytochemical screening

Preliminary phytochemical analyses were conducted to identify the presence of major bioactive compounds in *V. negundo* L., including alkaloids, flavonoids, tannins, saponins, phenolic compounds, and glycosides, at Biotechnology research center – Al-Nahrain University.

Quantification of phytochemicals

Determined total flavonoid content and total phenolic content from methanolic extraction

Total flavonoid content (TFC)

Total flavonoid content determined by Sakanaka [9], (3mg) of *V. negundo* L. methanolic extract in (5mL) of methanol 50%, followed; added (1mL) $NaNO_3$ 5% and waited (6min.). Then, added (1mL) of $AlCl_3$ and leaved (5min) before added (10mL) of NaOH 10%. After that, mixed well with same time made up to (50mL) with D.W. Lastly, the mixture was left for 15 minutes, then the absorption was measured at 510nm for all replicates. TFC was calculated based on a rutin calibration curve [y=0.0012x+0.1109 R^2=0.9317] and expressed as mg rutin equivalents per gram of extract.

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Total phenolic content (TPC)

TPC was measured by the Folin–Ciocalteu method [10]. (1mL) from *V. negundo* L. methanolic extracts were mixed with (2.5mL) Folin–Ciocalteu reagent and after (5min) added (2mL) of sodium carbonate solution, followed by incubation at (50°C) for (10min). The absorbance was recorded at (765nm), and TPC was expressed as mg gallic acid equivalents (GAE) per gram of extract using a standard curve prepared with gallic acid [y=0.8101x-0.7595 R^2=0.99958].

Antioxidant activity assays

Antioxidant activity was evaluated through 2-2-Diphenyl-1-picrylhydrazyl (DPPH) assessing radical scavenging capacity and ferric reducing antioxidant power (FRAP) determining ferric ion reducing power.

2-2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay was performed to evaluate the radical scavenging activity [12] of *V. negundo* L. extracts among vitamin C. Different concentrations of the methanolic extract (0.0625, 0.125, 0.250, 0.500mg/mL) were prepared by (1mL) of DPPH to the four tubes, and then incubated for (30min.) at (37°C); The absorbance was measured at (517nm), fig. 2, and the radical scavenging activity was calculated as follows:

$$DPPH\ scarving\ activity = \frac{absorbance\ of control - absorbance\ of\ sample}{absorbance\ of\ control} \times 100$$

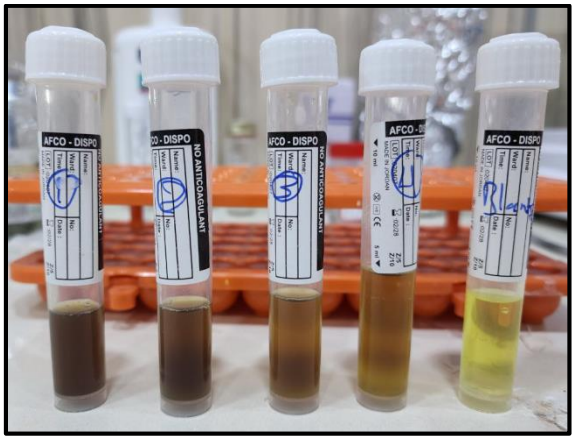

Figure 2 Preparation of DPPH for *Vitex negundo* L. methanolic extraction

Ferric reducing antioxidant power (FRAP) Assay

The FRAP assay was conducted according to Fu [12], to assess the ferric reducing capacity of the extracts. Briefly, samples of *V. negundo* L. extract was prepared in concentrations of (0.64, 0.32, 0.16, 0.08mg/mL). Each sample was combined with phosphate buffer (0.2M, pH6.6) and (1%) potassium ferricyanide and incubated at (50°C) for (20min). After adding trichloroacetic acid (10%) and centrifuging, the supernatant was mixed with distilled water and freshly prepared ferric chloride (1%) solution. Absorbance was measured at (700 nm). Results were compared with a Trolox standard curve in concentrations of (0.64, 0.32, 0.16, 0.08mg/mL), and data were expressed in Trolox equivalents, fig 3.

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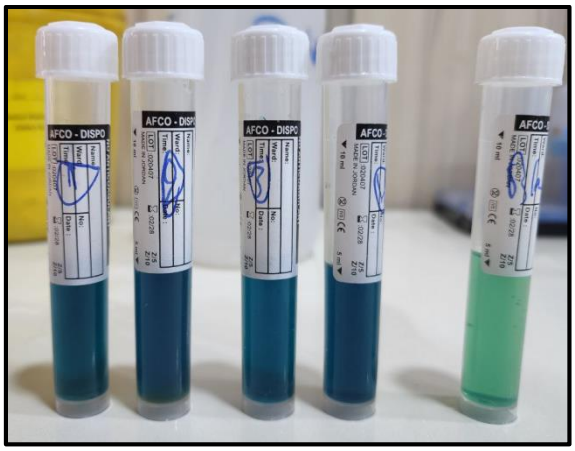
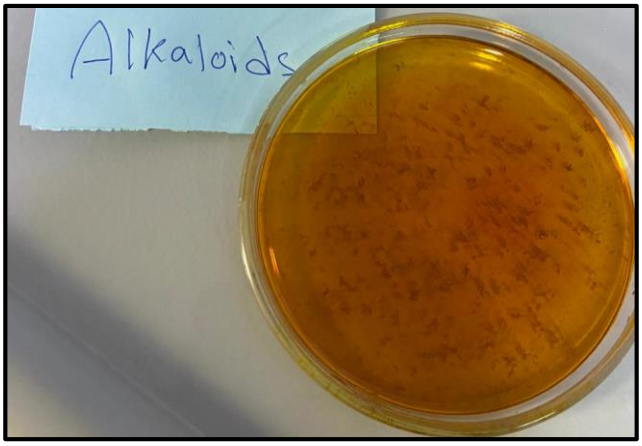
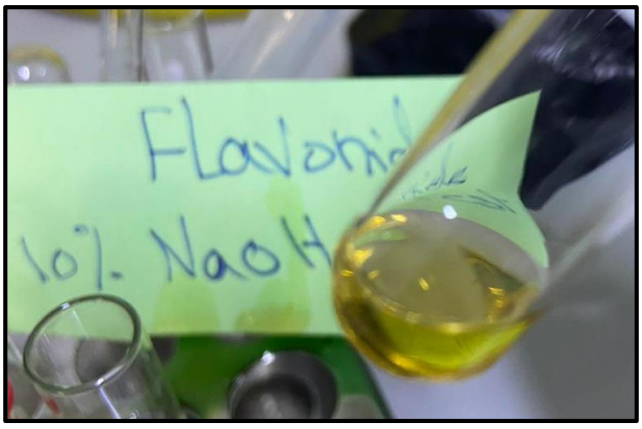


Figure 3 Preparation of FRAP for *Vitex negundo* L. methanolic extraction

Results and Discussion

Phytochemical analysis showed;

Detection test	Result	
Alkaloids by • Mayer reagent • Dragendroff reagent	+	
Flavonoids	+++	

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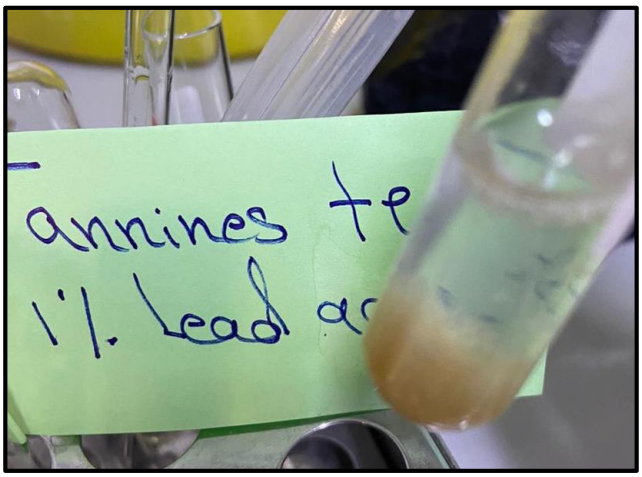
Polyphenolic	+	
Polysaccharides	++	
Tannins	++	

** + mild, ++ moderate, +++ strong

The appearance of alkaloids, although in mild levels, is significant due to literature pharmacological effects, as ex. analgesic, antimicrobial, and anti-inflammatory activities. These combinations can react with cellular pathways, intensifying the herb's potential as a therapeutic agent [13].

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The plenty of flavonoids indicates rich antioxidant capacity, as these flavonoids are known for their capacity to donate a lot of electrons with stabilizing free radicals. This activity is important in lightening oxidative stress and save cells from damage [14].

Polyphenols supply extra antioxidant activity, aid to reduction of ROS and intensifying the overall cellular structures stability. Both Polyphenols and flavonoids, generating a synergistic antioxidant outcome [15].

Polysaccharides share in immune effects, that's support immune in its responses and serve health. Due to that, *V. negundo* may aid in improving immune function, further more exceed visibility range its potential medicinal uses [16].

The moderate level of tannins assists special properties, that can help in healing of wound and reduction the inflammation. In addition, tannins possess antimicrobial properties, given more benefit to *V. negundo* therapeutic protocol [1].

Quantitative assay for Total Flavonoids and Total phenolic compounds

Total flavonoid content was spectro-photochemically determined in methanolic extract of *V. negundo* as rutin equivalent. The extract was found to contain (513.34±23.15µg/mL) flavonoids, in compared with stander curve fig (2-A) [19], while total phenolic content was (350.67±27.68mg/mL), in compared with stander curve fig (2-B) [18].

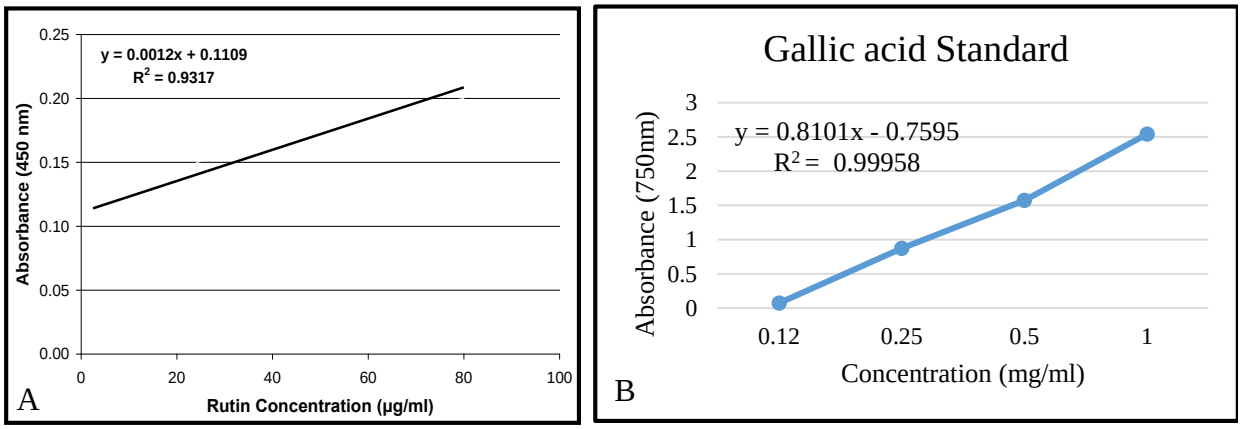

Figure 2. A – stander curve of Rutin, B – stander curve of gallic acid

The Antioxidant Activity

Result of DPPH radical scavenging activity

Methanolic extract of *V. negundo* was more effective in DPPH radical scavenging activity than vitamin C at the four concentrations tested [19]. The concentrations (0.500 and 0.250mg/mL) of plant extract shared an approximated radical scavenging activity (83.34±3.51 and 74.01±2.64%, respectively) and shown low activity at the concentrations (0.125 and 0.0620mg/mL) as (47.01±5.02 and 32.67±5.50%, respectively), as explain in fig. 3-A.

Result of FRAP

While, methanolic extract of *V. negundo* was more effective in FRAP than Trolox (vitamin E) at the four concentrations tested [20]. The concentrations (0.64 and 0.32mg/mL) of plant extract shared an approximated radical scavenging activity (1.92±0.30 and 1.33±0.29mg/mL, respectively) and shown low activity at the concentrations (0.16 and 0.08mg/mL, respectively) as (1.14±0.04 and 0.85±0.03mg/mL, respectively), as explain in fig. 3-B.

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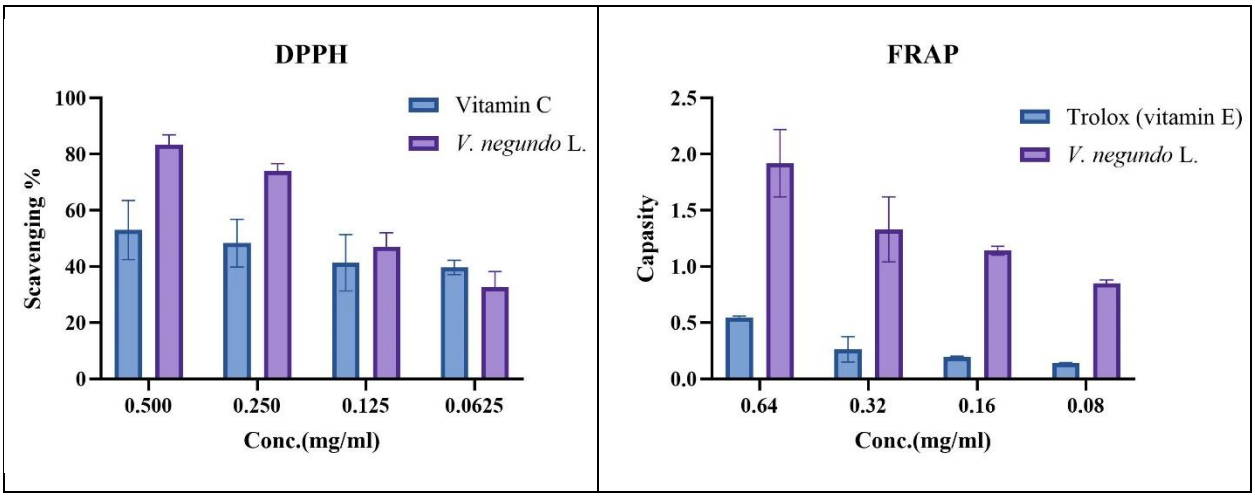

Figure 3. A – DPPH radical scavenging action of *V. negundo* L.
B – Reductive ability over *V. negundo* L. methanolic banish then trolox (vitamin E)

Conclusions

Result indicated that the scavenging ability of *V. negundo* L. methanolic extract was achieved in relative to vitamin C the potential antioxidant agent at concentration (83.34±3.51µg/mL). In the FRAP experiment conducted on *V. negundo* L. plant samples showed a ferric reduction capacity of (1.92±30mg/mL) at the concentration (0.545±15mg/mL) in relative to Trolox standard. These significant findings highlight *V. negundo* L. potential as a natural source of antioxidants. These results indicate that *V. negundo* L. may be an option, for medical purposes in addressing conditions linked to oxidative stress.

Acknowledgments

We express our gratitude to Al-Nahrain University and the College of Biotechnology, namely the head of the College, for their consent to proceed with this study. Gratitude is expressed to the researchers who contributed to the successful completion of this study.

Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

[First Author]: Conducted some experiments, data rearrangement and drafted the initial manuscript.

[Second Author]: Contributed to the conception and design of the study, and conducted some characteristics of the products.

[Third Author]: Facilitated processes to ensure the project ran smoothly.

References

1. Goswami, S., and Roy, B. (2024). Vitex Negundo L., an Indigenous Plant: A Systematic Review on Traditional Use, Bioactives, and Pharmacological Activities. *Bioactives and Pharmacology of Lamiaceae*, 309–330.
2. Nguyen, T. T., Do, P. T., Nguyen, L. T., and Pham, A. T. (2024). Acute and Sub–Chronic Toxicity Studies of Aqueous Ethanol Leaf Extract of *Vitex negundo* L in Experimental Animals. *Tropical Journal of Natural Product Research*, **8**(1).
3. Sharma, D., Radha, Kumar, M., Andrade-Cetto, A., Puri, S., Kumar, A., and Lorenzo, J. M. (2023). Chemical Diversity and Medicinal Potential of *Vitex negundo* L.: From Traditional Knowledge to Modern Clinical Trials. *Chemistry and Biodiversity*, **20**(11), e202301086.
4. Mutlag, S. H. (2023). Evaluation the possible Genotoxic Effect of *Vitex Negundo* Ethyl Acetate Fraction on Chromosomal Aberration in Mouse Spleen and Bone Marrow Cell. *Journal of Population Therapeutics and Clinical Pharmacology*, **30** (5), 288–294.
5. Ngandjui, Y. A. T., Tchamgoue, J., Kagho, D. U. K., Msagati, T. A. M., Ngameni, B., and Kouam, S. F. (2024). Chemical Characterization of Phytoconstituents for Lifestyle Diseases. *Role of Herbal Medicines: Management of Lifestyle Diseases*, 59–87. Singapore: Springer Nature Singapore.
6. Kalachaveedu, M., Senthil, R., Azhagiyamanavalan, S., Ravi, R., Meenakshisundaram, H., and Dharmarajan, A. (2023). Traditional medicine herbs as natural product matrices in cancer chemoprevention: A trans pharmacological perspective (scoping review). *Phytotherapy Research*, **37**(4), 1539–1573.
7. Ruckmani, K., Krishnamoorthy, R., Samuel, S., and Kumari, H. L. J. (2014). Formulation of herbal bath soap from vitex negundo leaf extract. *Journal of Chemical and Pharmaceutical Sciences International Standard Serial Number*, **974**, 2115.
8. Richard J. Cannell, Methods in Biotechnology, Natural Products Isolation, 2000, New Jersey
9. Sakanaka, S., Tachibana, Y., and Okada, Y. (2005). Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). *Food chemistry*, **89**(4), 569-575.
10. Khatiwora, E., Adsul, V. B., Kulkarni, M. M., Deshpande, N. R., and Kashalkar, R. V. (2010). Spectroscopic determination of total phenol and flavonoid contents of Ipomoea carnea. *International Journal of ChemTech Research*, **2**(3), 1698-1701.
11. Patel Rajesh, M., and Patel Natvar, J. (2011). In vitro antioxidant activity of coumarin compounds by DPPH, Super oxide and nitric oxide free radical scavenging methods. *Journal of advanced pharmacy education and research*, *1*(1), 52-68.
12. Fu, W., Chen, J., Cai, Y., Lei, Y., Chen, L., Pei, L., and Ruan, J. (2010). Antioxidant, free radical scavenging, anti-inflammatory and hepatoprotective potential of the extract from Parathelypteris nipponica (Franch. et Sav.) Ching. *Journal of ethnopharmacology*, **130**(3), 521-528.
13. Gandhimathi, R., & Aanandhi, V. (2023). A Review of Phytochemical and Pharmacological Activities of Vitex negundo. *International Journal of Research in Phytochemistry and Pharmacology*, **13**(1), 1-6.
14. Das, S., Parvin, S., Islam, M. M., Rahman, A., Mohi-Ud-Din, M., Ahmed, M., and ALMunqedhi, B. M. A. (2024). Morpho-physiological and biochemical responses of Vitex negundo to seawater induced salt stress. *South African Journal of Botany*, **166**, 648-662.
15. Sivapalan, S., Dharmalingam, S., Ashokkumar, V., Venkatesan, V., and Angappan, M. (2024). Evaluation of the anti-inflammatory and antioxidant properties and isolation and characterization of a

new bioactive compound, 3, 4, 9-trimethyl-7-propyldecanoic acid from Vitex negundo. *Journal of Ethnopharmacology*, **319**, 117314.

16. Pal, D., and Lal, P. (2023). Plants Showing Anti-Viral Activity with Emphasis on Secondary Metabolites and Biological Screening. *Anti-Viral Metabolites from Medicinal Plants*, 29-95.
17. Kalaivani, J., Jegadeeswari, V., KR, V., Arulmozhiyan, R., and Padmadevi, K. (2024). Nutraceutical Value and Therapeutic Applications of Indian Heritage Fruits: A Review. *Journal of Scientific Research and Reports*, **30**(7), 303-312.
18. Shettar, P. S., Hiremath, M. B., and Kumbar, V. M. (2024). Anti-proliferative Activity of Vitex negundo Leaf Extracts on PA1 Human Ovarian Cancer Cell Lines. *Archives of Razi Institute*, **79**(2), 426.
19. Alans, A., Al-Anesi, A. A. Q. F., Alrabie, A., Farooqui, M., & Kayande, D. D. (2024). phytochemical screening, gc-ms, lc-ms assisted metabolite profiling, and antioxidant and antibacterial activity of natural products from medicinal plant Vitex negundo. *European Journal of Biomedical*, **11**(10), 370-381.
20. Salmi, D., Alenazy, R., Nabti, E. H., Alqasmi, M., Almufarriji, F. M., Alhumaidi, M. S., and Houali, K. (2024). Endophytic fungal diversity and bioactive potentials: investigating antimicrobial and antioxidant activities. *Cellular and Molecular Biology*, *70*(8), 226-234.

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Investigation the antibacterial activity on bacterial isolates isolated from alopecia areata and skin injuries

Noor A. Kshash[1*]; Ali S. Ahmed[2]
[1] Plant Biotechnology, College of Biotechnology, Al-Nahrian University, Baghdad, Iraq
*Corresponding Author. noora.mbp23@ced.nahrainuniv.edu.iq
https://orcid.org/0009-0009-2910-2311
[2] Plant Biotechnology, College of Biotechnology, Al-Nahrian University, Baghdad, Iraq
*Corresponding Author. ali.shihab@.nahrainuniv.edu.iq
https://orcid.org/0000-0002-1679-9262.
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Abstract

The study aims to conduct a screening of the bacteria colonizing the alopecia areata disease and *Pseudomonas aeruginosa* bacterium that infect skin wounds and burns. Alopecia areata is an autoimmune condition characterized by localized hair loss, often influenced by both genetic and microbial factors. Patients with burns and wounds are more likely to contract an infection in the hospital than other patients due to the loss of the protective barrier (skin) and immune system disorders that appear in these patients. This study investigated bacterial colonization and antimicrobial resistance in alopecia-affected areas and burns/wounds infections. Specimens were collected between October 2023 and February 2024 from three hospitals in Baghdad, including Number of 180 specimens were collected, 116 specimens from them collected from alopecia cases and 64 specimens from wound and burn infection. Half of alopecia cases was collected from the affected areas and the other half from the healthy areas (control) of the same patients. The selected predominance bacterial infection according to the confirmation tests were subjected to identification and antimicrobial susceptibility test using the VITEK-2 system. For alopecia the most commonly isolated species were *Staphylococcus species* which appeared in 10 isolates from 27 positive growth (35.48%). *Pseudomonas aeruginosa* isolates were the most commonly isolated species from burns and wounds appeared in 10 from 25 positive growth. The results revealed high levels of resistance, with 90% of *P. aeruginosa* isolates resistant to ticarcillin and 85% to aminoglycosides, while *Staphylococcus species* exhibited 80% resistance to oxacillin and 70% to vancomycin. Comparing the bacterial profiles of the affected and healthy parts of the same patient's scalp showed big differences. This shows how microbial communities play a part in how diseases progress.

Keywords: Alopecia areata, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Minimum inhibitory concentration

Introduction

The skin is the body's primary defense against microbial invasion, but injuries or burns compromise this barrier, leading to infections that can spread to internal tissues and cause severe complications[1]. Common pathogens in such infections include *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae,* and *Acinetobacter baumannii*[2]. Initially, Gram-positive bacteria like *S. aureus*

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dominate, while Gram-negative bacteria, including *P. aeruginosa*, emerge later and can cause sepsis if they reach the bloodstream. Chronic wounds often harbor biofilms, microbial communities that resist treatment due to a protective extracellular matrix, requiring higher antimicrobial concentrations for eradication[3,4].

Alopecia areata, an autoimmune disease influenced by genetics, is the second most common cause of non-scarring hair loss. It presents as localized hair loss with no follicular destruction and can have unpredictable remissions and relapses. Inflammation during the hair follicle's growth phase and potential microbial influences, including external bacteria and immune responses, play roles in its pathogenesis[5].

The integrity of the skin is compromised when a wound is present, which makes it easier for organisms to penetrate the tissues under the surface[6]. The severity of the wound and the likelihood that the agent would act as a vector for infectious organisms are two factors that can be used to classify the agents that are responsible for wounds[7]. Trauma that penetrates the skin, whether it be from plants, animals, weapons, knives, or other items, can lead to the development of wound infections. As stated by Chhabra *et al*. (2017)[8], numerous plant compounds have the capability of penetrating the skin, which might result in the development of wound infections. Due to the fact that plants are made up of porous materials, they may allow thorns or other similar items to aid the entry of *S. aureus* or other organisms into their tissues. In many cases, cellulitis develops as a consequence of conditions that affect the skin[9,10].

According to Fayisa and Tuli (2023)[11], the commensal and opportunistic microbe known as Staphylococcus aureus has the ability to colonise the skin and mucous membranes of persons, which presents a huge challenge to the public health of the entire world. The bacterium is the dominant representative of the genus *Staphylococcus* and has been identified as the agent responsible for a number of diseases that affect both people and animals[12]. *S. aureus* is a multipurpose microbe that is capable of effortlessly adjusting to a wide range of environmental circumstances[13]. This microbe is capable of producing a variety of virulence factors that are associated with its pathogenicity. Additionally, it is able to penetrate regions within the host that are generally sterile[12,13] . The bacterium *Staphylococcus aureus* can cause diseases not only by direct tissue invasion, but also through the activity of more than thirty exoproteins that are encoded by the pathogen on its own [14].

Pseudomonas aeruginosa is a member of the Pseudomonadaceae bacterial family, a member of γ-proteobacteria. "Schroeter" was the first to propose the scientific name *Bacterium aeruginosa* for *P. aeruginosa* in 1872, after he isolated it from suppurating wounds basis on its phenotypic characteristics. It is one of twelve subtypes of bacteria seen seldom as a component of the human micro-flora in healthy individuals. *P. aeruginosa* is widespread in nature and it is an opportunistic pathogen causing nosocomial infection in humans that causes a number of diseases like inflammation of urinary tract, burns, respiratory infections, and septicemia[15];[16] .

Materials and Methods

Skin Specimen Collection

Between October 2023 and February 2024, specimens were collected from three hospitals in Baghdad: Al Yarmouk Teaching Hospital, Baghdad Hospital and Burns Specialist from both gender with different age, 64 samples from burn and wound infections from patients at Hospital, 116 samples from alopecia patients 58 (50%) of the affected area and 58 (50%) of the healthy areas (control) of the same patients. The specimens were transferred using sterile swabs immersed in transport medium and stored in a cooling box to the college of biotechnology labs at Al-Nahrain University. We inoculated each swab into nutrient broth and incubated it at 37 °C for 24 hours.

Isolation and Identification of bacterial isolates

Swabs of cultures from transmitted medium were streaked onto nutrient agar and incubated for 24-48 h at 37 °C. Fungi were excluded and the bacterial colonies were transferred and streaked on nutrient agar medium. The growth colonies were cultured on MacConkey and Manmedia. The colonies growing on the two media were selected and examined under the microscope and stained with Gram stain. The golden and non-golden colonies were selected from the Menthol culture and transferred to the slant nutrient agar medium. The colonies growing on MacConkey and not fermenting lactose were selected and transferred to

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the slant nutrient agar medium for subsequent studies. And further analysis, including Gram staining and microscopic assessment for shape and motility and to identify the isolation, VITEK 2 system compact ID gNB cards were used.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC for each isolate was determined using the VITEK-2 system (Biomerieux, USA).

Results and Discussion

Isolation of bacterial isolates

A total of 180 clinically significant samples from patients with wounds, burns and alopecia were collected, Among them, 64 swabs of burn/ wounds patients, 116 swabs were for alopecia patients (affected area and control). This cultivated sample may produce bacterial communities indicative of these disturbances, perhaps including dangerous strains like *Staphylococcus spp*, which flourish in inflamed or damaged skin conditions. Control Sample (Healthy Region), This specimen is derived from the unaffected, healthy skin of the same subject. It signifies the baseline microbiome or the "normal" configuration of skin microorganisms in the absence of pathological alterations. The culture results often indicate a steady equilibrium of commensal bacteria, including less harmful species, which contribute to skin health maintenance. The results showed that from a total of 58 affected area of alopecia specimens, only 27 (46.5%) were clinical positive growth samples, while the rest 31 were negative growth samples (53.4 %). And from 64 of burns and wounds specimens, only 25 (39%) were positive growth samples, while the rest 39(56.25 %) were negative growth samples as show in Table 1. Negative growth results may suggest to absence of infection at the time of sample collection or successful treatment of infection, some organisms may not grow easily in standard lab conditions**.**

Identification

Ten isolates (35.71%) from the positive growth were primary identified as *Staphylococcus spp* which collected from alopecia areata, Some strains possess the golden appearance suggests the presence of *S. aureus*, a species known for producing staphyloxanthin, a carotenoid pigment. This pigment gives *S. aureus* its distinctive golden color and serves as a virulence factor, enhancing its ability to evade host immune responses[17]. It also has the ability to ferment mannitol, which results in a change in the color of the medium as a result of acid production during the fermentation process[18]. The presence of *S. aureus* and *S. epidermidis* and some undefined isolates in the infected area, as shown in figure (1), aligns with the hypothesis that opportunistic pathogens might exploit the disrupted skin barrier and inflamed microenvironment characteristic of alopecia areata. while Colorless Colonies from the control area, a common commensal bacterium found on healthy skin[19].

For burn and wound samples also ten isolates identified as *P. aeruginosa* from both gender with different age: using the traditional culture method MacConkey agar and nutrient agar and microscopic examination As shown in figure (2), as well as VITEK-2 compact as a confirmatory test. The isolates were diagnosed as *Staphylococcus* for alopecia isolate and *P. aeruginosa* for wound and born isolates.

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Table 1: Isolation and identification of *Staphylococcus* spp. and *P. aeruginosa* isolates using VITEK-2 system

Sample Type	Total No. of sample	No. of grown sample	No of *Staphylococcus*	Number of *P. aeruginosa*
Alopecia	116	27	10*	-
Burn/Wound Infection	64	25	-	10
Total	**180**	**52**	**10**	**10**

**S. aureuS (3)isoaltes;*Coagulase nagative *Staphylococcus*—> (4); *S.epidermides* (1); *S. hominis ssp hominis*(2)

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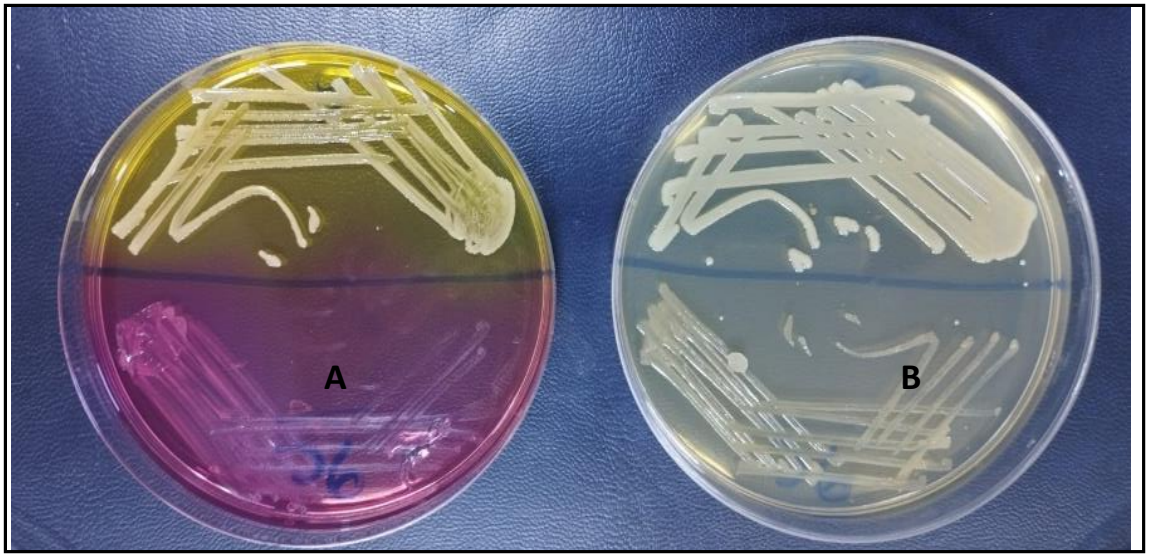

Figure 1. Comparison of bacterial isolates from Alopecia-affected (A) and healthy Scalp (B) regions in the same patient.

Figure 2. Growth of *Pseudomonas aeruginosa* on MacConkey agar.

On the other hand, *P. aeruginosa* bacteria were cultivated on the medium of the MacConkey aquarium in the form of pale colonies. While it formed in the shape of irregular, opaque colonies, it had a glossy buttery texture and gave off a fruity smell[20] . It was a pale green color, smooth, flat, and regular, and it did not ferment the lactose, G-ve, oxidase-positive, and catalase-positive The isolates of *S. aureus* were G+ve, catalase-positive, and oxidase-negative.

Minimum Inhibitory concentration (MIC) determination using VITEK-2 system

The VITEK 2 compact system was utilized to determine the (MIC) values for eighteen antibiotics against 10 isolates from alopecia and 10 isolates from burn and wound infection. The findings demonstrated a significant bacterial resistance and minimum inhibitory concentration of Utilized Antibiotics The resistance of bacteria, as illustrated in the in the subsequent table 2 and table 3.

Table (2): MIC determination of *Staphylococcus spp* against antimicrobial agent using VITEK-2

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N.	Antibiotic	MIC (µg/ml)[1]	R	S	I
1	Cefoxitin Screen	≥0.5	9	1	
2	Benzylpenicillin	-	10		
3	+Piperacillin/Tazobactam		10		
4	Oxacillin	≥1	10		
5	Gentamicin	≥16	3	7	
6	Tobramycin	≥16	3	7	
7	Levofloxacin	≥4	4	5	1
8	Moxifloxacin	≥2	1	7	2
9	Inducible Clindamycin Resistance				
10	Erythromycin	≥8	9		1
11	Clindamycin	≥4	8	2	
12	Linezolid	≥8	6	4	
13	Teicoplanin	≥32	6	3	1
14	Vancomycin	≥16	7	2	1
15	Tetracycline	≥16	6	2	2
16	Tigecycline	≥1	1	9	
17	Nitrofurantoin	≥78	5	4	1
18	Fusidic Acid	≥32	9	1	
19	Rifampicin	≥4	6	4	
20	Trimethoprim/ Sulfamethoxazole	≥76		10	

Table (3) : MIC determination of *Pseudomonas aeruginosa* against antimicrobial agent using VITEK-2

N.	Antibiotic	No.of isolates	MIC Mg/ml	R	S	I
1	Ticarcillin	10	≥ 128	7	2	1
2	Amikacin	10	≥ 128	6	4	
3	Ticarcillin/Clavulanic Acid	10	≥ 128	6	4	
4	Gentamicin	10	≥ 16	5	5	
5	Piperacillin	10	≥ 128	6	4	
6	-Netilmicin	10		5	5	
7	+Cefixime	10		4		6
8	Tobramycin	10	≥ 16	5	5	
9	+Cefpodoxime	10		4		6
10	Ciprofloxacin	10	≥ 4	6	4	
11	+Cefotaxime	10		4		6
12	-Levofloxacin	10		4		6
13	Ceftazidime	10	≥64	3	6	1
14	+Norfloxacin	10		4	4	2
15	+Ceftriaxon	10		8		2
16	+Ofloxacin	10		4	4	2
17	Cefepime	10	≥8	3	5	2
18	+Ertapenem	10		4		6
19	Colistin	10	≥2	3	6	2
20	Imipenem	10	≥ 16	4	5	1

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The resistance and sensitivity percentages of antibiotics for *S. aureus* isolates from alopecia, as determined by VITEK device tests, indicate a 76% resistance rate to erythromycin, as reported by Giulieri *et al* (2022)[21]. The current investigation identified a 70% resistance rate to oxacillin, in contrast to Shaker (2018)[22], who documented a 100% resistance rate to this antibiotic. Additionally, the current study revealed a 70% resistance rate to trimethoprim/sulfamethoxazole, contrasting with Jameel (2018)[23], who indicated a 100% resistance rate to trimethoprim/sulfonamide.

Al-Azzawi (2018) [24] reported that in her investigation of 69 isolates of *P. aeruginosa*, 78% were resistant to Cefotaxime, 70% to Meropenem, and 66% to Imipenem. A study by Ameen et al. (2015) [25] on 230 isolates of *Pseudomonas aeruginosa* revealed that 49.9% of the isolates were resistant to Imipenem, while a study by Musafer et al. (2013)[26] on 58 isolates indicated a 15.52% resistance to Meropenem. A primary factor contributing to *P. aeruginosa* resistance to the beta-lactam class is its synthesis of beta-lactamase enzymes (Penicillinase), which target the beta-lactam ring present in penicillin's and cephalosporins, rendering them ineffective, alongside its possession of additional virulence factors. The isolates exhibited resistance to the class of anti-aminoglycosides due to the bacterial synthesis of mutated enzymes, including phosphotransferase and N-acetyltransferase, with other virulence factors.

The MIC results from *S. aureus* and *P. aeruginosa* isolates present clinically significant multidrug resistance profiles, which have direct implications in the management of infections associated with alopecia, wound and burn infection. Bacterial colonization in skin infection sites is increasingly recognized as a factor that not only influences disease progression but also introduces considerable challenges in therapeutic intervention due to antimicrobial resistance. Effectively managing such cases necessitates a tailored antibiotic regimen based on susceptibility testing, ensuring that therapeutic efforts are directed towards reducing bacterial burden and preventing further complications associated with infection-driven inflammation and tissue damage.

Conclusions

This study shows that *Staphylococcus* species that are resistant to antibiotics may make alopecia worse, so it's important to use specific antimicrobial drugs based on accurate susceptibility testing for effective treatment. The fact that resistant *Staphylococcus* species are found in the scalp microbiome suggests that the presence of microbes may make alopecia worse by making treatment more difficult. This study stresses how important it is to use specific antimicrobial treatments based on thorough susceptibility testing in order to help people with alopecia, especially when there are resistant bacterial strains present. The results show that a planned, evidence-based approach is needed to lower the number of bacteria, reduce inflammation caused by infections, and improve therapeutic outcomes in people with alopecia. The occurrence of clinically significant *P. aeruginosa* in burn and wound infections is negligible in this facility. Nevertheless, *P. aeruginosa* is a common causative agent of infection in our burn centers, aligning with global observations. The isolates of *P. aeruginosa* that were tested were most sensitive to Ticarcillin and least sensitive to Gentamicin. This suggests that Gentamicin is less effective at treating *P. aeruginosa* infections in burn wounds. Most *P. aeruginosa* isolates demonstrated significant sensitivity to most of the antibiotics tested.

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Express our gratitude to Al-Nahrain University and the Collage of Biotechnology, namely the head of the collage, head of plant biotechnology department, for their consent to proceed with this study. Gratitude is expressed to the researchers who contributed to the successful completion of this study.

Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

[First Author]: Contributed to the conception and design of the study, conducted some experiments, data rearrangement and drafted the initial manuscript.

[Second Author]: conducted some experiments, collection a part of literature review and conducted some characteristics of the products.

References

1. Coates, M., Blanchard, S. & MacLeod, A. S. Innate antimicrobial immunity in the skin: A protective barrier against bacteria, viruses, and fungi. *PLoS Pathog.* **14**, e1007353 (2018).
2. Percival, S. L., Emanuel, C., Cutting, K. F. & Williams, D. W. Microbiology of the skin and the role of biofilms in infection. *Int. Wound J.* **9**, 14–32 (2012).
3. Durand, B. A. R. N. *et al.* Bacterial Interactions in the Context of Chronic Wound Biofilm: A Review. *Microorganisms* **10**, (2022).
4. Minasyan, H. Sepsis: mechanisms of bacterial injury to the patient. *Scand. J. Trauma. Resusc. Emerg. Med.* **27**, 19 (2019).
5. McElwee, K. J. *et al.* What causes alopecia areata? *Exp. Dermatol.* **22**, 609–626 (2013).
6. Zhang, H. *et al.* Developing natural polymers for skin wound healing. *Bioact. Mater.* **33**, 355–376 (2024).
7. Mofazzal Jahromi, M. A. *et al.* Nanomedicine and advanced technologies for burns: Preventing infection and facilitating wound healing. *Adv. Drug Deliv. Rev.* **123**, 33–64 (2018).
8. Chhabra, S., Chhabra, N., Kaur, A. & Gupta, N. Wound Healing Concepts in Clinical Practice of OMFS. *J. Maxillofac. Oral Surg.* **16**, 403–423 (2017).
9. Morguette, A. E. *et al.* The Antibacterial and Wound Healing Properties of Natural Products: A Review on Plant Species with Therapeutic Potential against Staphylococcus aureus Wound Infections. *Plants* vol. 12 at https://doi.org/10.3390/plants12112147 (2023).
10. O'Dell, M. L. Skin and wound infections: An overview. *Am. Fam. Physician* **57**, 2424–2432 (1998).
11. Fayisa, W. O. & Tuli, N. F. Review on Staphylococcus Aureus. *Int J Nurs. Care Res.* **1**, 1–8 (2023).
12. Głowacka, P., Zakowska, D., Naylor, K., Niemcewicz, M. & Bielawska-Drózd, A. Brucella – Virulence factors, pathogenesis and treatment. *Polish J. Microbiol.* **67**, 151–161 (2018).
13. Qin, S. *et al.* Pseudomonas aeruginosa: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. *Signal Transduct. Target. Ther.* **7**, 199 (2022).

14. Colautti, A., Orecchia, E., Comi, G. & Iacumin, L. Lactobacilli, a Weapon to Counteract Pathogens through the Inhibition of Their Virulence Factors. *J. Bacteriol.* **204**, (2022).

15. Al-yasiri, S. & Risan, M. H. Identification , characterization and antibiotic susceptibility testing of Pseudomonas aeruginosa isolated from clinical sources. (2024).

16. Ahmed, A. S. Production of silver nanoparticles from the aerobic and anaerobic cultures supernatants of Pseudomonas aeruginosa. 1–10 (2024).

17. Liu, G. Y. *et al.* Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J. Exp. Med.* **202**, 209–215 (2005).

18. Thakur, P., Nayyar, C., Tak, V. & Saigal, K. Mannitol-fermenting and Tube Coagulase-negative Staphylococcal Isolates: Unraveling the Diagnostic Dilemma. *J. Lab. Physicians* **9**, 65–66 (2017).

19. Gradmann, C. Medical Bacteriology. *Routledge Hist. Dis.* 378–401 (2018) doi:10.4324/9781315543420-21.

20. Aldaraghi, W., Albadrawi, M. & RidaJassim, H. Molecular Detection of Pseudomonas aeruginosa and its Relationship with Multidrug Resistance and Transposons. *Indian J. Forensic Med. Toxicol.* **14**, 300 (2021).

21. G., G. S. *et al.* Comprehensive Genomic Investigation of Adaptive Mutations Driving the Low-Level Oxacillin Resistance Phenotype in Staphylococcus aureus. *MBio* **11**, 10.1128/mbio.02882-20 (2020).

22. Shaker, M., Zaki, A., Asser, S. L. & Sayed, I. El. Trends and predictors of antimicrobial resistance among patients with urinary tract infections at a tertiary hospital facility in Alexandria, Egypt: a retrospective record-based classification and regression tree analysis. *BMC Infect. Dis.* **24**, 1–15 (2024).

23. Zahraa Jaafar Jameel, Sura Hameed Nayef & Muthana Abdulkhader Al-Mahdawi. Study Some Virulence Factors of CO- agulase Positive Staphylococci and CO-agulase Negative Staphylococci Isolated from Different Parts of Human Body. *Tikrit J. Pure Sci.* **20**, 47–54 (2023).

24. Al-Azzawi, S. N. A. & Abdullah, R. M. Study of the resistance of p. Aeruginosa isolated from wounds and burns for some disinfects and antiseptic from some baghdad hospitals. *J. Pharm. Sci. Res.* **10**, 1481–1484 (2018).

25. Ameen, N., Memon, Z., Shaheen, S., Fatima, G. & Ahmed, F. Imipenem Resistant Pseudomonas aeruginosa: The fall of the final quarterback. *Pakistan J. Med. Sci.* **31**, 561–565 (2015).

26. Hadeel, K. M., Harith, J. F. A.-M. & Majeed, A. S. OprD Expression and Imipenem Resistance in Pseudomonas aeruginosa. *Greener J. Biol. Sci.* **3**, 325–329 (2013).