Sys Rev Pharm 2020;11(12):1483-1494
A multifaceted review journal in the field of pharmacy

DETECTION OF EXOA, NAN1 GENES, THE BIOFILM PRODUCTION WITH THE EFFECT OF OYSTER SHELL AND TWO PLANT EXTRACTS ON PSEUDOMONAS AEROGINOSA ISOLATED FROM BURN' PATIENT AND THEIR SURROUNDING ENVIRONMENT

Raghad J. Mahdi Alhamdani and Yasin Y. Y. Al-Luaibi

Department of Biology, College of Science, University of Basrah, Basrah, Iraq

Abstract

Burns are big issue worldwide, can be caused by electricity, nuclear radiation, heat, and chemicals. Infections with multi-drug resistant bacteria delay the burn's healing and make it more complex. Many bacteria can cause burn infection including *Pseudomonas aeroginosa*.The present study included collection of 112 samples from Al-Fyahaa General Hospital \ Burn Center in Basra \ Iraq; 68 samples from burn' patients and 44 samples were collected from the burn patients' surrounding environment; included (air, bed, patient companion, and nursing room). The present study recorded that the highest cases of burns (48.53%) were due to hot water whereas the lowest were by electricity (10.29%). Using biochemical tests, Vitek2 and *16S rRNA* gene, 41 isolates were diagnosed as *Pseudomonas aeruginosa*, 26 isolates from burn patients and 15 isolates from the surrounding environment.

Among the virulence factors genes produced by *P. aeruginosa*, two genes encode for virulence factors; *exoA* and *nan1* were investigated. The results showed the presence of the *exoA* gene in 38 isolates out of the 41 (92.68%). While the *nan1*was found in 4 isolates only (9.75%). Biofilm production as one of the virulence factors using Congo red agar method was also studied, it was found that 36/41 (87.80%) were producing biofilm while (12.19%) only 5/41 were not.

The sensitivity of *P. aeruginosa* isolated in the present study to 10 antibiotics belonging to different groups were explained, the results showed that 38/41 (92.68%) with multiple resistance to antibiotics. It has been found that most of biofilm' producing *P. aeruginosa* was found to be resistant to up to 6 antibiotics from different groups. The high resistance *P. aeruginosa* isolated were then subjected to Oyster shell and natural plant extracts. Stem bark of *Ziziphus spina-christi var.malaci* gave high efficacy toward isolated *P. aeruginosa* from burn patients, the cold extract of stem bark were effective toward all isolates with the high inhibition diameter up to 30 mm comparing to the most effective antibiotic toward *P. aeruginosa*. The highest inhibition diameter for Oyster shell was 16 mm while the lowest efficacy was to the cold aqueous extract of Oyster shell. However, in an attempt to point the source of infection, it has not been found that the source of infection was from the patient' surrounding area and more studies are needed (under investigation).

Keyword: *P. aeroginosa*, *exoA* and *nan1* genes, Biofilm production, Oyster shell and natural extracts.

INTRODUCTION

Burns, is an injury that affects the skin, can be caused by several factors, including electricity, nuclear radiation, heat, and chemical properties. Burn' infection is a common health problem in many countries around the world (Mabrouk et al., 2016). The burn usually hurts the skin, the first streak of defense and the main barrier to infection (Lee et al., 2014). Patients with burns who entered hospital, 72 hours later are more likely to develop infections by antibiotics resistant and multidrug resistant bacteria (MRD), the patient survival become more complex and its life in dangerous. The microorganisms that exist at the beginning of the patient's entry commonly are Gram positive bacteria while Gram-negative bacteria will be found later (Lachiewicz et al., 2017). One of the most gram-negative pathogenic bacteria found in burn wounds is *Pseudomonas aeruginosa* due to its ability to adapt different environmental conditions where it exists in water, soil and humid environment (Singh et al., 2010). Due

to the numerous virulence factors created by the bacteria *Pseudomonas aerugnosia*, it is considered one of the most important and dangerous organisms in human infections. These genes recorded the highest prevalence of *Pseudomonas spp* in burn injuries more than urinary tract infections. The most produced genes in most *Pseudomonas* strains are the toxin factor encoded by toxin gene is *tox A*, followed by *exo S*, and *oprI gene* (Al-Kaaby., 2015). Furthermore, due to the diverse nature of *Pseudomonas aeruginosa* and its ability to use various energy sources and attaching to different surfaces, it can survives in low-nutrient environments, and can combine to each other to form biomass named biofilms (Ghanbari et al., 2016).

Neuraminidases encoded by the nan1 gene are among the main enzymes also produced by *Pseudomonas* strains. They break down the association between peripheral sialic acid to generate the free sialic acid present on the various ends of the sialoglycoconjugates, neuraminidases (Vimr et al.,2004 and Severi et al., 2007).

The aims of the present study was to determine the of genes of virulence factors (ExoA and Nan1) in *Pseudomonas aeruginosa*, biofilm formation, determine the sensitivity of bacteria to a number of antibiotics belonging to different groups. In addition to studying the efficacy of two plant extracts and oyster shell against resistant *Pseudomonas aeruginosa* isolated from the patient's burn and the environment surrounding him.

MATERIALS AND METHODS

Samples collection

Total of 112 samples were collected between 18 November 2018 to 10 March 2019 from Al-Fayhaa General Hospital in Basrah / Iraq. 68\112 samples were collected from burn patients using the sterile cotton swab, and 44\112 samples were collected from surrounding environment of the patient from the air using the Settle Plate Method, (Singh et al., 2013). In addition, sterile cotton swab were also used to collect samples from bedding, companion with the burn patients, and Nursing room. Samples were inoculated in Brain Heart

Infusion Broth for 24h at 37ºC.

Identification of *Pseudomonas aeruginosa*

Inoculated samples were cultured on MacConkey agar medium and *Pseudomonas* chromogenic agar medium. After incubation for 24-48 h, the growing bacteria were cultured on Nutrient agar to do biochemical tests including Gram stain, Oxidase test, Catalase, Citrate utilization, Kliger iron agar as well as its ability to produce pyocyanin and pyomelanin pigment. For more characterization confirmation, Vitek 2 was performed on some isolates and detection, sequencing of gene *16S rRNA* was done for the rest of the isolated strains.

DNA Extraction

DNA was extracted according to the manufacturer's instructions; QIAamp® DNA Mini Kit (Germany) from bacteria grew on a nutrient agar (broth) for 24 hours at 37 ° C. The product was loaded into 1% agarose gel for electrophoresis and visualizing under UV transiluminater.

Polymerase Chain Reaction (PCR)

Amplification of targeted genes using primers designed previously for *Pseudomonas's 16S rRNA* (Spilker et al., 2004) was done. In addition, the genes encoded for ExoA and Nan1 were amplified using their specific primers as shown in **(Table, 1).** The total volume of PCR reaction for the three genes was 50µl including 25µl of master mix (Bioneer), 1.5µl of forward and reverse primers for each gene (10pmol), and 5-10 ng of DNA sample, the volume was completed into 50 µl with Nuclease-free distilled water (Bioneer). PCR reaction conditions for each gene was as shown in **(Table,2).**

Agarose Gel Electrophoresis

A 5µl of PCR products and 3µl of 1kb DNA ladder (iNtRON company/ Korea) were loaded into 1% agarose gel (prepared in 1X TBE with 0.2µl of ethidium bromide) for 1h in 60V in casting tray, then it was visualized under UV transilluminator (Sambrook and Russel., 2001).

Sequencing of *16S rRNA* gene

22 samples of *16S rRNA* gene PCR products were sent to Macrogen Inc Company in (Korea) and Yang ling tianrun aoke biotechnology co., ltd (China) for sequencing. The coming back results of *16S rRNA* gene sequence was processed and then analyzed in the National Center for Biotechnology Information Database (NCBI) using the Basic Local Alignment Analysis Tool ' BLAST to identify homologous sequences.

http://www.blast.ncbi.nlm.nih.gov . The genes sequences were identified by matching a sequence with the GenBank database's highest identity score (Jenkins et al., 2012).

Table 1. Primers used in the study

Primer target		Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Reference
16S rRNA	F	5' GGGGGATCTTCGGACCTCA 3′	956bp	(Spilker et al., 2004)
	R	5' TCCTTAGAGTGCCCACCCG 3′		
ToxA	F	5' CAG AAC TGG ACG GTG GAG C 3′	535 bp	(Neamah., 2017)
	R	5' CCT GTT CCT TGT CGG GGA TG 3′		
Nan1	F	5' ATG AAT ACT TAT TTT GAT AT 3′	1316 bp	(Mitov, Strateva, and Markova., 2010)
	R	5' CTA AAT CCA TGC TCT GAC CC 3′		

Table 2. The PCR conditions for amplifying *16S rRNA*, exoA and nan1 genes in *P. aeruginosa*

The PCR Condition of Bacteria by used 16S rRNA gene					The PCR Condition of Bacteria by using specific primer for ExoA gene			The PCR Condition of Bacteria by using a specific primer for Nan1 gene			For ExoA and Nan1
No.	Steps	Temperature (°C)	Time	No.of cycles	Steps	Temperature (°C)	Time	Steps	Temperature (°C)	Time	No.of cycles
1.	Denaturation	94	4 min.	1	Denaturation	95	5 min.	Denaturation	95	3 min.	1

2. 3. 4.	Denaturation Annealing Extension	94 60 72	30 sec. 30 sec. 2 min.	35	Denaturation Annealing Extension	95 57.2 72	30 sec. 30 sec 1 min.	Denaturation Annealing Extension	94 55 72	30 sec. 30 sec 1 min and 30 sec.	30
5.	Final Extension	72	5 min.	1	Final Extension	72	5 min.	Final Extension	72	5 min.	1
Store											8 °C

Biofilm formation experiment

The Congo Red Agar (CRA) method was used to detect the ability of bacteria to form biofilms. This medium was prepared by adding sucrose 50g/L, Congo red 0.8g/L stain to Brain Heart Infusion agar, (Deka., 2014).

Antimicrobial susceptibility Test

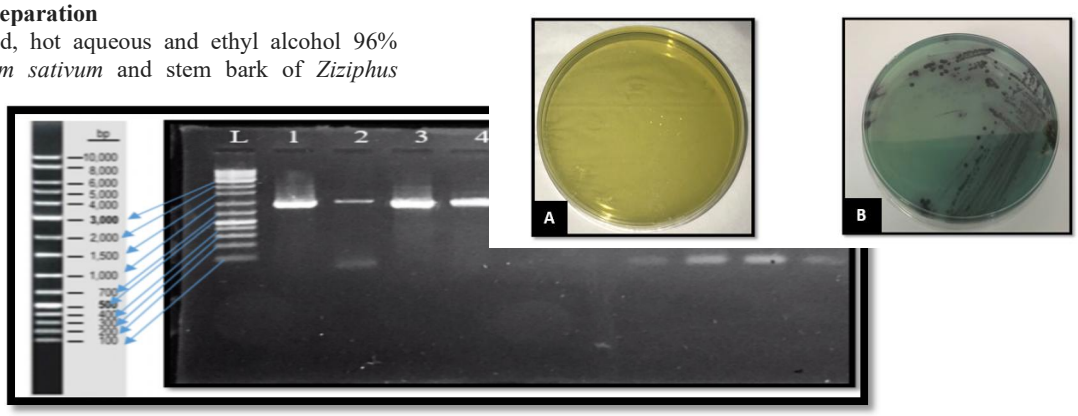
10 antibiotics were selected and used in the study based on the Clinical and Laboratory Standards Institute (CLSI 2019) **(Table 3)**. Using the diffusion method (Nichollas.,2000) on Muller-Hinton Agar.

Table 3; Antibiotic discs used in the present study

NO.	Groups	Antibiotic disc	Content	Company
1	Aminoglycosides	Amikacin (AK)	30 µg	Bioanalyse(Turkey)
2		Gentamicin (CN)	10 µg	Bioanalyse(Turkey)
3	Carbapenems	Imipenem (IPM)	10 µg	CONDA(Spain)
4		Meropenem (MEM)	10 µg	Bioanalyse(Turkey)
5	Cephem	Cefepime (FEP)	30 µg	Bioanalyse(Turkey)
6		Ceftazidime (CAZ)	30 µg	CONDA(Spain)
7	Fluoroquinolones	Ciprofloxacin (CIP)	5 µg	Bioanalyse(Turkey)
8		levofloxacin (LEV)	5 µg	Bioanalyse(Turkey)
9	Monocyclin	Aztreonam (ATM)	30 µg	Bioanalyse(Turkey)
10	Penicillin's	Piperacillin (PRL)	100µg	Bioanalyse(Turkey)

Natural extracts preparation

Natural extract; cold, hot aqueous and ethyl alcohol 96% extract from (*Allium sativum* and stem bark of *Ziziphus spina-christi var.malaci*) plant , oyster shell extract were prepared based on the method followed by Ahmed et al., (1998). The effectiveness of plant and oyster shell extracts against *Pseudomonas aeruginosa* was investigated using Agar well diffusion method (Padhi and Tayung .,2015).



Statistical analysis

All the results were analyzed statistically using SPSS program

RESULTS

Isolation and identification of *P. aeruginosa*

In the present study, 41 isolates of *Pseudomonas aeruginosa* were identified including; 26 from patients and 15 from the patients surrounding environment. Magenta colonies can be seen on *Pseudomonas* chromogenic agar medium after incubation for 24 h where the medium color changed from light green to bluish-green **(Figure 1)**.

Figure 1. (A); *Pseudomonas* chromogenic agar medium without culture. (B): growing colony of *Pseudomonas spp* in a magenta color on *Pseudomonas* chromogenic agar.

Further identification was done for the suspected *Pseudomonas spp* as mentioned in materials and methods. Bacteria diagnosed based on the positive results of the biochemical tests used in the present study. The results also recorded that 14 isolates of *Pseudomonas aeruginosa* produced melanin and 8 isolated produced pyocyanin pigment. 20 of the 41 isolates were diagnosed with vitek2 assay that showed with a rate about 97-99% as bacteria *Pseudomonas aeruginosa*.

Genetic work

The results of the gel electrophoresis were demonstrated clear DNA bands of extracted from all isolates (41 isolates) when they were electrophoresed in 1% agarose, the gel showed one
band in each hall respuseartied isolates the genomics DNA**.**
16S rRNA gene for the 41 isolates when they were amplified by PCR in the present study and then subjected for gel electrophoresis, sharp bands corresponding to a 956 bp which

belong to *Pseudomonas aeruginosa* can be noticed in electrophoresed agarose gel compared to the molecular ladder 10000bp **(Figure 2)**, 21 isolates were sent for sequencing.

Figure 2. Patterns of 1% of agarose gel electrophoresis shows bands of *16S rRNA*. L; DNA ladder, the numbers 1,2,3,4,5,6,7,8,9; are examples of *16S rRNA* bands and C; control negative (contain primers forward and reverse, nuclease-free water and master mix without DNA template) which showed no band.

Presence of Exo A and Nan1 genes in *Pseudomonas aeruginosa*

After doing PCR for amplification exoA and nan1 genes, PCR products were electrophoresed and visualized under UV transilluminater, approximately 535bp bands when compared with the DNA ladder as shown in **Figure (3)** could be seen in the gel which corresponds to the exoA gene of *Pseudomonas aeruginosa*. The results showed that 38\41 (92.68%) of isolated *Pseudomonas aeruginosa* harboring the exoA gene. However, after many attempt to optimize the PCR conditions for nan1 gene, the present study recorded only four isolates 4 (9.75%) isolates of *Pseudomonas aeruginosa* having this gene, 2 (4.87%) isolates from patient' burn and 2 isolates from the surrounding environment with band size approximately 1316 bp that is represent the requested gene as it can be seen in the 1% agarose gel visualized with UV light **(Figure 4).**

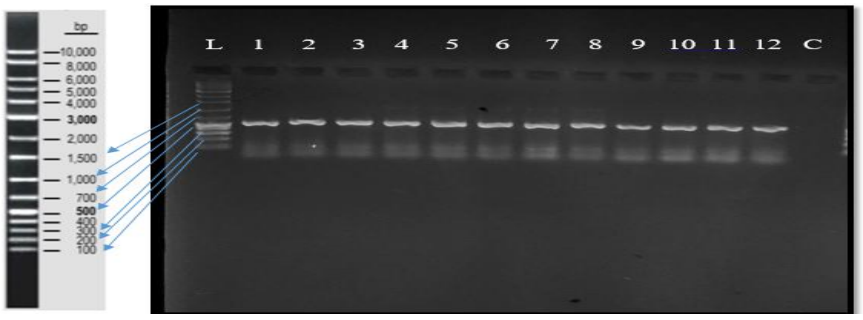
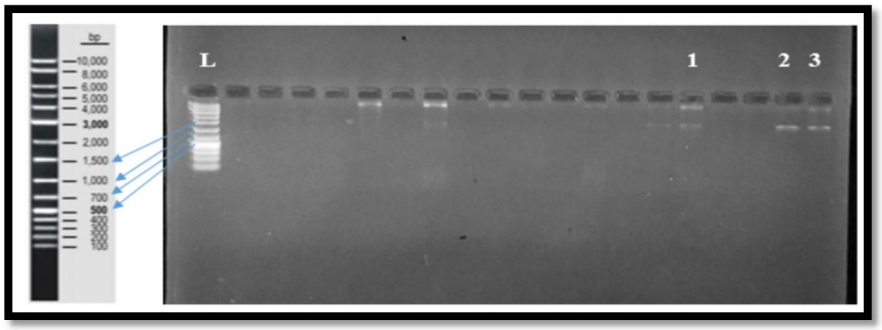


Figure 3. The exoA gene in *P. aeruginosa* using a specific primer with bands nearly 535 bp when compared with DNA ladder. L; DNA ladder, nember 1,2,3,4,5,6,7,8,9,10,11,12, represent isolates of *Pseudomonas aeruginosa* isolated from burn, C; negative control contains all contents for PCR except DNA template.

Figure 4. Gel electrophoresis showing bands corresponding to *P. aeruginosa* nan1gene. 1, 2, 3. isolates of *Pseudomonas aeruginosa*



isolated from burn showed bands with size 1316bp (as expected for nan1 gene using specific primers). L; DNA ladder (iNtRON company/ Korea).

Sequencing of Bacterial genes

Where 20 isolates were identified using Vitek2, 23 (5 isolates identified by Vitek and *16S rRNA* for comparing) from the rest of isolates were more identified by subjecting them for *16S rRNA* sequencing alongside with Vitek2, the coming back results and after treatment were confirmed the identification by being *P.aeroginosa* **(Table 4).** The results also showed the efficiency of the vitek2 system, as there was close ratio in the diagnosis **(Table 5).**

Table 4. *16S rRNA* nucleotides sequencing data for isolates which sent to Macrogen Corporation in Korea

Name of isolates	Bacterial species	Identical to strain	Query cover	Accession number
R26	Pseudomonas aeruginosa	99.89% strain BD0603	97%	MT109313.1
R27	Pseudomonas aeruginosa	99.66% strain BD0603	100%	MT109313.1
R28	Pseudomonas aeruginosa	100% strain BD0603	100%	MT109313.1
R29	Pseudomonas aeruginosa	100% strain BD0603	100%	MT109313.1
R30	Pseudomonas aeruginosa	100% strain BD0603	100%	MT109313.1
R31	Pseudomonas aeruginosa	100% strain CUPR29	100%	MT107139.1
R32	Pseudomonas aeruginosa	99.89% strain CUPR29	100%	MT107139.1
R33	Pseudomonas aeruginosa	100% strain BD0603	100%	MT109313.1
R34	Pseudomonas aeruginosa	100% strain BD0603	100%	MT109313.1
R35	Pseudomonas aeruginosa	100% strain BD0603	99%	MT109313.1
R36	Pseudomonas aeruginosa	99.75% strain BD0603	100%	MT109313.1

R37	Pseudomonas aeruginosa	99.43% strain BD0603	100%	MT109313.1
R38	Pseudomonas aeruginosa	99.76% strain BD0603	100%	MT109313.1
R39	Pseudomonas aeruginosa	100% strain BD0603	100%	MT109313.1
R40	Pseudomonas aeruginosa	100% strain BD0603	100%	MT109313.1
R41	Pseudomonas aeruginosa	100% strain BD0603	100%	MT109313.1
R42	Pseudomonas aeruginosa	100% strain BD0603	100%	MT109313.1
R43	Pseudomonas aeruginosa	99.65% strain YEBL3	100%	MK367616.1
R44	Pseudomonas aeruginosa	100% strain BD0603	100%	MT109313.1
64 *	Pseudomonas aeruginosa	99,78% strain BD0603	96%	MT109313.1

***16S rRNA* nucleotides sequencing data was done in a Yang ling tianrun aoke biotechnology co., ltd company in China**

Table 5. Bacteriological and molecular methods performed on bacterial isolates

Name of isolates	**Vitek 2**	***16S rRNA* gene sequence**	
13 environment	99% probability	R40	100%
3 environment	99% probability	R42	100%
64 Patient	99% probability	64	99.78%
40 Patient	97% probability	R33	100%
44 Patient	97% probability	R34	100%

Biofilm production using Congo Red Agar method

The total number of *Pseudomonas aeruginosa* isolated from burn patients and the environment surrounding him, which produced biofilm were 36/41 (87.80%). Whereas 5\41 (12.19%) unproductive, one of them only from the surrounding environment. 20\36 (55.55%) produced strong biofilm with black-colored, 16\36 (44.44%) were intermediate-produced **(Figure 5).**

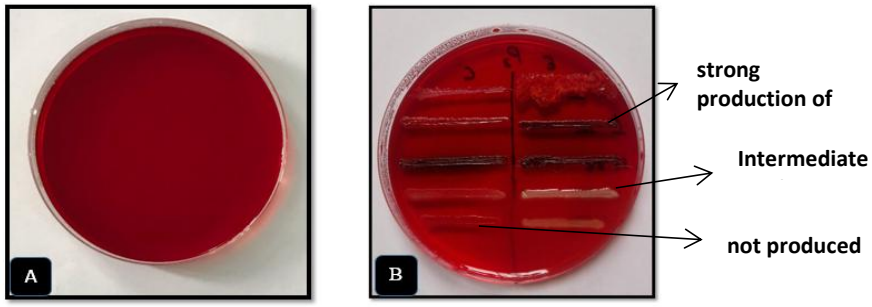


Figure 5. A Congo red agar medium uncultured. B Congo red agar medium with cultured bacteria shown biofilm production for different isolates *Pseudomonas aeruginosa*. Isolated from patients with burns and the surrounding environment, while black indicates strong production of biofilm, white color is intermediate production of biofilm and red is not produced biofilm.

Resistance of *P.aeruginosa* to antibiotics

The antibiotics susceptibility pattern among the *P.aeruginosa* isolated from the patient's burn and their surrounding environment were shown in **(Table 6 and 7)** respectively**.** The results indicated that the *Pseudomonas aeruginosa* isolated from burn patients differ in their sensitivity and resistance to antibiotics from the *P.aeruginosa* isolated from the surrounding environment. Where the bacteria isolated from patient's burns showed high resistance toward piperacillin antibiotic (88.46%). Besides (84.61%) resistance to cefepime and ceftazidime, while it showed the highest sensitivity against gentamicin (80.76%), and imipenem with (73.07%).

Whereas the *Pseudomonas aeruginosa* isolated from the environment surrounding the patients showed the highest resistance in a rate (100%) to piperacillin and Ceftazidim . Also, it was resistance (93.33%) toward cefepime. They were recorded (73.33%) sensitive to the gentamicin and (66.67%) to Imipenem.

The results indicated that 38\41 (92.68%) *Pseudomonas aeruginosa* bacteria have a resistance of more than three antibiotics for different groups, which means that they are Multi Drug Resistant (MDR). In the present study, all antibiotics sensitivity results were compared with the CLSI Updated in 2019 inhibition diameters and have been followed in Al-Fyhaa General Hospital /Basrah.

Table 6. Antibiotics sensitivity of *Pseudomonas aeruginosa* isolated from burn patients toward different antibiotics belong to different groups

Groups	**Antibiotics**	**No.of isolates Sensitive**	**No.of isolates Intermediate**	**No.of isolates Resistant**
Aminoglycosides	Amikacin	4 (15.38%)	8 (30.76%)	14 (53.84%)
	Gentamicin	21 (80.76%)	0	5 (19.23%)
Carbapenems	Imipenem	13 (50%)	5 (19.23%)	8 (30.76%)
	Meropenem	19 (73.07%)	2 (07.69%)	5 (19.23%)
Cephem	Cefepime	2 (07.69%)	2 (07.69%)	22 (84.61%)
	Ceftazidime	1 (03.84%)	3 (11.53%)	22 (84.61%)
Fluoroquinolones	Levofloxacin	17 (65.38%)	5 (19.23%)	4 (15.38%)

	Ciprofloxacin	9 (34.61%)	7 (26.92%)	10 (38.46%)
Monocyclin	Aztreonam	2 (07.69%)	7 (26.92%)	17 (65.38%)
Penicillin's	Piperacillin	0	3 (11.53%)	23 (88.46%)
P value =		0.0103861	0.0049046	0.000150
Among the results of statistical analysis and test the least significant difference, p<0.05were shown significant differences between the sensitivity of the studied bacteria towards the antibiotics.				

Table 7. Antibiotics sensitivity of *Pseudomonas aeruginosa* isolated from the environment surrounding burn patients toward different groups of antibiotics

Groups	Antibiotics	No.of isolates Sensitive	No.of isolates Intermediate	No.of isolates Resistant
Aminoglycosides	Amikacin	5 (33.33%)	8 (53.33%)	2 (13.33 %)
	Gentamicin	11 (73.33 %)	4 (26.67 %)	0
Carbapenems	Imipenem	10 (66.67 %)	1 (06. 66 %)	4 (26.67%)
	Meropenem	14 (93.33 %)	0	1 (06. 66%)
Cephem	Cefepime	1 (06. 66 %)	0	14 (93.33%)
	Ceftazidime	0	0	15 (100%)
Fluoroquinolones	Levofloxacin	7 (46.67 %)	6 (40 %)	2 (13.33 %)
	Ciprofloxacin	1 (06. 66 %)	9 (60%)	5 (33.33%)
Monocyclin	Aztreonam	0	5 (33.33%)	.10 (66.67%)
Penicillin's	Piperacillin	0	0	15 (100 %)
P value =		0.7595176	0.1539272	0.5635829
The statistical analysis showed that there were no significant differences between the sensitivity of the bacteria and the antibiotics used in the present study.				

The relation between biofilm production and antibiotics resistance of *Pseudomonas aeruginosa* isolated from patient's burn and their surrounding environment

The results showed that there was a different relation between biofilm production and the resistant *Pseudomonas aeruginosa* to antibiotics **(Figure 6 and Figure 7**). The present study recorded that the strong biofilm-producing bacteria were resistance to 6 antibiotics belong to different groups that were used in this study. While the non- biofilm producing bacteria found to be resistance to 3 antibiotics **(Table 8)**

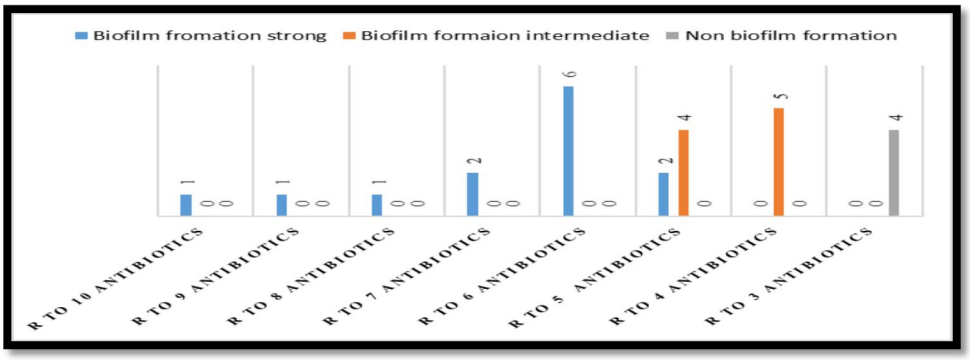


Figure 6. Relationship between biofilm production and resistance to antibiotics for *Pseudomonas aeruginosa* isolated from burn patients. R; Resistance.

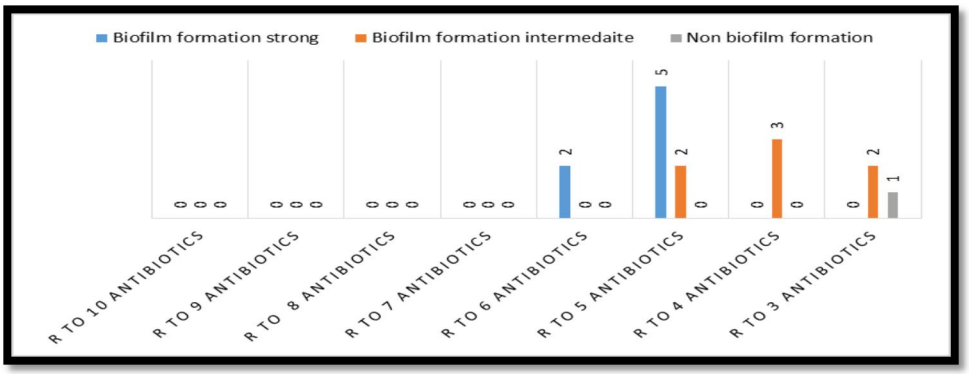


Figure 7. Relationship between biofilm production and resistance to antibiotics for *Pseudomonas aeruginosa* isolated from the environment surrounding burn patients, R means Resistance.

Table 8. The relationship between the production of biofilm and resistant *Pseudomonas aeruginosa* to antibiotic isolated from burning patients and environment surrounding them.

NO.	Antibiotics	Strong biofilm formation n=20	Intermediate biofilm formation n= 16	Non biofilm formation n= 5
		%)No. (	%)No. (	%)No. (
1.	Amikacin	14 (70%)	6 (37.5%)	4 (80%)
2.	Gentamicin	3 (15%)	1 (06.25%)	1 (20%)
3.	Imipenem	9 (45%)	2 (12.5%)	0
4.	Meropenem	6 (30%)	0	0
5.	Cefepime	19 (95%)	15 (93.75%)	1 (20%)
6.	Ceftazidime	20 (100%)	15 (93.75%)	1 (20%)
7.	Levofloxacin	6 (30%)	0	0
8.	Ciprofloxacin	11 (30%)	3 (18.75%)	1 (20%)
9.	Aztreonam	16 (80%)	10 (62.5%)	2 (40%)
10.	Piperacillin	20 (100%)	16 (100%)	1 (20%)
P value =		39.8	43.7	1.3
The study showed the value of p<0.01 with a significant difference for the strong and intermediate produced bacteria of the biofilm and a non-significant difference for the bacteria unproduced.				

Activity of plant extracts and oyster shell on *P. aeruginosa*

The present study demonstrated the inhibitory efficacy with different levels of plant extracts and oyster shell towards *Pseudomonas aeruginosa* isolated from burn patients and their surrounding environment, **(Table 9A ,and B)** respectively. Where the following concentrations were used (100,50,25,15,10,5,3,2) mg\ml. The results showed the cold aqueous extract of stem bark of *Ziziphus spina-christi var.malaci* was with high efficacy towards bacteria recording the highest inhibition diameter of about 30mm. While the cold and hot aqueous extract of *Allium sativum* showed no effect on all isolates of the present study, whereas the present study recorded the effectiveness of the alcohol extract of *Allium sativum* on many isolates. Cold and hot aqueous extract of Oyster shell

recorded inhibitory activity against few isolates, while the alcohol extract did not record any biological activity against all isolates **(Figure 8).** Also, the minimum inhibitory and bactericidal concentrations (MIC and MBC) for plant and oyster shell extraction were investigated and recorded on *Pseudomonas aeruginosa* as shown in **(Table 9A and B)**.

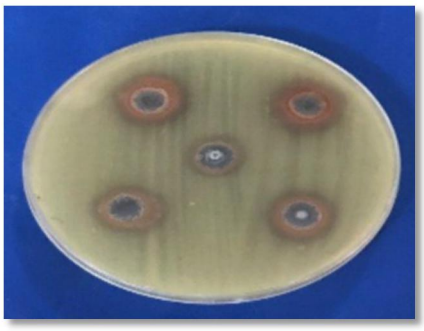


Figure 8. Shows effect of cold and hot aqueous extract stem bark on one isolate of Pseudomonas *aeruginosa*.

Table 9A. The inhibitory effect extract cold and hot of stem bark of *Ziziphus spina-christi var.malaci* against *Pseudomonas aeruginosa* isolated from burn patients and environment surrounding them at a concentration of 100 g / ml.

The inhibitory effect cold and hot extract of stem bark of *Ziziphus spina-christi var.malaci* against *Pseudomonas aeruginosa* isolated from burn patients at a concentration of 100 mg/ml.								**The inhibitory effect cold and hot extract of stem bark *Ziziphus spina-christi var.malaci* against *Pseudomonas aeruginosa* isolated from environment surrounding patients with burns at a concentration of 100 mg/ml.**							
Cold extract of stem bark				**Hot extract of stem bark**				**Cold extract of stem bark**				**Hot extract of stem bark**			
NO.of sample	**100**	**MIC**	**MBC**	**NO.of sample**	**100**	**MIC**	**MBC**	**NO.of sample**	**100**	**MIC**	**MBC**	**NO.of sample**	**100**	**MIC**	**MBC**
3 isolates	21mm	75	100	8 isolates	20mm	100	100>	6 isolates	20 mm	100	100>	4 isolates	19mm	100	100>
3 isolates	20mm			5 isolates	19mm			2 isolates	21mm			2 isolates	21mm		
2 isolates	23mm			3 isolates	16mm			3 isolates	19mm			2 isolates	22mm		
3 isolates	16mm			2 isolates	17mm			28e	29mm			2 isolates	20mm		
5 isolates	19mm			2 isolates	18mm			13e	17mm			10e	17mm		
2 isolates	17mm			2 isolates	21mm			8e	21mm	10	15	16e	18mm		
3 isolates	18mm			67 Patient	15mm			22e	20mm			29e	15mm		

60 Patient	26mm			34 Patient	17mm	10	15	P value	0.15	0.23	0.28	8e	20mm	15	25
67 Patient	19mm			57 Patient	21mm							22e		10	15
46 Patient	16mm	15	25	46 Patient	20mm	15	25					P value	0.68	1061.6	3329.8
57 Patient	19mm			P value	8.513	2303.6	2416.6								
54 Patient	30mm	50	75												
P value	6.64	0.77	2.6												
The results of the statistical analysis showed P>0.01 significant differences for each of the MBC values and the rate of inhibition diameters at 100mg\ml concentrations and no a significant difference for the MIC values.				**The results of the statistical analysis showed P>0.01 significant differences for each of the MIC values , the rate of inhibition diameters at 100mg\ml concentrations MBC values.**				**The results of the statistical analysis showed no significant differences for each of the MIC values , the rate of inhibition diameters at 100mg\ml concentrations and MBC values.**				**The results of the statistical analysis showed P>0.01 significant differences for each of the MIC and MBC values.**			

Determined MIC and MBC values. e; environment.

Table 9B. The inhibitory effect alcohol extract of stem bark of *Ziziphus spina-christi var.malaci* and alcohol extract of *Allium sativum* against *Pseudomonas aeruginosa* isolated from burn patients and environment surrounding patients with burns at a concentration of 100 g / ml.

The inhibitory effect alcohol extract of stem bark on *Pseudomonas aeruginosa* isolated from burn patients at a concentration of 100 mg/ml.				**The inhibitory effect alcohol extract of stem bark against *Pseudomonas aeruginosa* isolated from environment surrounding patients with burns at a concentration of 100 mg/ml.**				**The inhibitory effect alcohol extract of *Allium sativum* against *Pseudomonas aeruginosa* isolated from burn patients at a concentration of 100 mg/ml.**			
No. of sample	100 mg/ml	MIC	MBC	No. of sample	100 mg/ml	MIC	MBC	NO. of sample	100 mg\ml	MIC	MBC
2 Patient	19mm	75	100	3e	18mm	5	10	13 isolates	0	0	>100
14 Patient	17mm			29e	21mm			4 Patient	19	50	75
22 Patient	23mm			2 isolates	20mm	75	100	2 isolates	11	75	100
3 isolates	20mm			6e	21mm			2 isolates	20		
3 Patient	20mm	100	>100	10e	18mm			15 Patient	12		
6 Patient	12mm			7e	20mm	100	>100	30 Patient	14		
2 isolates	17mm			3 isolates	15mm			34 Patient	16		
2 isolates	16mm			2 isolates	17mm			67 Patient	21		
34 Patient	21mm			35e	25mm			53 Patient	17		
2 isolates	19mm			41e	18mm			2 isolates	0	100	>100
47 Patient	15mm			30e	20mm	10	15	63 Patient	20		
49 Patient	18mm			P value	0.131	2.1	61.05	P value	0.0000526	0.002356	0.00000001
65 Patient	14mm			**The results of the statistical analysis showed no significant differences for each of the MIC values and the rate of inhibition diameters at 100mg\ml concentrations and P>0.05 a significant difference for the MBC values.**				**The results of the statistical analysis showed P>0.01 significant differences for each of the MBC values and the rate of inhibition diameters at 100mg\ml concentrations and MIC values.**			
								The inhibitory effect alcohol extract of *Allium sativum against Pseudomonas aeruginosa* isolated fromnvironment surrounding patients with burns at a concentration of 100 mg/ml.			
4 isolates	20mm	5	10					NO. of sample	100 mg\ml	MIC mg\ml	MBC mg\ml
2 isolates	20mm	10	15					3e	15	75	100

28 Patient	19mm				10e	18		
46 Patient	21mm				13 isolates	0	0	>100
P value	908.219	51.532	59.419		P value	0.0037314	0.5707052	6.44939
The results of statistical analysis showed at P>0.01 value significant differences for each of the MBC values , the rate of inhibition diameters at 100mg\ml concentrations and MIC value.					**The results of the statistical analysis showed P<0.01 significant differences for each of the MBC values and the rate of inhibition diameters at 100mg\ml concentrations and no a significant difference for the MIC values.**			

Determined MIC and MBC values. e; environment.

DIISCUSSION

Frequency of exoA and nan1 genes in *Pseudomonas aeruginosa*

Gene *16S rRNA* has been used in the diagnosis, because its length is appropriate, you can follow the sequences of each bacterium, it is a fixed gene and cannot be changed (Skow et al., 2005). In the present study the primers used were designed by (Spilker et al., 2004) specifically for identification of *Pseudomonas spp*. Total of 41 isolates were showed bands corresponding to 956bp which is belong to *Pseudomonas spp* and this is in consistence with Jalil., (2018).

Virulence factors of the *P. aeruginosa* lead to impairment of the immune system and destruction of the skin barrier in patients with burning, which overstates pathogenesis and improves dissemination (Alhazmi., 2015). Recorded 93% of *P. aeruginosa* isolated from burn and environment surrounding patients with burns who possess this gene, the present study was compatible with Elmaraghy et al ., (2019), who found (95.7%) of the gene presence in the *P. aeruginosa* collected from hospitalized patients with urinary tract infection (UTI), respiratory tract infection (RTI), burning infection, and bed ulcers. exoA gene encodes for ExoA that plays a significant role in the spread of *P. aeruginosa* within the burned skin and the emergence of endogenous septicemia. Moreover, this had a special role in retarding wound healing and contraction (El-Din et al., 2008).

The gene known as nan1 encodes sialidase that is responsible for adherence to the respiratory tract which plays a major role in bacterial association and subsequent invasion of host cells, in particular epithelial ones (Bradbury et al., 2010). In the present study gene nan1 was recorded with rate (9.75%) of the *P. aeruginosa*. Although the present study close to the study of Thamir and Al-Jubori ., (2014) who showed that only 1\26 (3.8%) isolates having this nan1 gene it was not in agreement with Lanotte et al., (2004) who found that nan1 was in 86 isolates (53%).

Biofilm formation and resistance *P. aeruginosa* to antibiotics

Most of the bacteria likely form a biofilm as a virulence factor that plays different important roles in pathogenicity. Bacteria that form biofilm have been associated with chronic and recurrent infections, difficult to treat and eliminate, when bacteria form biofilm become more resistance to changeable environment condition such as pH, antibiotics, disinfectants, oxygen radicals and phagocytosis (Lewis ., 2001 ; Aparna and Yadav .,2008).

The present study is similar to Abd El-Galil et al.,(2013) who was found that 84% were producers of biofilms ,4 isolates (8%) were moderate and 4 isolates (8%) were weak. The appearance of the black color is due to the interaction of the pigment Congo red with the polysaccharide, which is evidence of the formation of biofilm. The red color, which is usually flat, indicates that bacteria cannot produce polysaccharides, meaning they are not composed of biofilm. The formation of bacteria in the biofilm was closely related to the bacterial resistance to antimicrobial drugs, which increases the spread of antibiotic resistance through the transmission of the genes responsible for them and increases in mutation rates in bacterial cells (Niveditha et al., 2012).

The antibiotic sensitivity test is one of the key tests to determine the resistance and sensitivity of bacteria, which are critical for clinical purposes (Aruna et al., 2010). The results of the present study recorded the highest, of *P. aeruginosa* isolated from patients with burns towards the piperacillin antibiotic (88.46%). This result was agreed with Corehtash et al., (2015).

The present study showed that there was an increase in the resistance isolates *Pseudomonas aeruginosa* towards the third generation of cephalosporins represented by Ceftazidime and cefepime. In addition, the present study recorded a high sensitivity of the *P. aeruginosa* to the gentamicin that belong to the aminoglycosides group at (80.76%). This result was consistent with the study of the Corehtash et al., (2015), but did not agree with Salimi et al., (2010) who recorded bacterial sensitivity to gentamicin (22.9%) and resistance (75.6%).

The multiple resistance to antibiotics may be due to the presence of mutations plasmids that carry the characteristics gene of resistance to a number of antibiotics combined, which may play an important role in the spread of the phenomenon of multiple resistance to antibiotics and thus its spread among bacteria in a large way (Jacoby and Archer., 1991) or may be due to the widely indirect use of antibiotics in treatment of conditions that are caused by these bacteria (Marthez and Baquero .,2002). However, according to the response of the *P. aeruginosa* isolated from the patient's burn and from their surrounding environment, the antibiogram showed a various react to the different antibiotics belong to different group which may refer of being the *P. aeruginosa* isolated from the patient did not transfer to him from the surrounding area and more experiment are needed (under investigation).

Effect of different extracts on *P. aeruginosa*

Recently the World Health Organization (WHO) announced an unprecedented increase in Multidrug-resistant (MDR) diseases occurring worldwide (Unemo t al., 2019). Many researchers suggesting the use of alternative treatment for multidrug resistant bacteria such as natural extract. However, despite the widespread use of medicinal plants, their effect is electoral based on the type of bacteria and the type of disease. Al Rawi and Chakravorty (1988) were indicated that the variation in the effect of plant extracts is controlled by many factors, especially those affecting the plant's antagonist effectiveness.

Oyster shell is one of the most popular traditional anti-drugs that are widely used in many Asian countries because it contains a large amount of calcium carbonate (CaCO3),

(Thamyres et al.,2019), relatively low amounts of calcium sulfate (CaSO4), calcium phosphate (CaPO4), and amino acids (Fujita et al.,1998; Wang., 2013). Therefore, the effect of oyster shell extract on the vital efficacy of inflammatory burns has been studied. The results of the present study showed of the effectiveness of the oyster shell cold aqueous extract since it recorded a highest inhibitory activity for the one patient at a concentration of 75mg\ml with a diameter of inhibition 16mm, and with diameter of 12mm for two isolates at a the same concentration.

The results of the effectiveness of the hot and cold aqueous extract of *Allium sativum* were explained since no inhibiting activity was recorded for all isolates, whether for patients or their surrounding environment. This may be due to the inefficiency of the aqueous extract, which in turn is due to many reasons, including the type of extract, the ideal method of extraction and the test method used to evaluate abstract (Nostro et al.,2000). Medicinal plants have been recognized worldwide (Jamshidi et al .,2018). The present study showed also the efficacy of cold-water extract of stem bark on all isolates of *Pseudomonas earuginosa*. The effectiveness of the extract varied depending on the concentration used, where the highest inhibition diameter recorded was 30 mm compared to the antibiotics and the hot aqueous extract which reveals efficacy but also with inhibition diameters less than the cold aqueous extract.

The present study was incompatible with Makhawi et al .,(2020) in their study, who recorded an inhibition diameters 20 mm on *Pseudomonas earuginosa* for the 100mg\ml concentration. Perhaps the reason of the inhibitory action of the aqueous extract for the stem bark of *Ziziphus spina-christi* may be due to in the presence of varying proportions of tannins, flavonoid, coumarins, alkaloid, triturbin and sterol, cardic glycoside and Saponins, all of them are known as anti-oxidants, that act due to contain affect free radicals that attack DNA and thus prevent mutations (Duthie et al., 1996). The present study showed that cold aqueous extract is more effective than hot water extract.

The inhibitory action of an alcohol extract is due to several reasons, as the extract contains active substances obtained using ethanol, which included both saponin glycosides, carbohydrate, flavonoids, terpernoids, and alkaloids, (Ads et al .,2018) . The antibacterial activity of tannins is attributable to its ability to react with protein to form stable water-insoluble components (Dangoggo et al., 2012). Or, it may inhibit the enzymes in microbes (Chung et al., 1998). However, more investigation is needed to point the active compounds in these crude extract before recommended them as alternative treatment.

CONCLUSION

exoA gene was with high frequency among *Pseudomonas aeruginosa* whereas nan1 gene has low frequency. There is a relation between biofilm production and multi-drug resistance in *P.aeruginosa*, biofilm producing bacteria was MDR. The is another source for the bacteria in addition to their presence as opportunistic bacteria in the hospitals. The crud extract of stem bark *Ziziphus spina-christi var.malaci* provide promising results in inhibition of MDR growth.

REFERENCES

1. Abd El Galil, K., AbdelGhani, S. M., Sebak, M. A. and El-Naggar, W., (2013). Detection of biofilm genes among clinical isolates of *Pseudomonas aeruginosa* recovered from some Egyptian hospitals. Journal. Microbiology, 36, 86-101.
2. Ads, E. N., Rajendrasozhan, S., Hassan, S. I., Sharawy, S. M. S. and Humaidi, J. R., (2018). Phytochemical screening of different organic crude extracts from the stem bark of *Ziziphus spina-christi (L).* Journal of Biomedical Research, 29(8), 1645-1652.
3. Alhazmi, A., (2015). *Pseudomonas aeruginosa*-pathogenesis and pathogenic mechanisms. International Journal of Biology, 7(2), 44.
4. Al-Kaaby, W. A. (2015). Molecular Detection of Virulence Factors Genes in Pseudomonas aeruginosa Isolated from Different Infections Cases in Al-Diwaniya Hospital. journal of al-qadisiyah for pure science (quarterly), 2(20), 53-58.
5. Al-Rawi, A. and Chakaravarty, H. L., (1988). Medicinal plants of Iraq. 2nd. Ministary of Agriculture and Irrigation. Baghdad, Iraq.
6. Aparna, M. S. and Yadav, S., (2008). Biofilms: microbes and disease. Brazilian Journal of Infectious Diseases, 12(6), 526-530.
7. Aruna, B., Jitendra, S., Anjali, P. and Shobha, G., (2010). Bacteriological profile of burns, in tertiary care referral center, bangalore. Pharmaco, 1, 556-560.
8. Bradbury, R. S., Roddam, L. F., Merritt, A., Reid, D. W. and Champion, A. C., (2010). Virulence gene distribution in clinical, nosocomial and environmental isolates of *Pseudomonas aeruginosa*. Journal of medical microbiology, 59(8), 881-890.
9. Chung, K. T., Wong, T. Y., Wey, C.I., Huang, Y. W. and Lin, Y., (1998). Tannins and human health, a review. *Critical* reviews in food science and nutrition, 38(6), 421–464.
10. Corehtash, Z. G., Ahmad Khorshidi, F. F., Akbari, H. and Aznaveh, A. M., (2015). Biofilm formation and virulence factors among *Pseudomonas aeruginosa* isolated from burn patients. Jundishapur journal of microbiology, 8(10).
11. Dangoggo, S.M., Hassan, L.G., Sadiq, I.S. and Manga S.B., (2012) Phytochemical analysis and antibacterial screening of leaves of diospyros espiliformis and *Ziziphus spina-christi*. Journal of Chemical Engineering, 1(1), 31-37.
12. Deka, N., (2014). Comparison of Tissue Culture plate method, Tube Method and Congo Red Agar Method for the detection of biofilm formation by Coagulase Negative *Staphylococcus* isolated from Non-clinical Isolates. International Journal of Current Microbiology and Applied Sciences, 3(10), 810–815.
13. Duthie, S. J., Ma, A., Ross, M. A. and Collins, A. R., (1996). Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. Cancer Research, 56(6), 1291-1295.
14. El-Din, A. B., EL-Nagdy, M. A., Badr, R. A. W. I. A. and EL-Sabagh, A. M., (2008). *Pseudomonas aeruginosa* exotoxin A: its role in burn wound infection and wound healing. Egypt Journal Plast Reconstr Surg, 32, 59-65.
15. Elmaraghy, N., Abbadi, S., Elhadidi, G., Hashem, A. and Yousef, A., (2019). Virulence Genes in *Pseudomonas Aeruginosa* Strains Isolated at Suez Canal University Hospitals with Respect to the Site of Infection and Antimicrobial Resistance. International Journal of Clinical Microbiology and Biochemical Technology, 2, 8-19.
16. Fujita, T., Fukase, M., Nakada, M. and Koishi M., (1998). Intestinal absorption of oyster shell electrolysate. Bone Miner 11, 85-91.
17. Ghanbari, A., Dehghany, J., Schwebs, T., Müsken, M., Häussler, S. and Meyer-Hermann, M., (2016).

Inoculation density and nutrient level determine the formation of mushroom-shaped structures in Pseudomonas aeruginosa biofilms. Scientific reports, 6(1), 1-12.
18. Jacoby, G. A. and Archer, G. L., (1991). New mechanisms of bacterial resistance to antimicrobial agents. New England Journal of Medicine, 324(9), 601-612.
19. Jalil, M.B., (2018). Isolation and Bacteriophages Against MDR *Pseudomonas aeruginosa* Isolated from Burn Patients in Basrah Governorate-Iraq. Ph.D. Thesis, Biology, College of Science. University of Basrah, 128p.
20. Jamshidi-Kia, F., Lorigooini, Z. and Amini-Khoei, H., (2018). Medicinal plants: Past history and future perspective. Journal of herbmed pharmacology, 7(1), 1-7.
21. Jenkins, C., Ling, C. L., Ciesielczuk, H. L., Lockwood, J., Hopkins, S., McHugh, T. D. and Kibbler, C. C., (2012). Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. Journal of medical microbiology, 61(4), 483-488.
22. Lachiewicz, A. M., Hauck, C. G., Weber, D. J., Cairns, B. A. and van Duin, D., (2017). Bacterial infections after burn injuries: impact of multidrug resistance. Clinical Infectious Diseases, 65(12), 2130-2136.
23. Lanotte, P., Watt, S., Mereghetti, L., Dartiguelongue, N., Rastegar-Lari, A., Goudeau, A. and Quenn, R., (2004). Gene ganglioside content affect the binding of *Clostridium perfringens* epsilon-toxin to detergent-resistant membranes of madin-darby canine kidney cells. Microbiology and immunology, 49, 245–253.
24. Lee, K. C., Joory, K. and Moiemen, N. S., (2014). History of burns: The past, present and the future. Burns and trauma, 2(4), 2321-3868.
25. Lewis, K., (2001). Riddle of biofilm resistance. Antimicrobial agents and chemotherapy, 45(4), 999-1007.
26. Mabrouk, M. I., El-Hendawy, H. H., Basha, A. M. and Saleh, N. M., (2016). Prevalence, antibiotic and oil resistance pattern of some bacterial isolates from burns. Journal of Applied Pharmaceutical Science, 6(06), 123-130.
27. Makhawi, A. M., Mustafa, M. I. and Uagoub, H. A., (2020). Phytochemical Screening and Antimicrobial Activity of *Ziziphus spina-christi* Stem Barks. bioRxiv. doi: https://doi.org/10.1101/2020.02.24.963157.
28. Martínez, J. L. and Baquero, F., (2002). Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. Clinical microbiology reviews, 15(4), 647-679
29. Mitov, I., Strateva, T., and Markova, B., (2010). Prevalence of virulence genes among bulgarian nosocomial and cystic fibrosis isolates of *Pseudomonas aeruginosa*. Brazilian Journal of Microbiology, 41(3), 588-595.
30. Neamah, A. A., (2017). Molecular Detection of virulence factor genes in Pseudomonas aeruginosa isolated from human and animals in Diwaniya province. Kufa Journal For Veterinary Medical Sciences, 8(1), 218-230.
31. Nichollas, C., (2000). Aromatic medicine in the treatment of infections British Journal of Phytotherapy, 5 (1), 22-24.
32. Niveditha, S., Pramodhini, S., Umadevi, S., Kumar, S. and Stephen, S., (2012). The isolation and the biofilm formation of uropathogens in the patients with catheter associated urinary tract infections (UTIs). Journal of clinical and diagnostic research: JCDR, 6(9), 1478.
33. Nostro, A., Germano, M. P. and Marino, A., (2000). Plant antimicrobial activity. Letters in applied microbiology, 30(1), 379- 384.
34. Padhi S. and Tayuung K., (2015). In vitro antimicrobial potentials of endolichenic fungi isolated from thalli of Parmaelia lichen against some human pathogen. Beni-Suef Journal of Basic and Applied Science, 4(4), 299-306.
35. Salimi, H., Yakhchali, B., Owlia, P. and Lari, A. R., (2010). Molecular epidemiology and drug susceptibility of *Pseudomonas aeruginosa* strains isolated from burn patients. Laboratory Medicine, 41(9), 540-544.
36. Sambrook, J., Russell, D. W. and Russell, D. W., (2001). Molecular cloning: a laboratory manual (3-volume set). Immunology, 49, 895-909.
37. Severi, E., Hood, D. W. and Thomas, G. H., (2007). Sialic acid utilization by bacterial pathogens. Microbiology, 153(9), 2817–2822.
38. Singh, G., Wu, B., Baek, M. S., Camargo, A., Nguyen, A., Slusher, N. A. and Lynch, S. V., (2010). Secretion of *Pseudomonas aeruginosa* type III cytotoxins is dependent on *Pseudomonas* quinolone signal concentration. Microbial pathogenesis, 49(4), 196-203.
39. Singh, K., Dar, F. A. and Kishor, K., (2013). Bacterial contamination in operating theatres of district hospital Budgam in Kashmir division. International Journal of Pharma Sciences and Research, 3 (2), 62-63.
40. Skow, A., Mangold, K. A., Tajuddin, M., Huntington, A., Fritz, B., Thomson, R. B. and Kaul, K. L., (2005). Species-level identification of staphylococcal isolates by real-time PCR and melt curve analysis. Journal of clinical microbiology, 43(6), 2876-2880.
41. Spilker, T., Coenye, T., Vandamme, P. and LiPuma, J. J., (2004). PCR-based assay for differentiation of Pseudomonas *aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. Journal of clinical microbiology, 42(5), 2074-2079.
42. Thamir, E. and Al-Jubori, S. S., (2014). Genetic detection of some virulence genes in *Pseudomonas aeruginosa* isolated from cystic fibrosis and no-cystic fibrosis patients in Iraq. Journal of Gene c and Environmental Resources Conservation, 2(3), 380-387.
43. Thamyres, H. S., Mesquita-Guimarães, J., Henriques, B., Silva, F. S. and Fredel, M. C., (2019). The potential use of oyster shell waste in new value-added by-product. Resources, 8(1), 13.
44. Unemo, M., Lahra, M. M., Cole, M., Galarza, P., Ndowa, F., Martin, I. and Wi, T., (2019). World Health Organization Global Gonococcal Antimicrobial Surveillance Program (WHO GASP): review of new data and evidence to inform international collaborative actions and research efforts. Sexual health, 16(5), 412-425.
45. Vimr, E., Kalivoda, K., Deszo, E. L. and Steenbergen, S., (2004). Diversity of microbial sialic acid metabolism. Microbiology and molecular biology reviews, 68(1), 132-153.