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Antimicrobial Susceptibility of *Enterococcus faecium* Isolated from Bee-gut on *PhzM* Gene of *Pseudomonas aeruginosa* Isolates from Infected Wounds

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Abstract

The aim of this study was to isolate and characterize Fructophilic lactic acid bacteria (FLAB) species from the honeybee gut. Based on the results of this study, it was found that the FLAB species obtained from honey were Gram-positive and catalase-negative, and this identification was confirmed through *16S rRNA* gene sequencing. It was performed a primary screening to evaluate the effect of the cell-free supernatant (CFS) obtained from *Enterococcus faecium* (E5), and it was observed that the CFS showed a high inhibition zone of 23 mm against multidrug-resistant Pseudomonas aeruginosa, as determined by the agar well diffusion assay. This study also conducted further investigation to determine the optimal conditions for the production of cell-free supernatant (CFS). The results indicated that yeast extract was the most effective nitrogen source, while glucose was the preferred carbon source for CFS production. The optimal pH for CFS production was 5, and the incubation period of 72 hours was determined to be the most suitable for obtaining a high yield of CFS. Another aspect of the study aimed to identify multidrug-resistant *Pseudomonas aeruginosa* isolates from burn wound infections. The isolates were identified using the VITEK 2 system, and the presence of the *phzM* gene was detected in all nine strains. Furthermore, the study evaluated the effect of the cell-free supernatant (CFS) of the selected strain (E5) on the expression of the *phzM* gene. According to the study, the cell-free supernatant (CFS) significantly decreased the expression of the *phzM* gene in isolates of multidrug-resistant *Pseudomonas aeruginosa*. *Enterococcus faecium* could be a useful antimicrobial agent for treating *P. aeruginosa* infections that are resistant to multiple drugs.

Keywords: *Pseudomonas aeruginosa*, *phzM* gene, bee-gut, multi-drug resistance.

1. Introduction

Enterococcus faecium is found in the gut flora of both humans and animals and is also known as an opportunistic pathogen. They are mainly associated with hospital-acquired infections in humans as well as many in animals, such as mastitis in cattle, diarrhea in swine and cattle, and septicemic diseases in poultry [1]. Certain virulence factors can be produced by *E. faecium* that potentially increase their pathogenicity, thereby contributing to the development of diseases [2]. The present study aimed to analyze the antagonistic activity of lactic acid bacteria (LAB), which are present in the honeybee environment, against both known honeybee pathogens and opportunistic pathogens. The LAB strains used in the experiments were previously isolated and characterized from the honeybee environment [3]. This study examined the postbiotic properties

of lactic acid bacteria (LAB) metabolites in inhibiting the growth of honeybee pathogens at both physiological and neutral pH levels. The authors believe that the topic of LAB's antagonistic activity against honeybee pathogens has not been extensively studied, noting that no research has been conducted to assess the postbiotic impact, like cell-free supernatants, on the growth inhibition of pathogenic microorganisms [3]. *Pseudomonas aeruginosa* is most frequently found in patients with compromised immune systems who had an extensive medical operation or those with diseases such as diabetes. It is a non-fermenting, Gram-negative pathogen and is considered one of the most hospital-associated infections [4]. *P.aeruginosa* Infections usually develop in connection with medical devices such as central lines, ventilators, and urine catheters, and more recently, coinfection in patients hospitalized with COVID-19 has been discovered to be prevalent with *P.aeruginosa* [5]. Without the existence of new therapeutic agents, the problem of drug resistance may worsen. Recently, targeting virulence factors has appeared to be a new line of action against MDR *P.aeruginosa* strains. [6]. *P.aeruginosa* has the ability to synthesize several compounds, including pyocyanin. It stimulates the production of reactive oxygen species (ROS) by host cells and inhibits the expression of catalase, which is an enzyme that neutralizes ROS by oxidizing NADPH [7]. In addition, a decrease in lung function and a contribution to the dominance of *P.aeruginosa* in the CF lung are related to pyocyanin [8]. The aim of this research is to detect the effect of FLAB suspension on upregulation or downregulation of the pyocyanin gene.

2. Materials and Methods

2.1. Sample collection

Ten female worker bees were collected during the summer foraging season and transported to the laboratory for analysis. To avoid contamination by external microbes, the bees were treated with 70% ethanol for 60 seconds. In a laminar flow hood, the researchers dissected the bees and isolated the stomach and midgut from the rest of the alimentary canal for further analysis [9].

2.2. Isolation and identification of *E. faecium* from honeybee gut

The procedure of isolation was done according to [3]. To isolate bacterial cultures from the nectar stomach of each bee, the process involved dissecting under sterile conditions within a laminar flow hood, using sterile forceps. The nectar stomach was separated from the remaining gut contents and placed in sterile tubes containing MRS broth supplemented with 2% fructose and 0.1% cysteine at a volume of 10 ml. Incubation of the tubes occurred for 24-48 hours at a temperature range of 28-30°C anaerobically to promote lactic acid production [10]. The pure cultures obtained were regrown in MRS broth, and after incubation, 200 µl of glycerol was added to 800 µl of MRS broth culture in a 2 ml microtube.

2.3. 16S rRNA gene sequencing

The characterization of unidentified bacteria using 16S rRNA gene sequencing was carried out according to previous work [11], with some modifications that are described here briefly. Colonies of each unidentified bacteria were re-cultured for 24–48 hours, depending on their growth conditions. The genomic DNA of bacteria was extracted using the ABIOpureTM Total DNA Kit (USA) before PCR amplification of 16S rRNA genes. Amplification of isolates was performed using universal primers (5’-AGAGTTTGATCCTGGCTCAG-3' and 5'-TACGTTACCTTGTTACGACTT-3’) [12]. PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation, Korea. The results were received by email and then analyzed using Geneious software.

2.4. Antimicrobial spectrum analyses

The antimicrobial activity of Fructophilic lactic acid bacteria suspension was obtained by growing FLAB in tubes with MRS broth, which contains 0.1% cysteine and 2% fructose, anaerobically in an anaerobic jar at 28–30 °C for 24-48 hours, then centrifuged at 6000 rpm for fifteen minutes, after which it was transferred to fresh tubes, filtered with sterile syringe filters with a pore size of 0.22 µm, and kept at 4°C in sterilized test tubes [13].

2.5. Screening for CFS production

After obtaining CFS, the antimicrobial activity of the cell-free supernatant was determined using the agar-well diffusion assay. 50µl of the supernatant was placed in 6mm-diameter wells cut with a cork borer into cooled Mueller Hinton agar plates inoculated with $1.5*10^8$ (cell/ml) that confronted McFarland tube (0.5 turbidity) of the MDR *P. aeruginosa.* The supernatant was allowed to diffuse by leaving the plates at room temperature for 1h before incubation at 37 ºC for 24h and examined for the presence of an inhibition zone [14].

2.6. Synthesis of bacteriocins is optimized numerous factors were examined to determine the ideal settings for high-level bacteriocin synthesis, and these are [15]

2.6.1. Determination of optimum pH

The production and growth of cell-free supernatants from various isolates were examined to determine the impact of pH. To achieve different pH values of 5, 6, 7, and 8, MRS broth was prepared in 10 ml tubes and adjusted with 0.5 N HCl or 0.5 N NaOH after autoclaving. The tubes were then inoculated with selected strains and incubated at 37°C under anaerobic conditions for 24 hours.

2.6.2. Determination of optimum incubation

To investigate the impact of the incubation period, 10 ml of a selected strain (1.5×10^9 CFU/ml) was incubated in a MRS broth medium for different durations (18, 24, 48, and 72 hours) at 37ºC under anaerobic conditions.

2.6.3. Effect of Nitrogen Source & carbon source (sugars)

Included (Tryptone, yeast extract, sucrose, and glucose) with concentrations were used, with 1% of each one of them added to MRS broth.

2.7. *P. aeruginosa* isolation

Ten samples were collected from patients suffering from wound infections in an indoor setting after obtaining ethical approval from the Ethical Committee in the Department of Biology, College of Science, University of Baghdad. Before sample collection, signed consent was obtained from the patients. The samples were cultured under sterile conditions on nutrient agar, followed by culturing on cetrimide agar and MacConkey agar [16]. The VITEK 2 system was then used to confirm the identification of the bacterial isolates [17].

2.8. Detection of *phzM* gene

The tested gene was amplified by conventional PCR using primers obtained from [18]. PCR amplifications were carried out in 20 µl volumes containing 10 µl of GoTaq Green Master Mix (2X), 1µl of primer (10 pmol), 6 µl of nuclease-free water, and 2 µl of template DNA. The PCR cycling was performed using a PCR Express (Thermal Cycler, Thermo Fisher Scientific, USA), following this temperature program: initial denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55, 58, 60, 63, or 65°C for 30 seconds, and extension at 72°C for 30 seconds. The final extension step was carried out at 72°C for 7 minutes, followed by a 10-minute incubation at 4°C to stop the reactions. The primer sequence for phzM used in this study is mentioned in **Table 1**.

Table 1. Primer used in PCR and real-time PCR.

Primer Name	Sequence 5`-3`	Annealing Temp.(°C)	Product size (bp)	Reference
phzM-F	ACGGCTGTGGCGGTTTA	60	~180	[18]
phzM-R	CCGTGACCGTCGCATT			
fbp-F	CCTACCTGTTGGTCTTCGACCCG	53	53	[19]
fbp-R	GCTGATGTTGTCGTGGGTGAGG			

2.9 RT-qPCR protocol

Real-time quantification of cDNA was carried out on the GoTaq® 1-Step RT-qPCR System (Promega, USA) using the SYBR green PCR master mix. Real-time PCR was used to investigate the expression levels of the *phzM* and *fbp* genes. In order to assess the gene expression of the *phzM* gene, the results were normalized using the *fbp* gene, which is considered a housekeeping gene. Primers of these genes **Table 1.** were provided in a lyophilized form and dissolved in sterile nuclease-free water to give a final concentration of 100 pmol/µl. Afterwards, they were stored in a deep freezer until used in qPCR. The reaction mixture was summarized in **Table 2.**

Table 2. The components of master mix in qRT-PCR**.**

Master mix components	Unit	Volume / 1 Sample µl
qPCR Master Mix	X	5
RT mix	x	0.25
MgCl2		0.25
Forward primer	µM	0.5
Reverse primer	µM	0.5
Nuclease Free Water		2.5
RNA	ng/µl	1
Total volume	10	

3. Results and Discussion

3.1. Identification of Bacterial Isolates

The isolation FLAB species (E_1 –E_{10}) characterization such as Gram-positive, catalase-negative, and appearing as large, white colonies were isolated on MRS**.** The result agrees with Elzeini *et al*. [20]. LAB was isolated from the gastrointestinal tract of the bee.

After sequencing the 16S rRNA gene, the isolate was identified, and the resulting sequence was submitted to the NCBI database. The accession number showed a high level of similarity to *E. faecium*. This finding is consistent with the identification of enterococci isolates from both dairy and honeybee samples using 16S rRNA gene sequencing, as depicted in **Figure 1** [21].

Samples were collected from patients with burn and wound infections, and several isolates of *P. aeruginosa* were identified. The colony shapes of these isolates were determined using selective media. On MacConkey agar, the isolates that were able to grow were observed as pale colonies, while on Cetrimide agar, *P. aeruginosa* isolates appear as mucoid, smooth colonies with flat edges and elevated centers and exhibit the ability to produce a blue-green pigment after 72 hours of incubation.

The confirmation of all isolates was done and diagnosed using the VITEK 2. From the 10 clinical isolates named (Z1, Z2, Z3, Z5, Z4, Z9, 72, 77, and 79), the current results agree with [17]. The results of the Vitek 2 automated system show a probability of about 95%–99% belonging to the genus *Pseudomonas aeruginosa*.

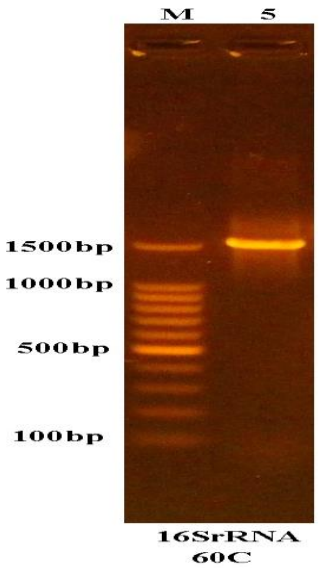

Figure 1. Results of amplification of 16SrRNA gene of bacterial samples were fractionated on 1,5% agarose gel electrophoresis stained with E th..Br. M: 100 bp ladder marker. Lanes 5 resemble 1500 bp PCR.

3.2. Screening of antimicrobial activity of LAB isolated from honey-bee

E. faecium strain screening the best CFS crude antimicrobial activities' spectrum as each of them had activity against multidrug-resistant *P. aeruginosa* shown in **Figure 2**. The cell-free culture supernatant (CFS) antibacterial activities were assessed by an agar-well diffusion assay [22]. The 23-mm inhibition zone diameter at the pH of the CFS was modified to 5. Other results agree that FLAB strains have been isolated from bees and selected for their broad antimicrobial activity against pathogenic bacteria [23, 24].

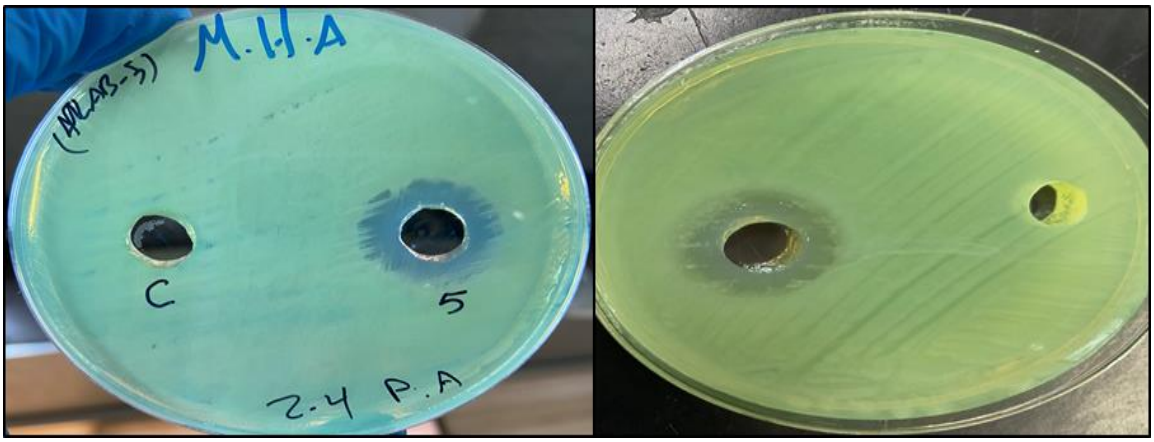

Figure 2. Effect of antimicrobial activity of FLAB on *P. aeruginosa* on MHA anaerobic condition at 37C.

3.3. Optimum conditions for bacteriocin production

3.3.1. Optimal pH

The results indicate that the diameter of the inhibition zone reached 22.5 mm, and pH 5 was the best value for production. The lower inhibition zone 15.12 mm at pH 8 is shown in **Figure 3**. According to the results that correlate with [25], the antimicrobial activity of both CFS and crude enterocin was stable at pH levels varying from 4 to 8.

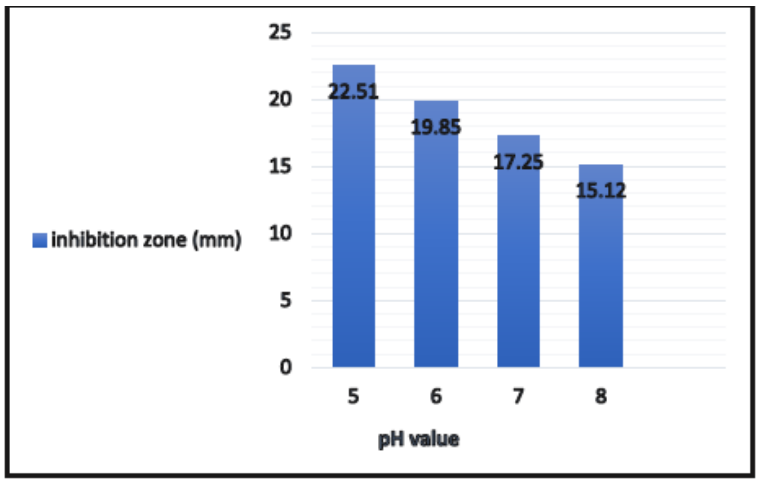

Figure 3. Effect of pH on CFS production**.**

3.3.2. Optimum incubation time

The production of CFS (cell-free supernatant) was observed at various incubation times, and it was noted that the highest production occurred after 72 hours of incubation, resulting in the largest inhibition zone measuring 24 mm. However, the activity of CFS decreased after 48 and 18 hours, with the lowest inhibition zone measuring 10 mm (as illustrated in **Figure 4**). These findings agree with [26] and [27]. The observations found the highest activity from *E. faecalis* was observed after a three-day incubation period.

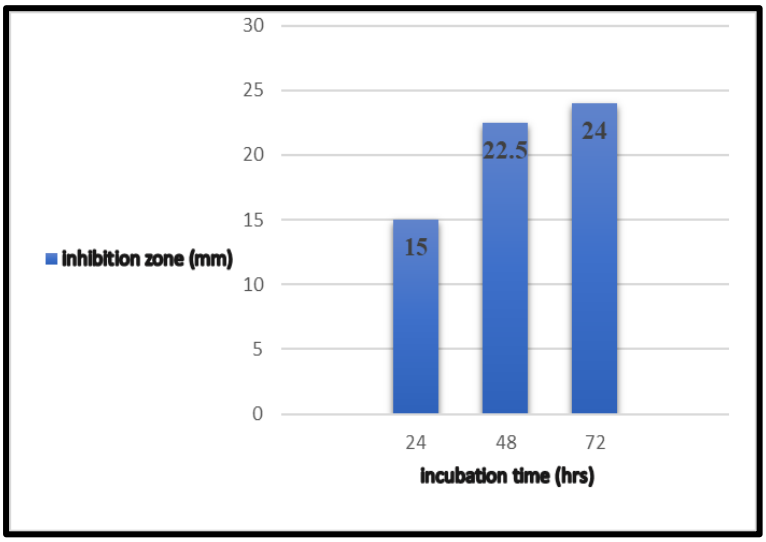

Figure 4. Effect of incubation time on CFS production**.**

3.3.3. Nitrogen &carbon source

The optimum nitrogen Sources such as yeast extract reached 24 mm zone and was the best nitrogen source than tryptone; scours best carbon source than glucose reached 22 and 21 mm, respectively, as in **Figure 5**. The results demonstrated that under specific production conditions, there was an increase in the antimicrobial activity produced [28].

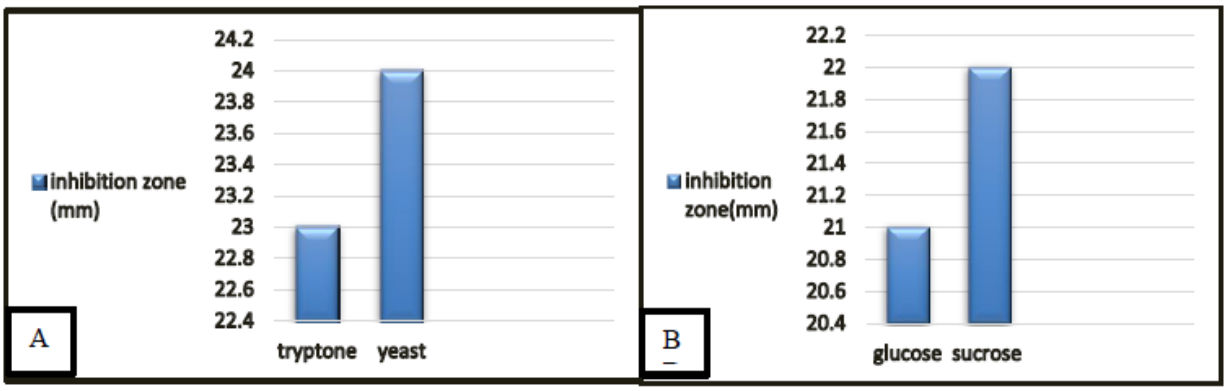

Figure 5. A. Effect of Nitrogen source, **B.** Effect of carbon source (sugars) on CFS production.

3.4. Polymerase Chain Reaction (PCR)Technique

The PCR results identified nine isolates of *P. aeruginosa* with the *phzM* gene, selected based on their multidrug resistance (MDR). The positive gene result was subsequently confirmed through electrophoresis on a 1.5% agarose gel stained with ethidium bromide, electrophoresed at 75 volts for 50 minutes, and visualized under an ultraviolet (UV) transilluminator. The present study revealed the presence of a sharp, singular, and non-dispersed 180 bp *phzM* gene band, which was clearly distinguished from the DNA ladder, as demonstrated in **Figure 6**. Notably, there was no evidence of DNA degradation, as indicated by the absence of any smearing of the gene band.

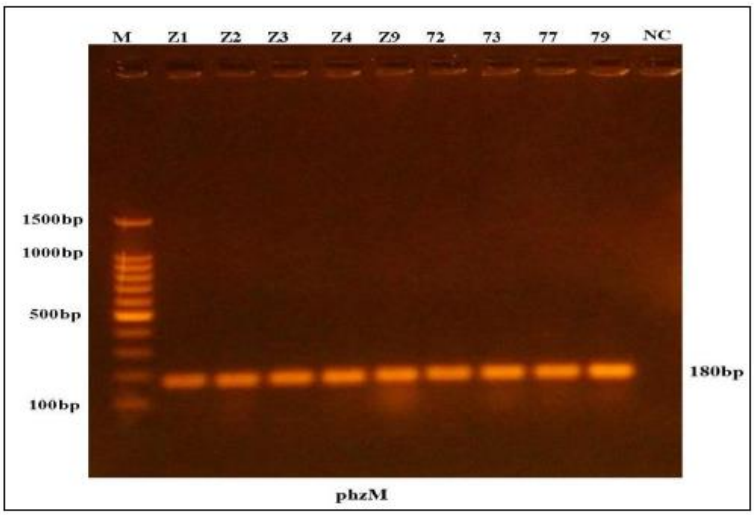

Figure 6. Results of the amplification of *phzM gene* of *Pseudomonas aeruginosa* bacterial samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes Z1-79 resemble 180bp PCR products**.**

To estimate the effect of CFS on *E. faecium* at concentrations of 8 µg/ml, two *P. aeruginosa* isolates were studied using the RT-qPCR technique. RT-PCR reveals a major downregulation in *phzM* expression after exposure to the CFS suspension of *E. faecium* compared to normal gene expression in bacteria. A fold change in gene expression reveals that *phzM* was downregulated in response to CFS in all isolates of *P. aeruginosa*, as entailed in **Table 3**. The result agrees with [29]. Mechanical exploration of different antimicrobial substances was performed with the loss of functional genes involved in pyocyanin biosynthesis strain-derived *phzM* 73 and z1. The other

gene was *P. aeruginosa.* At the molecular level, *amrZ* (a global regulator of multiple genes) and *rhl* (responsible for rhamnolipid production) [30].

Table 3. gene expression changes before and after treatment with CFS.

Sample	Fbp	phzM	dct	ddct	Folding
73(before)	18.52	15.28	-3.25	0.00	1.00
73(after)	19.00	16.08	-2.92	0.33	0.79
Z4(before)	21.76	17.58	-4.17	0.00	1.00
Z4(after)	19.76	16.17	-3.59	0.59	0.67

The results indicated a major down-regulation in *phzM* expression after exposure to CFS. The fold change in gene expression showed that *phzM* was down-regulated in response to CFS in all isolates of *P. aeruginosa*. These findings suggest that the CFS of *E. faecium* can have an inhibitory effect on *phzM* gene expression, which may lead to the loss of functional genes involved in pyocyanin biosynthesis.

5. Conclusion

The use of FLAB as an alternative to antibiotics can reduce the use of antibiotics and is important in the context of the global problem of antibiotic resistance. *E. faecium* bacteria isolated from the gut of honeybees have antimicrobial properties that make them a promising alternative to antibiotics and have been shown to be effective in inhibiting the expression of important virulence factors in *P. aeruginosa phzM* gene expression for pyocyanin. This inhibitory effect on pyocyanin gene expression may represent a potential strategy for controlling *P. aeruginosa* infections.

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Conflict of Interest

There are no conflicts of interest.

Funding

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Ethical Clearance

This research was subjected to ethical considerations, and the research was approved by the Committee of Ethical Standards in the college Science, University of Baghdad, in line with the form issued for this purpose by the Iraqi Ministry of Health.

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