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Impact of herbal plants and acetic acid treatment on bacteria isolated from burned patients.

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Abstract: Infection is a major cause of morbidity and mortality in burn patients. Loss in the protective skin barrier, reduced immunity most commonly *Pseudomonas aeruginosa which* grows both in nature and inside hospitals and its capacity to acquire resistance mechanisms to antibiotics, makes it one of the most significant causes of serious nosocomial infection, affecting mainly immunocompromised patients. A multidrug resistant (MDR) *P. aeruginosa* is a common and growing problem in most hospitals. It is distinct as a bacterium which is unaffected to three or more anti-pseudomonal anti-microbial classes; carbapenems, fluoroquinolones, penicillin /cephalosporins and aminoglycosides.Natural products and plant extract such as acetic acid (vinegar) , ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) have been used traditionally with pronounced protagonist in the *treatment* and managing of wounds. These natural medicines are harmless, inexpensive and affordable. Results of electron microscope showed cell deformation, membrane and cell wall rupture of isolate treated with ethanolic extracts of vinegar when compared to control treatment.

keywords: Infection, MDR, *Zingiber officinale, Curcuma longa*, Acetic acid.

Introduction

Wounds are a major health-related problem all over the world. Burns are thermal wounds brought on by biological, chemical, electrical, or physical factors and can have both local and systemic effects. First degree burns are superficial, second degree burns are partial thickness burns, and third degree burns are full thickness burns **[1]**. Infection is a major cause of illness and death in burn patients. Loss in the shielding skin hurdle, compact resistance and prolonged hospital stay are vital factors guilty for infection of burn wound with unprincipled pathogens most normally *Pseudomonas aeruginosa (P. aeruginosa)* **[2][3]**. *P. aeruginosa* is a gram-negative, aerobic, motile and non-fermenting bacterium that is broadly dispersed in nature **[4]**. It is one of the greatest mutual bacteria causing nosocomial contaminations, especially in burn units. It contributes to about 20% of infection of burn wound due to the company of late, denatured tissues and moist location that makes the burn wound vulnerable to infection by *P. aeruginosa* **[5]**. *P. aeruginosa* grows both in nature and inside hospitals and its ability to attain fighting mechanisms to antibiotics, makes it one of the most significant causes of serious nosocomial contamination, affecting mainly immunocompromised patients [6]. A multidrug resilient (MDR) *P. aeruginosa* is a common and growing difficult in most hospitals. Bacteria that are resistant to at least three anti-Pseudomonal anti-microbial classes, including carbapenems, fluoroquinolones, penicillin / cephalosporins, and aminoglycosides, are referred to be multidrug-resistant bacteria **[7][8][9]** found a high prevalence of MDR *Pseudomonas* from infected burn wound (76.8% and 93.1% respectively). *P. aeruginosa* secretes multiple virulence factors, either cell-

associated or secreted into the extracellular space. In addition to different exotoxin and enzyme production, *P. aeruginosa* has a great ability for biofilm creation which causes considerable problems in medical and industrial settings **[10]**. The extracellular polysaccharide matrix of biofilm, which is made up of multilayered cell clusters, promotes the adherence of these microorganisms to wound surfaces while shielding them from the host immune system and antibiotic treatment. **[11].** Burn wounds may cause major difficulties due to secondary microbial infections in poorer nations due to poor hygiene settings. Antibiotics are therefore important, along with good wound care. However, due to the overuse of antibiotics, microbial drug resistance has grown, which reduces the effectiveness of the therapy and causes a significant financial loss. **[12]**. Natural products and plant extract have been used traditionally with great role in the treatment and management of wounds. These natural medicines are harmless, inexpensive and affordable **[13].** Acetic acid (vinegar) was used as a topical agent for the treatment of pseudomonal wound infections as it lowers wound pH causing inhibition of bacterial growth and its protease activity **[14][15].** Also, it was shown that organic acids mainly acetic acid destroyed the bacterial outer membrane, inhibition of macromolecular synthesis and increase of bacterial intracellular osmotic pressure **[16].** The topical use of acetic acid dressing did not encourage the evolution of multiple drug-resistant nosocomial bacterial strains unlike the excessive use of antibiotics **[17].** Egyptian study conducted by **[18]** indicated that the removal of numerous antibiotic resistant strains of *P. aeruginosa* from soft tissue infected wounds may be done safely, effectively, and relatively affordably by applying 5% acetic acid. The Zingiberaceae family includes the therapeutic herb ginger (*Zingiber officinale*). **[19].** The ginger contains a variety of vitamins, including vitamins C, A, and B, lipids, and proteins that have a substantial impact on tissue regeneration and wound healing. **[20].** Ginger compounds such as shogaol, gingerol and volatile oils possess antioxidant, antibacterial and anti-inflammatory possessions **[21].** Ginger was found to be effective in killing MDR P. aeruginosa and inhibiting biofilm formation **[22].** One of the natural medications used traditionally is turmeric (*Curcuma longa*). It is a member of the family Zingiberaceae. The antibacterial and antioxidant properties of turmeric extracts are due to curcumin, a polyphenolic molecule. Therefore, curcumin's phenolic component is what gives it its antioxidant properties **[23]**. Zingiberene is a component of fresh turmeric, but curcumin is the most important curcuminoid present. Turmeric has an antimicrobial (antibacterial and antifungal) action, according to prior literature **[24]**. Growth of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *P. aeruginosa* were inhibited by the extract of curcumin plant **[25].**

Materials and methods

Plant Samples Collection:

This study was performed on two medicinal plants Ginger (*Zingiber officinale*), turmeric (*Curcuma longa*) and acetic acid (vinegar) were chosen. Fresh parts were removed from the Rhizome and transferred to the lab in clean, dry plastic sheets. Botany Lab, Faculty of Science, Mansoura University). These plants will be dried at room temperature (20-25°C) and ground into a powder using a blender. The dried plants powder will be macerated with methanol (80%) with continuous shaking for 48 h at room temperature **[26].**

2.2. Media Used for Isolation and Identification of Fungi:

1-Blood Agar medium (Oxoid, England)

2-MacConkey agar medium (Oxoid, England)

3-Muller Hinton Medium **[27]**.

4-Nutrient Broth Medium (Oxoid, England)

5-Cystine Lactose Electrolyte Deficient (CLED) agar medium (Oxoid, England)

6-Kligler Iron Agar (KIA) (Oxoid, England)

7-Lysine Iron Agar (LIA) (Oxoid, England)

8-Motility, Indole, Ornithine medium (MIO) (Oxoid, England)

9-Urea Agar Base (Christensen) (Oxoid, England)

10-Koser Citrate broth (Oxoid, England)

2.3. Isolation and Culturing of *P. aeruginosa*

Samples collection Clinical, wound swabs

samples were collected from patients admitted in Mansoura Burn Center Samples were collected under aseptic conditions after 3 days of stopping of antibiotics if it was taken. These samples were cultured using the standard media (CLED, Blood and MacConkey agar medium agar media) and incubated aerobically at 37 °C overnight.

Pseudomonas aeruginosa was identified using a variety of biochemical assays (IMVIC, TSI, and Urease), culture features, Gramme stain, catalase and oxidase tests, and growth at 42 °C. **[28].**

2.4. Morphological Identification of the Recovered *P. aeruginosa*:

Pyoverdine, a green fluorescent pigment, is produced by some strains. Additionally, some strains can create the blue pigment pyocyanin. **[29][30][31].**

3. Results

3.1. Identification of *P. aeruginosa* isolates:

P. aeruginosa is regular and greenish groups (2-4 mm in diameter) with asymmetrical edges and typical metallic luster. The color is most noticeable on Mueller- Hinton agar. Sometimes, a clear hemolysis zone is obtained on blood agar **(Fig. 1)**. It typical smell (Grape-like or tortilla-like odour).

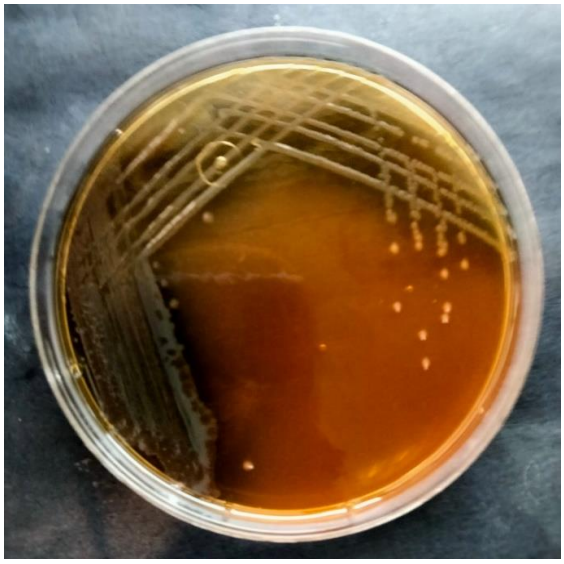

Fig. (1) *P. aeruginosa* colonies on blood agar.

Microscopic examination of *P.aeruginosa* showed a typical of character of Gram-negative, rod-shaped bacterium This finding was observed in all isolates **Fig. (2)**.

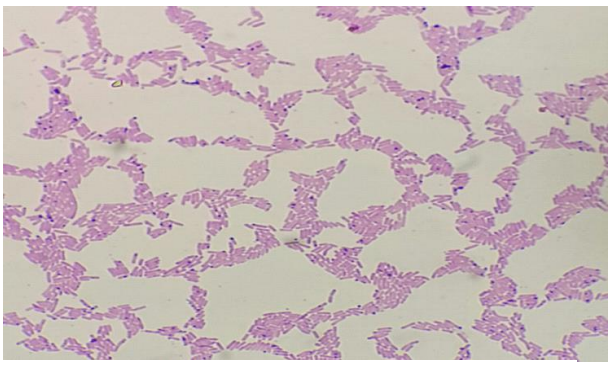

Fig (2): Gram Negative *P. aeruginosa* under bright field microscope.

3.2. Biochemical characterization of *P. aeruginosa.*

An oxidase positive reaction of *P. aeruginosa* is indicated by a deep blue colour appearing within 10 sec presented in **Table (1)** and Fig (3) the other biochemical reactions (KIA, LIA, MIO, Citrate and Urease) presented in **Figs. (3 & 4).**

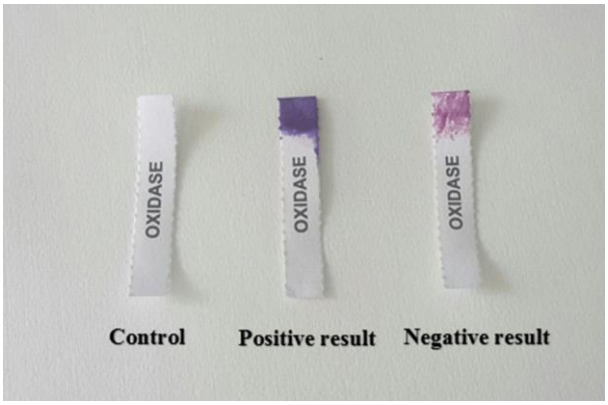

Fig (3) *P. aeruginosa* oxidase test.

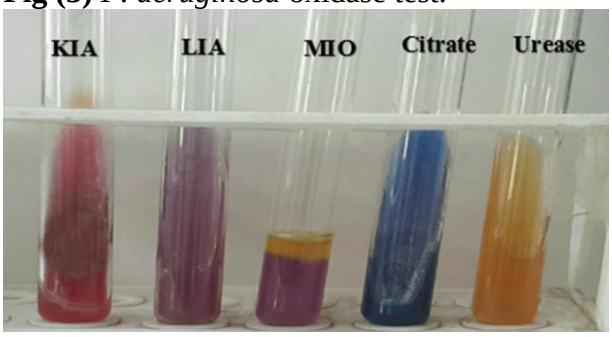

Fig (4): Biochemical reactions of *P. aeruginosa.*

3.3. Antibiotics susceptibility testing

Fifty *P. aeruginosa* isolates, recorded resistance to different antibiotic categories were obtained. The results in antimicrobial showed antimicrobial susceptibility of 50 *P. aeruginosa* to 12 antibiotics belonging to 7 antimicrobial categories. The highest resistance was shown to ceftazidime (96.0%) as shown in **Fig. (5)** and **Table (2)**.

Table 1. Morphological and biochemical characteristic of *P. aeruginosa*

Test	***P. aeruginosa***
Gram stain	**Morphological characters:**
Cell shape	Gram negative straight rods
Arrangement	Bacilli
Colony colour	Pairs or single short rods
Odour	Bluish Green colonies on nutrient agar (bright-blue to blue-green diffusible pigment), clear hemolysis zones on blood agar and pale yellow colonies on MacConkey's agar.
Motility	Grape-like or tortilla-like odour
+ve	
Oxidase test (OXI)	**Physiological characters:**
Hydrogen sulfide production (H_2S)	+ve
Gas production	-ve
Lysine decarboxylase (LDC)	-ve
Lysine deaminate (LDA)	-ve
Indole (IND)	-ve
Ornithine decarboxylase (ODC)	-ve
	-ve
Citrate utilization (CIT)	+ve
Urease (URE)	-ve
characteristic of *P. aeruginosa* isolates. -ve = negative, +ve = positive	

Fig (5): Comparative susceptibility of *P. aeruginosa* against antibiotics

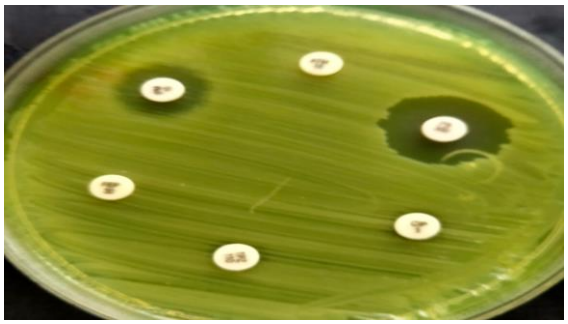

Table (2) show the antimicrobial susceptibility of 50 *P. aeruginosa* isolates.

Antimicrobial categories	**Antimicrobial Agents (Antibiotics)**	**Symbol**	**Resistance (R)**		**Intermediate (I)**		**Susceptible (S)**	
			No.	**%**	**No.**	**%**	**No.**	**%**
Aminoglycosides	Tobramycin	TOB	40	80	1	2	9	18
	Amikacin	AK	38	76	2	4	10	20
	Gentamicin	GN	38	76	4	8	8	16
Carbapenems	Imipenem	IPM	34	68	1	2	15	30
Cephalosporins	Ceftazidime	CAZ	48	96	1	2	1	2
	Cefepime	FEP	45	90	2	4	3	6
Fluoroquinolones	Ciprofloxacin	CIP	39	78	7	14	4	8
	Levofloxacin	LEV	33	66	7	14	10	20
Penicillins/ß-lactamase inhibitors	Piperacillin-tazobactam	TPZ	28	56	10	20	12	24
	Piperacillin	PRL	40	80	6	12	4	8
Monobactams	Aztreonam	ATM	40	80	6	12	43	86
Polymyxins	Colistin	CT	5	10	3	6	42	84
	Polymyxin B	BP	6	12	--	--	44	88

P. aeruginosa sample in **Fig. (6)** Is considered as MDR because it exhibits resistance to at least one agent in ≥3 antimicrobial categories, it was resistant to: Amikacin and tobramycin aminoglycoside, Ciprofloxacin and fluoroquinolones. Ceftazidime and cefepime cephalosporins, Aztreonam monobactams.

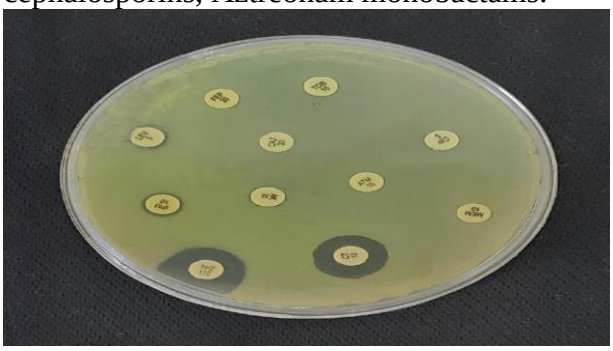

Fig (6): Multidrug resistant (MDR) P. aeruginosa isolate

3.4. Detection of biofilm

3.4.1. Microtitration plate (MTP) method

It was noted that, seven *P. aeruginosa* strains (14%) were non-producers' biofilm while, 32 were (64%) strong producer biofilm and 11 were (22 %) moderate producer in **Table (3)** and **Fig. (7)**.

Table (3): Detection of biofilm production among isolated samples by Microtiter Plate (MTP) method

Type of Samples	Non producer		Moderate producer		Strong producer	
	NO	%	NO	%	NO	%
Wound swab	7	14	11	22	32	64

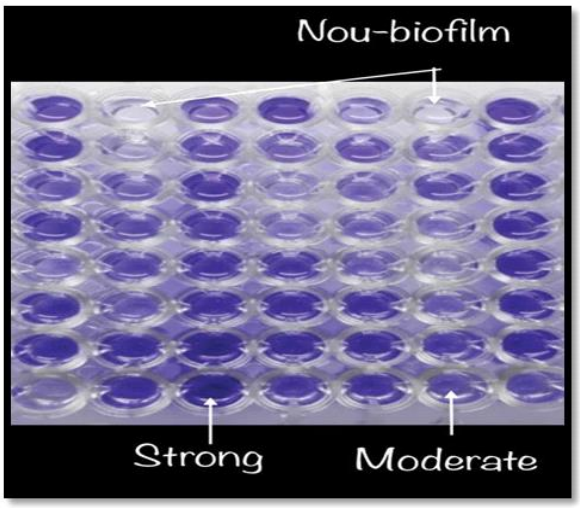

Fig (7): Biofilm production by *P. aeruginosa* strains using the quantitative microtiter plate method.

3.5. Antibacterial activity of medicinal plant extracts against the most resistant P. aeruginosa isolates

In the current study two ethanolic extracts derived from different parts of two medicinal plants traditionally used in Egypt folk medicine belong to different two families and acetic acid (vinegar) were screened for their antibacterial activity against clinical *P. aeruginosa* isolates by the agar well diffusion method. The diameter of the inhibition zones of ethanolic extracts were tabulated in **Table (4)** and shown in **Fig (8).** Of all extracts, vinegar (acetic acid) was the most active one with inhibition zones diameter ranging between (15 mm – 40 mm).

Table (4): Antibacterial activity of ethanolic plant extracts against the most resistant *P. aeruginosa* isolates

Resistant *P. aeruginosa* isolate No.	Diameter of inhibition zone (mm) of different ethanolic plant extracts		
	Ginger	**Tumeric**	**Vinegar**
1	**0**	**0**	**35**
5	**0**	**0**	**20**
10	**0**	**0**	**25**
15	**0**	**0**	**20**
20	**0**	**0**	**20**
25	**0**	**0**	**30**
30	**0**	**0**	**35**
35	**0**	**0**	**28**
40	**0**	**0**	**25**
45	**0**	**0**	**25**
50	**0**	**0**	**40**

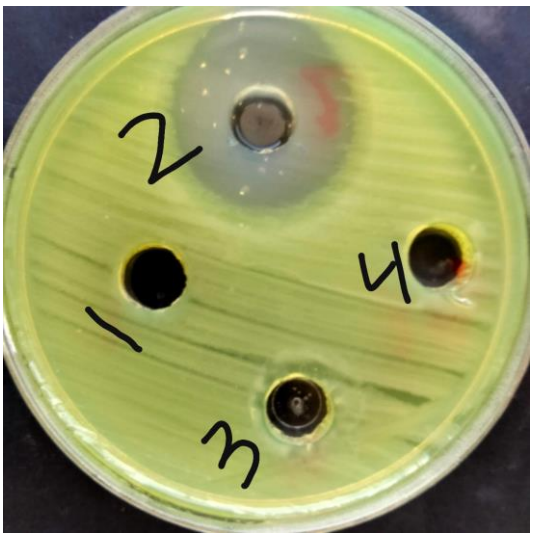

Fig. (8) shows the Inhibition zone of different plant extracts against *P.* aeruginosa isolates. 1: tumeric, 2: vinegar, 3: ginger 4: blank (Dimethyl sulfoxide (DMSO)).

3.6. Comparison between antibiotics and medicinal plants against the most resistant *P. aeruginosa* isolates by using antimicrobial activities

It is interesting to notice that Vinegar showed good activity against clinical *P. aeruginosa* isolates while the antibiotics treatment had limited effect as shown in **Table (5) and Fig. 9**

Table (5): Effective ethanolic plant extracts and vinegar against *P. aeruginosa* isolates.

P.aeruginosa isolates No.	Diameter of inhibition zone (mm)								
	Ginger	tumeric	vinegar	PB 300µg S≤12	ATM 30µg S≤22	IPM 10µg S≤19	FEP 30 µg S≤12	CIP 5µg S≤21	TP 30 µ S≤
1	0	0	35	0	0	12	12	0	0
2	0	0	30	0	0	0	0	0	0
3	0	0	37	0	0	12	0	20	0
4	0	0	22	10	10	0	0	0	0
5	0	0	20	0	0	0	0	19	0
6	0	0	25	0	0	0	0	15	0
7	0	0	20	0	0	0	0	14	0
8	0	0	16	0	0	0	0	14	0
9	0	0	20	0	0	0	0	0	0
10	0	0	25	0	0	0	0	20	0
11	0	0	27	0	0	0	12	18	0
12	0	0	30	0	0	0	0	18	0
13	0	0	25	11	13	0	0	20	18
14	0	0	20	0	0	0	0	0	0
15	0	0	20	0	0	0	0	0	0
16	0	0	17	12	0	0	0	0	0
17	0	0	20	12	0	0	0	0	0
18	0	0	25	0	0	0	0	0	0
19	0	0	30	0	0	0	0	0	0
20	0	0	20	0	0	0	0	0	15
21	0	0	30	0	18	0	0	0	10
22	0	0	30	0	19	0	0	0	0
23	0	0	26	0	20	0	0	0	0
24	0	0	30	0	0	0	0	0	0
25	0	0	30	0	0	0	0	0	0

Fig (9): shows comparison between the activity of some antibiotics, ethanolic plant extracts and vinegar against *P. aeruginosa isolates* (1: Tumeric, 2: Vinegar, 3: Ginger, 4: DMSO (Blank), 5: PB, 6: ATM, 7: IPM, 8: FEP, 9: TPZ, 10: CIP).

3.7. Antibacterial activity of medicinal plant extracts against the biofilm formation of resistant *P. aeruginosa* isolates

Table (6) and Fig (10) show that the high concentration of vinegar extracts (50mg/ml) lead to inhibit biofilm formation of *P. aeruginosa* isolates.

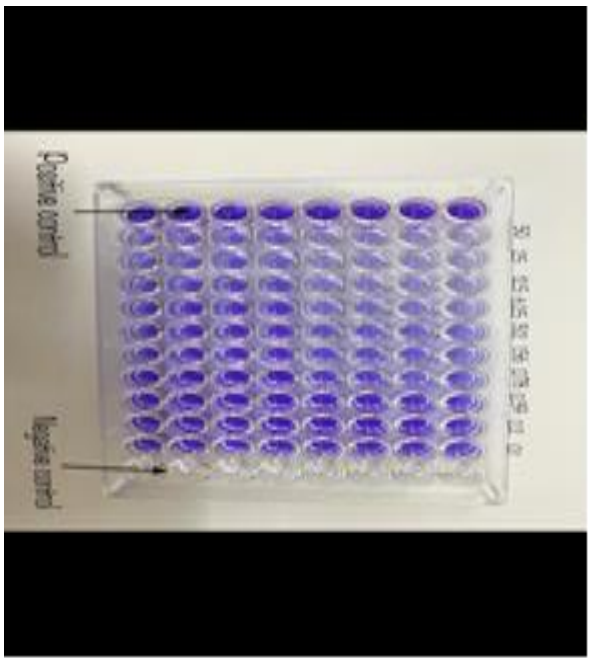

Fig (10): Effect of ethanolic plant extracts of vinegar against biofilm formation of *P. aeruginosa* isolates.

Table (6): Effect of vinegar on biofilm formation of *P. aeruginosa*.

Serial Conc. of vinegar (mg/ml)	Optical density readings of *P. aeruginosa at* 450-550nm					
	P. aeruginosa isolates					
	3	6	15	18	22	25
Negative control	0.1	0.1	0.1	0.1	0.1	0.1
50	0.18	0.234	0.314	0.549	0.203	0.548
25	0.16	0.102	0.224	0.399	0.064	0.071
12.5	0.205	0.139	0.244	0.202	0.083	0.112
6.25	0.547	0.222	0.157	0.137	0.097	0.072
3.1	0.244	0.279	0.170	0.390	0.046	0.075
1.56	0.618	0.229	0.174	0.068	0.058	0.042
0.78	0.141	0.258	0.165	0.227	0.046	0.150
0.39	0.283	0.272	0.120	0.180	0.046	0.038
0.2	0.279	0.224	0.141	0.188	0.066	0.046
0.1	0.304	0.227	0.601	0.103	0.036	0.044
Positive *P.aeruginosa*	1.05	1.05	1.05	1.05	1.05	1.05

3.8. Electron microscopic examination of plant-susceptible *P. aeruginosa* isolates

The most effective medicinal plant extract against antibiotic-resistant P. aeruginosa isolates was vinegar. This isolate was examined under the electron microscope before and after treatment with plant extract.

Results in **Fig. (11))** showed cell deformation, membrane and cell wall rupture of isolate treated with ethanolic extracts of vinegar.

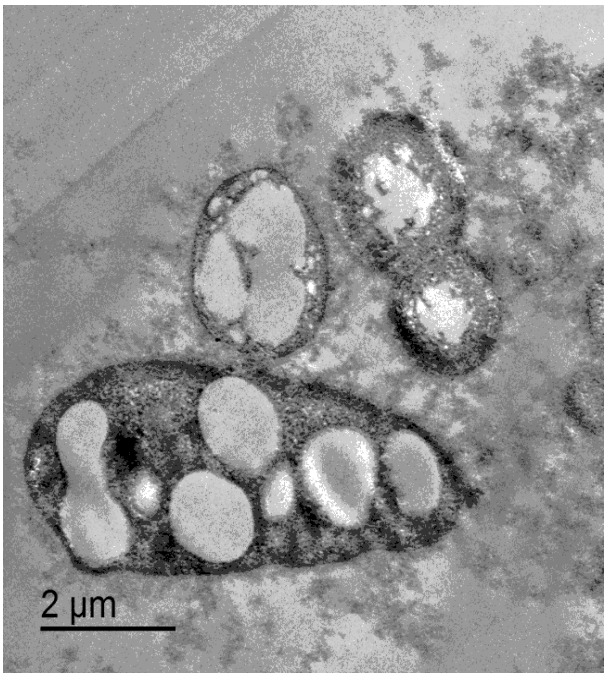

Fig. (11). Electron microscopic examination of *P. aeruginosa* isolate treated with ethanolic extract of vinegar.

.4. Discussion

P. aeruginosa is one of the most prominent causes of dangerous nosocomial infections, affecting primarily immunocompromised individuals. It grows both in nature and within hospitals, and it has the ability to develop antibiotic resistance mechanisms **[32]**. In the majority of hospitals, a multidrug-resistant (MDR) *P. aeruginosa* infection is widespread and getting worse. Bacteria that is resistant to at least three anti-Pseudomonal anti-microbial classes, including carbapenems, fluoroquinolones, penicillin/cephalosporins, and amino glycosides, are referred to be multidrug-resistant bacteria. **[33][34][35]** found high prevalence of MDR *Pseudomonas* from infected burn wound (76.8% and 93.1% respectively).

P. aeruginosa is a gram-negative, aerobic, motile and non-fermenting bacterium that is commonly disseminated in flora **[36].** It is one of the most common bacteria causing nosocomial infections, especially in burn units. It contributes to about 20% of infection of burn wound due to the company of lifeless, denatured tissues and humid situation that makes the burn wound susceptible to pollution by *P. aeruginosa* **[37].**

During this study, fifty swabs were collected using Levine's technique by rotating maneuver over 1 cm2 area of the wound with sufficient pressure to extract fluid from within the wound tissue **[38].**

Samples were transported to Microbiology Diagnostic and Infection Control Unite (MDICU) aseptically in Stuart's transport media **[39]**.

P. aeruginosa, isolated from burn wounds, were recognized by normal microbiological methods which comprised: colony morphology, Gram discoloration, pyocyanin pigment production, growth at 44°C, catalase, oxidase and Triple Sugar Iron (TSI) fermentation tests **[40].**

In the present study, showed that female (56%) had a higher infection rate than males (44%) and similar result was found in a study carried out by **[41]** who found that *P. aeruginosa* isolates were seen mostly in females (56.0%) than in males (44.0%). This result indicated that the female patients had higher prevalence of *P. aeruginosa* infections than in male patients.

Numerous human infections are brought on by *Pseudomonas aeruginosa*. Due to *P. aeruginosa* inherent resistance to numerous antibiotic classes and its ability to acquire practical resistance to all effective antibiotics, nosocomial infections caused by this organism are now recognized as a serious issue in hospitals **[42]**. *P. aeruginosa* is distinguished as a significant microbe to detect antibiotic resistance in clinical specimens by all these characteristics. A full description of the characteristics of strains isolated from clinical and environmental wards can be obtained thanks to genetic approaches supplemented by phenotypic testing, which are important to assess the role of hospital equipment and staff in the diffusion route of resistance genes [**43].**

The bacterial fight to antibacterial agents (such as antibiotics) is a threat to community health throughout the world. Multi-drug resistant (MDR) is a bacterium mounting in presence of several drugs or may be carry several resistances genes **[44].**

Drug hardy microbes show condensed or nonexistent weakness to antibiotic drugs, thus allowing the infections to continue in patients and growth the numbers of fatalities. When first choice antibiotics do not work to treat an pollution, a second or third, often more toxic “drug of last resort” is managed in an effort to luxury the drug resistance infection **[45].**

The extracellular polysaccharide matrix of biofilm, which is made up of multilayered cell clusters, promotes the adherence of these microorganisms to wound surfaces while shielding them from the host immune system and antibiotic treatment **[46].**

Conclusion

Pseudomonas aeruginosa is a type of bacterium that has the skill to mature fight to antibacterial managers (such as antibiotics) rather quickly over several generations. This resistance present in some strains makes *P. aeruginosa* very difficult to treat. Increasing bacterial resistance is linked with the volume of antibiotic prescribed. Resistant bacteria are a bacterium growing in presence of a lot of drugs or carrying several resistance genes. It could be due to genetic or structural changes (cell wall

or enzymes), so this resistance is a major medical problem for patient and physician. Antibiotic misuse (such as increases with the duration of treatment) is extremely dangerous because bacterial resistance is not only to the same antibiotic, but also to a list of antibiotics of the same category.

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