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

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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 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).

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

No.	Source of isolates	No. of isolate	%

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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%**

MacConkey agar

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

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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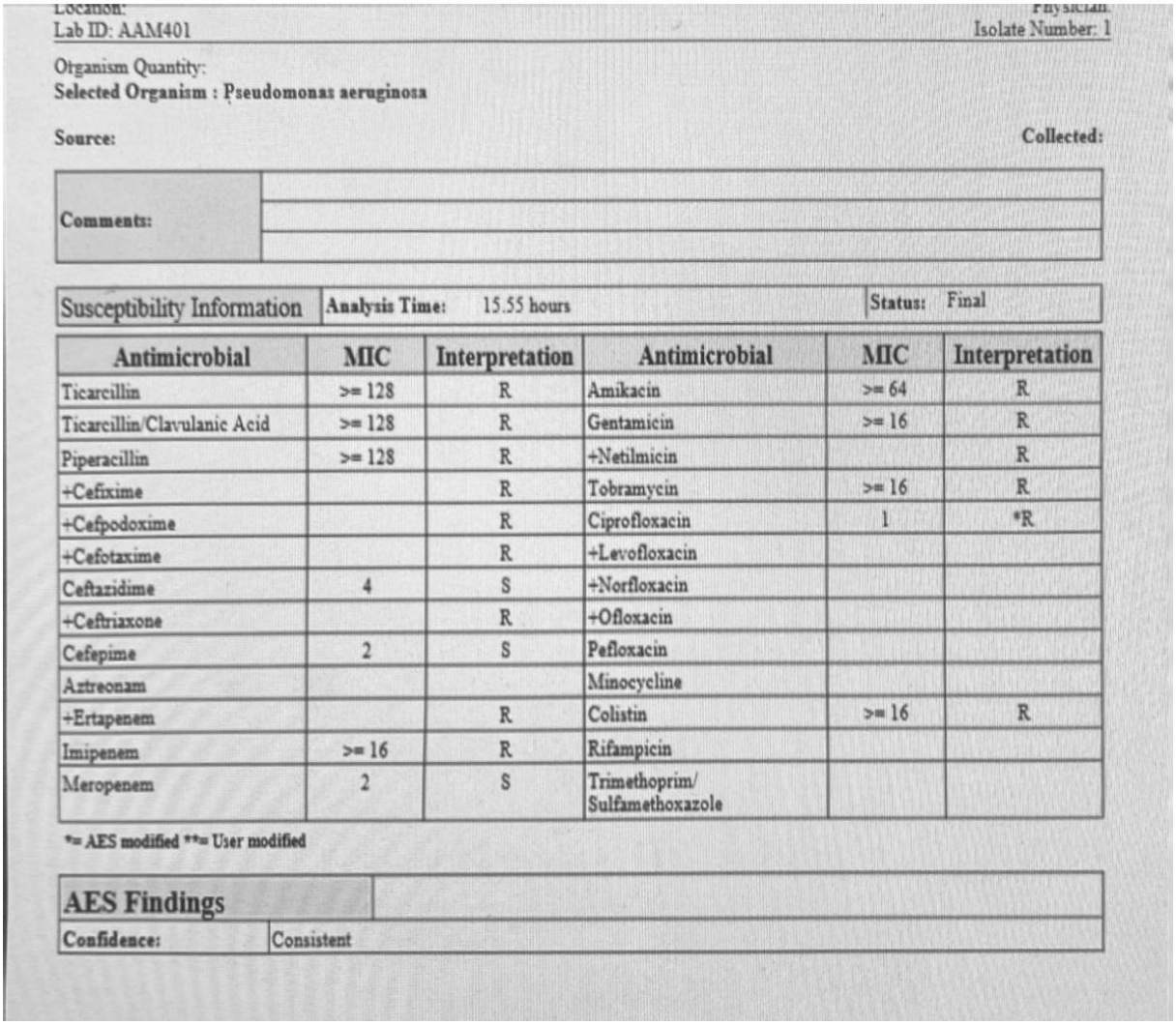

Location: Physician
Lab ID: AAM401 Isolate Number: 1

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.

Iraqi journal of Bioscience and Biomedical
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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).

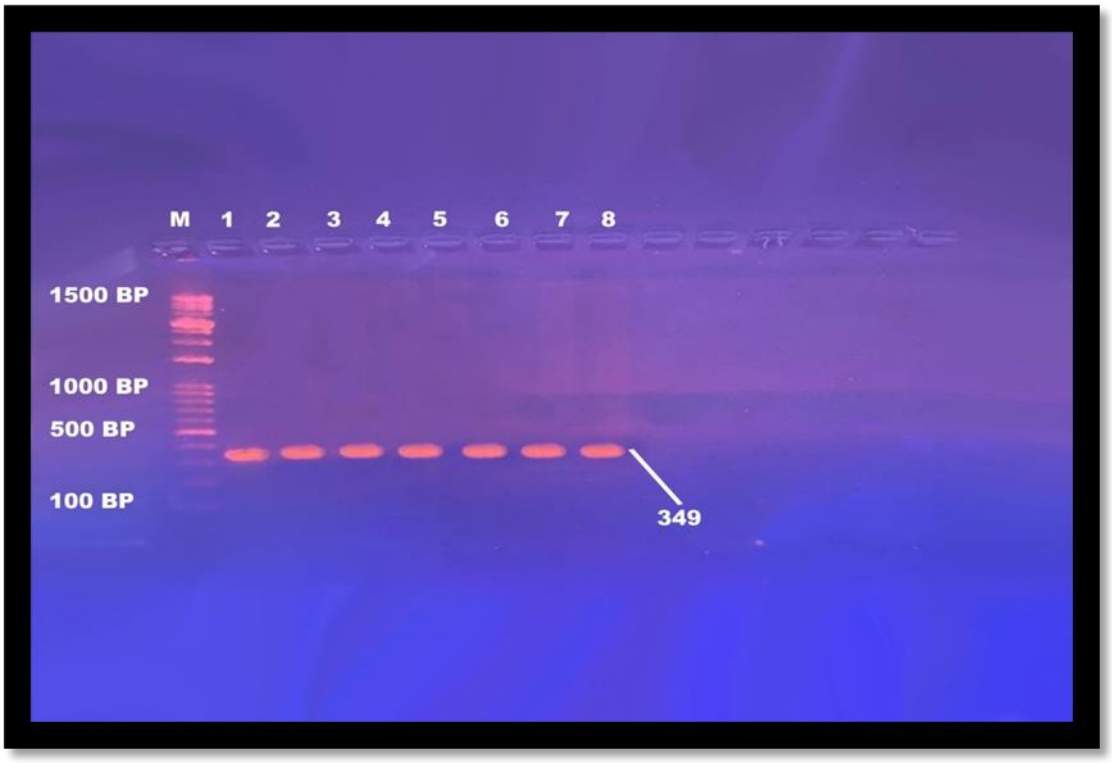

Figure 4: PCR profile for the amplified *lptE* gene bands on 349 bp of *Pseudomonas aeruginosa* isolates were fractionated on agarose 1.5% at 100v/m Amp for 60 minutes, Lane M: DNA ladder marker (100 bp-1500 bp).

Iraqi journal of Bioscience and Biomedical
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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].

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%).

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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.

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