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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(3):2937-2956. **OPEN ACCESS**

Microbiological studies on pathogenic bacteria isolated from burns and their susceptibility to some essential oils

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The objective of our study was to isolate and identify of bacteria that are prevalent in burn wounds, study their antibiotic resistance pattern and test the possibility of utilizing some essential oils in inhibition of these antibiotic resistant bacteria. This study was conducted on 100 patients who had sustained burn injury from outpatient and inpatient of Benha teaching Burn and Plastic Surgery department during the period from October 2016 to the end of May 2017. They were 60 female and 40 male and their ages were ranged from 1 to 70 years. One hundred burn wound swabs were subjected to isolation; identification; antimicrobial susceptibilities; antimicrobial activity of essential oils and combination between both antibiotics and essential oils. 16sr RNA PCR technique was used to confirm the most potent bacterial isolates. The effect of essential oils on bacterial cell were detected by scanning and transmission electron microscope. This study showed that the most common isolated organism from burn wounds were *Pseudomonas aeruginosa* (44.74%) followed by *Staph. aureus* (23.46%). 16sr RNA PCR technique was used to confirm the most potent bacterial isolates and it were *Pseudomonas aeruginosa* and *Staph. aureus*. thyme oil (66.7%) and tea tree oil (100%) were found to be the most effective essential oil against bacterial isolates. We also found that synergistic combination between Imipenem and Thyme oil has a high sensitivity effect (with inhibition zone of 25 mm diameter) against *Pseudomonas aeruginosa* than everyone alone, and also combination between Piperacillin and Thyme oil (with inhibition zone of 21 mm diameter).16S RNA-based PCR assays provide rapid, simple and reliable identification of *P. aeruginosa* and *S. aureus*, and its differentiation from other phylogenetically. Essential oils possess antibacterial activity against gram positive bacterial than gram negative bacterial isolates. Thyme oil considered one of the most important oils in the antimicrobial activity. Combination between antibiotics and essential oils were more effective against different bacterial isolates than using both individually. Both SEM and TEM are needed as complementary techniques to gain insight into AMP action, by revealing not only cell surface but also intracellular alterations.

Keywords: Burns, wound infections, antibiotic resistance, essential oils, 16s r RNA, electron microscope Original Article

INTRODUCTION

Burns are skin damage caused by variety of non-mechanical sources including chemicals, electricity, heat, sunlight or nuclear radiation. Thermal injury is a serious type of trauma requires care in a specialized units. It has been estimated that approximately 2.5 million people sustain burns of which 100,000 are hospitalized and there are around 12,000 deaths per year due to thermal injuries (Mayhall, 2003).The burn wound surface is a protein rich environment consisting of a vascular necrotic tissue that provides a favorable

niche for microbial colonization and proliferation (Church et al., 2006).The cause of nosocomial infections in burn patients might be endogenous or exogenous. Endogenous infections are caused by organism present as part of the normal flora of the patient, while exogenous infections are acquired through exposure to the hospital environment, hospital personnel or medical devices (Samuel et al., 2010).The most dominant bacteria in burn wounds is *Pseudomonas aeruginosa*, followed by *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumanii , Escherichia coli* and *Proteus mirabilis* isolates were also detected (Mona et al., 2016). 16S rRNA is a good method used in identification and confirmation of bacterial isolates (Momen, 2016). The emergence worldwide of antimicrobial resistance among a wide variety of human bacterial and fungal burn wound pathogens, particularly nosocomial isolates, limits the available therapeutic options for effective treatment of burn wound infections (Taneja et al., 2004). Some essential oils (EOs) are known for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties .They are widely used in folk medicine and food industry. They are also known as volatile oils, being complex mixtures of volatile constituents biosynthesized by plants (Silva et al., 2010). EOs contain 2twobiosynthetically related groups. These main groups include terpenes, terpenoids and aromatic, aliphatic constituents and some hydrocarbons also exhibit antimicrobial effects. Studies have shown that EO bacterial cell targets include the cell wall and membrane, thereby disturbing ATP production and pH homeostasis (Faleiro, 2011). The most famous essential oils of medical and antimicrobial activities are lemon oil, cinnamon bark oil, garlic oil, caraway oil, peppermint oil, tea tree oil, thyme oil, clove oil, camphor oil, olive oil, anise oil, dill oil, ginger oil, orange oil, chamomile oil and rose marry oil (Sonam et al., 2017) . Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are agood techniques used to examine the ultrastructural changes in bacteria induced by antimicrobial peptides (AMPs) (Nanis et al., 2015) . Sometimes the use of single antibiotic does not produce the desired effective inhibitory effects and to overcome this problem, a combination of essential oils (EO's) and antibiotics together are more strongly antimicrobially active than their major components individually (Abobakr et al.,2016).

MATERIALS AND METHODS

This work was carried out in Microbiology and Immunology Department, Faculty of Medicine, Benha University from October 2016 to the end of May 2017.

a-Sambles: This study was conducted on 100 patients who had sustained burn injury from outpatient and inpatient of Benha Teaching Burn and Plastic Surgery Department . They were 60 female and 40 male atients of ages ranging between 1 and 70 years

Burns were cleaned with sterile normal saline. The specimen was collected from either burn surface tissue or burn fluid (sampling by needle aspiration) with sterile cotton swabs.

Multiple samples from several areas of the burn were collected in order to obtain the most accurate assessment. They were taken before dressing changes and before administration of antibiotics. The samples were immediately transported to the laboratory (Mona et al., 2016).

b-Isolation and identification:

The sample was directly cultured on nutrient, blood and MacConkey agar plates. The plates were incubated aerobically at 37ºC for up to48 hours (Murray et al., 2003). Grown colonies were isolated and identified by biochemical reaction tests (Rasha, 2011).

c- Preparation of the bacterial suspension

The Inoculum was prepared by picking 5-10 colonies of each isolate with a sterile wire loop and suspended in into 2.5 ml of sterile distilled water. The density of suspension to be inoculated was determined by comparison with opacity standard on McFarland 0.5 Barium sulphate solution (CLSI, 2015) .

d-Antibiotic susceptibility testing :

Using Muller Hinton agar (Oxoid) and antibiotic discs ((Oxoid) including Imipenem(10 µg), cefotaxime(30 µg), ciprofloxacin(5 µg), ceftazidime(30 µg), Amoxicillin clavulanate(30 µg) , Levofloxacin(5 µg) , gentamycin(10 µg), Amikacin (30 µg) and Piperacillin (10 µg) for Gram negative bacteria and vancomycin(30 µg) , Erythromycin(15 µg), Clindamycin(2 µg) ,Ampicillin (10 µg) ,Gentamycin(10 µg) , Levofloxcin(5 µg) and Methicillin(5 µg) for Gram positive bacteria .Bacterial suspension was taken by a sterile cotton swab then streaked the surface of all the plate in three different planes. Using sterile forceps, the antimicrobial discs were evenly distributed. The discs should be about 15mm from

the edge of the plate and no closer than about 25 mm from disc to disc. After overnight incubation at 37˚C, inhibition zone diameters were read. The results of a disc diffusion test are interpreted by comparing the measured zone diameter with the interpretive criteria recommended by (CLSI, 2011)

e- Antimicrobial activity of essential oils:

Nine types of essential oils tested on bacterial isolates were purchased from local supermarkets and stored in full dark vials at 4 ºC. These oils were selected according to (Baser and Buchbauer, 2010) and (Abobakr et al., 2016) as mentioned in table (1)

Table (1): Essential oils tested for their antimicrobial activities against Gramnegative and Gram positive bacterial isolates

Family name	Scientific (Latin)name	English Name
Lamiaceae	*Ocimumbasilicum L.*	Basil
Lamiaceae	*Thymus vulgaris* L.	Thyme
Lauraceae	*Cinnamomumverum*	Cinnamon oil
Myrtaceae	*Melaleucaalternifolia*	Tea tree oil
Lamiaceae	*Menthapiperita L.*	Peppmint oil
Compositae	*Matricariachamomilla*	Chamomile oil
Apiaceae	*Cuminumcyminum* L.	Cumin oil
Lamiaceae	*OriganumVulgare*	Origanum oil
Oleaceae	*Oleaeuropaea L.*	Olive oil

The antibacterial activities of nine essential oils were assayed by agar disc diffusion. Suspension was taken by a sterile cotton swab then streaked on the surface of all the plate in three different planes. Filter paper discs were impregnated with 50 ul of different essential oils and then distributed on the inoculated agar medium with a sterile forceps . Plates were left for one hour at 4 ºC and then incubated for 24 h at 37ºC (Heba et al., 2015).

Inhibition zones were measured in mm and the organism were classified as sensitive, intermediate and resistant according to the standardized table supplied by approved National Committee for Clinical Laboratory Standards for any antimicrobial agent (NCCLS 2012).

F- Determination of the Minimum Inhibitory Concentration (MIC)

Serial two fold dilutions were performed for essential oils by using viscous liquid tween 80 (0.2 %). Filter paper discs were impregnated with 50ul .of oils dilutions and then were distributed on inoculated Mueller hinton agar plates. the plates were incubated overnight at 37°C for 24 hrs , then collected and zones of inhibition that developed were measured (CLSI, 2015).

***g*-Effect of combination between both antibiotics and essential oils against Gram negative and Gram positive bacterial isolates**

The antimicrobial activity of five commercial essential oils in combination with antibiotics was performed by using Disk diffusion test (indirect contact of essential oils) (Rodrigues et al., 2009).

By using the sterile forceps, the disks of selected antibiotics impregnated with 50 ul of oils were placed on the inoculated plates and then incubated at 37 ºC for 18-24 hrs. Inhibition zones were measured in mm and classified as sensitive, intermediate and resistant according to the standardized table supplied by approved (NCCLS 2012).

h- Molecular identification of the test isolate using 16s r RNA technique (Nanis et el., 2015):

It done to the two isolates that are resistant to all antibiotics and most sensitive to some essential oil.

Polymerase chain reaction (PCR) was performed in a thermal cycler (Bio-Rad MJ Research, Hercules, USA). The 50 µL reaction mixture consisted of 20 ng of genomic DNA, 2.5 U of Taq DNA polymerase, 5 µL of 10 × Taq buffer (100 mMTris-HCl, 500 mMKCl pH 8.3), 200 µMdNTP, 10 p moles each universal primers (forward primer AGA GTT TGA TCC TGG CTC AG and reverse primer GGT TAC CTT GTT ACG ACT T), and 2.0 mM MgCl2. Amplification included initial denaturation at 94°C for 5 minutes, followed by 25 cycles of denaturation 94°C for 30 seconds, annealing temperature of primers at 50°C for 30 seconds, and extension at 72°C for 1 minute. A final extension at72°C for 15 minutes was used. A total of 5 µL of the amplified product was then analyzed by submarine agarose gel electrophoresis in 1.2% agarose gel with ethidium bromide at 8 V/cm, and the PCR product were visualized under a gel doc UV transilluminator. The amplified PCR product was gel purified using the QIAGEN gel extraction kit. A total of 100 ng/µL concentration of 16S rRNA amplified product was used for the sequencing (Singh et al., 2012) by GATC Company using ABI 3730xl DNA sequence using forward and reverse primers (Sigma Scientific Services Co., Cairo, Egypt).

i-The effect of essential oils on bacterial cells by scanning and transmission electron microscope:

A loopful of bacteria was transferred to a 10mL test tube containing MacConkey broth. Essential oils were incubated with bacteria at

37°C for 18 hours. Changes in morphology of the bacteria were photographed under a scanning electron microscope (model JEOL, JSM-5200 LV, Japan) in the electron microscope unit of Tanta University. Changes in the ultrastructure of bacteria were photographed under a transmission electron microscope (model JEOL-JEM-100 SX electron microscope, Japan) at the electron microscope unit of the Faculty of Medicine of Tanta University. Authors please include name, city and country of manufacture (Nanis et el., 2015).

RESULTS

The clinical data of all studied cases (100 cases) showed that the age of the patients ranged from 1 to70 years. They were 60 females (60%) and 40 males (40%). 30 cases (30%) were outpatients while 70 cases (70%) were admitted in Burn and Plastic Surgery department in Benha Teaching Hospital.

Bacteria isolated from only 81 burn wound swabs from the total 100 swab indicated that 81% of examined patients had invasive burn wound infections, and only 19 samples (19%) were negative in bacterial growth.

Table (2): Distribution of the studied group according to Mechanism of burn:

Persistent in hospital	No. of samples (all patient)		No. of Isolates (positive patient)		% of Isolates From each group	Z test	P
	No	%	No	%			
Heat	62	62	55	67.9	88.7	98.46	<0.001**
Chemical	25	25	19	23.46	76.0	53.52	<0.001**
Electrical	10	10	6	7.41	60.0	26.68	<0.001**
Radiation	3	3	1	1.23	33.3	8.05	<0.001**
Total	100		81	100	81.0	114.13	<0.001**

FET= 10.1 P=0.018*

There were significant differences in the proportion of bacterial positive and negative patients group according to Mechanism of burn (P <0.001). There also were significant differences in the proportio of Mechanism of burn groups (P=0.018).The highest proportion of burn infection due to heat (67.9%).

Table (3): Distribution of the studied group according to Degree of burn:

Degree of burn	No. of samples (all patient		No. of Isolates (positive patient)		% of Isolates From each group	Z test	P
	No	%	No	%			
First	36	36	30	37.04	83.3	70.43	<0.001**
Second	52	52	44	54.32	84.6	85.98	<0.001**
Third	12	12	7	8.64	58.3	28.39	<0.001**
Total	100	100	81	100	81.0	114.13	<0.001**

X^2= 4.58 P=0.102

The highest rate of burn infection showed in the secound degree 52 (54.32%) . There were significant differences in the proportion of bacterial positive and negative patients group according to Degree of burn (P <0.001).

Table (4): Presentation of different bacterial strains in the total number of isolates (81 isolates) :

Isolated organism	Total no. of Isolates	% of isolates	Z test	P
Pseudomonas aeruginosa	33	40.74	51.35	<0.001**
Staph aureus	19	23.46	29.3	<0.001**
E.coli	13	16.05	19.84	<0.001**
Klebsiella pneumonia	11	13.58	16.69	<0.001**
Citrobacterfreundi	3	3.7	4.08	<0.001**
Proteus mirabilis	2	2.47	2.51	0.01*
Total	81	100		

the most common cause of burn infection was *Pseudomonas aeruginosa* 33 (40.74%) followed by *Staph aureus* 19 (23.46%).

Antibiotic sensitivity tests showed that the most sensitive antibiotic to *Pseudomonas aeruginosa*is Imipenem (75.76%) while the most resistance antibiotic is Cefotaxime (78.79%), the most sensitive antibiotic to *Staph. aureus*is clindamycin (73.68%) while the most resistance antibiotic is Methicillin (78.95%) , the most sensitive antibiotics to *E.Coli*is Amikacin (76.92%) while the most resistance antibiotic is Cefotaxime (76.92%) , the most sensitive antibiotics to *klebseilla pneumonia*is Levofloxacin (81.82%) while the most resistance antibiotic is Ceftazidime (63.64%), the most sensitive antibiotics to *Citrobacter freundii*is Amikacin (100%) while the most resistance antibiotic is Ceftazidime (100%), the most sensitive antibiotics to *Proteus mirabilis*is Ciprofloxacin (100%) while the most resistance antibiotic is Ceftazidime and Cefotaxime (100%).

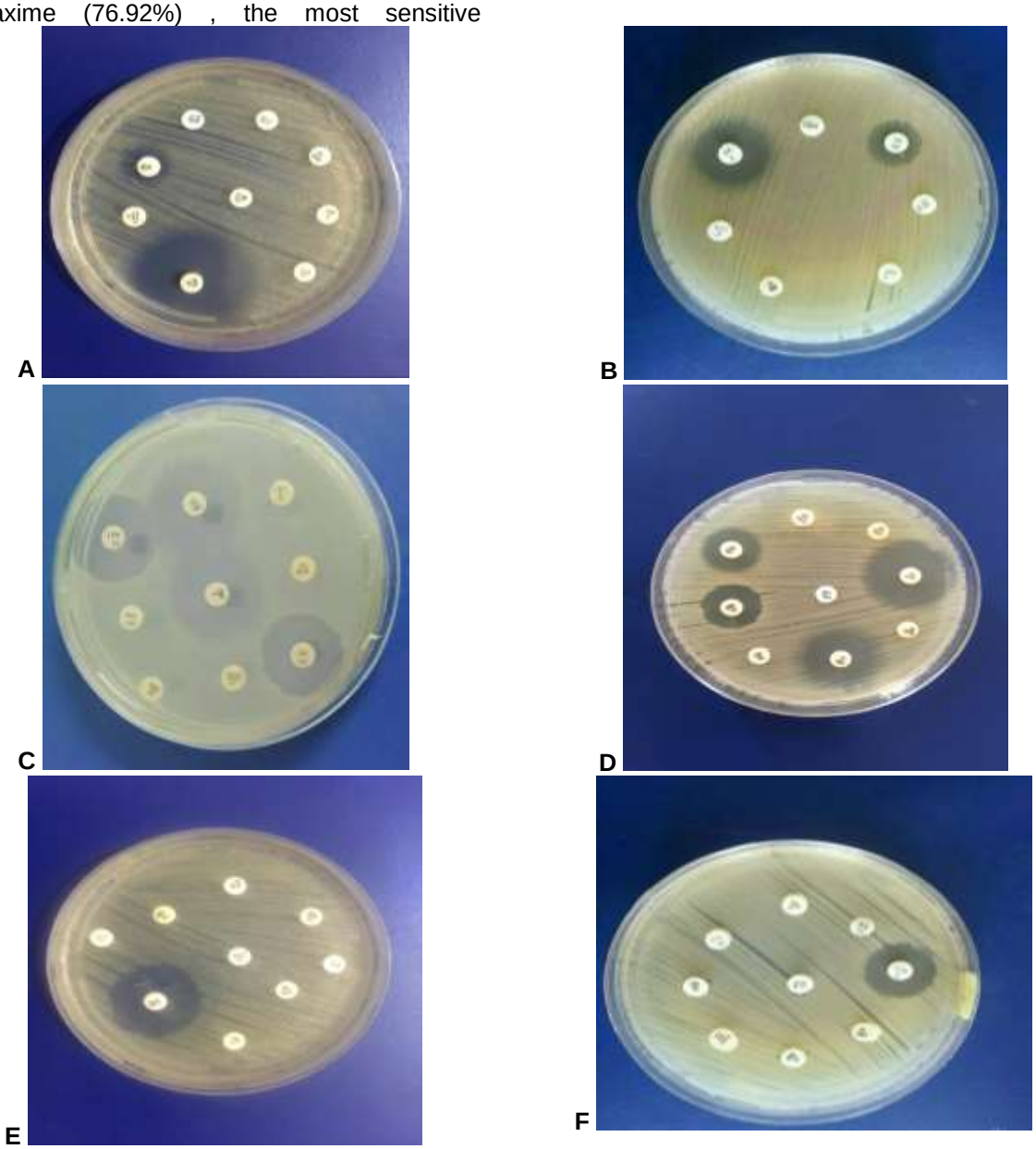

Figure 1; Photograph of antimicrobial sensitivity of antibiotics against bacterial isolates (Disk diffusion method)

A) *Pseudomonas aeruginosa*, (B) *Staph. areus* (c) *E.coli*, D) *Klebsiella pneumonia*, (E) *Citrobacterfreundii*, (F) *proteus mirabilis.*

Table 5: Antimicrobial sensitivity of essential oils against bacterial isolates by well diffusion method:

Essential oil sensitivity	staph areus (3 isolates)		Pseudomonas aeruginosa (3 isolates)		Klebseilla pneumonia (2 isolates)		FET	P
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)		
Cinnamon oil	1 (33.3%)	2 (66.7%)	1 (33.3%)	2 (66.7%)	0	2 (100%)	1.13	1.0
Thyme oil	1 (33.3%)	2 (66.7%)	2 (66.7%)	1 (33.3%)	0	2 (100%)	2.07	0.68
Tea tree oil	3 (100%)	0	1 (33.3%)	2 (66.7%)	0	2 (100%)	4.59	0.23
Pepprmint oil	0	3 (100%)	0	3 (100%)	0	2 (100%)		
Chamomile oil	0	3 (100%)	0	3 (100%)	0	2 (100%)		
Basil oil	0	3 (100%)	0	3 (100%)	0	2 (100%)		
Cumin oil	0	3 (100%)	0	3 (100%)	0	2 (100%)		
Origanum oil	0	3 (100%)	1 (33.3%)	2 (66.7%)	0	2 (100%)	1.9	1.0
Olive oil	0	3 (100%)	0	3 (100%)	0	2 (100%)		

The most effective essential oil to *Staph. areus*is Tea tree oil (%100) (with diameter 20 , 20.3 and 20.8) and the most effective essential oil to *Pseudomonas aeruginosa* is Thyme oil (66.7%)(with diameter 24 and 25.3) , while *Klebseilla pneumonia* isolates were resistant to all essential oil. There were no significance value.

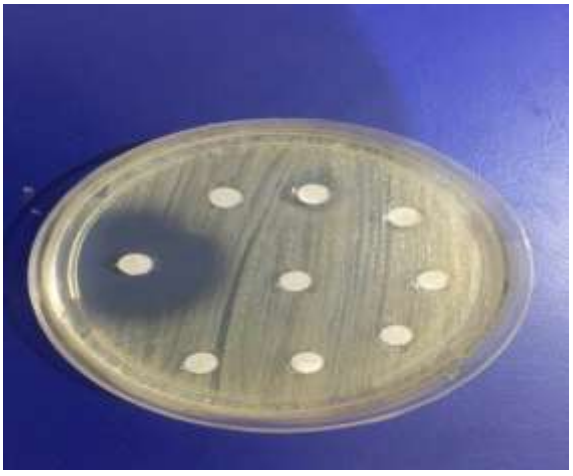

Figure (2): Disc diffusion for Pseudomonas *aeruginosa* and show that thyme oil is the most effective essential oil.

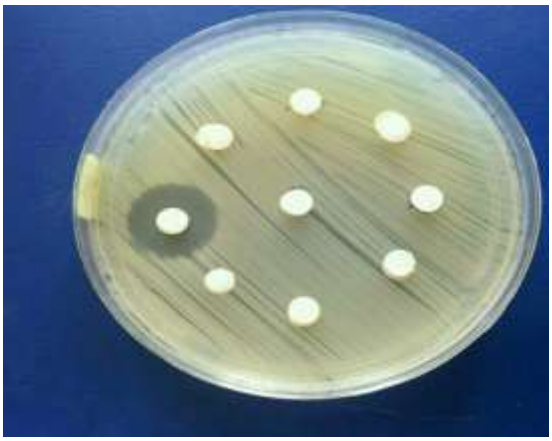

Figure (3): **Disc diffusion for Staph *aureus*and show that tea tree oil is the most effective essential oil.**

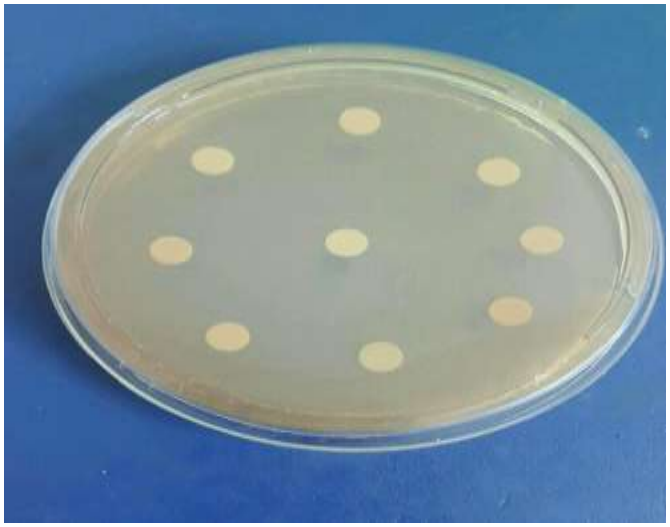

Figure (4) : Disc diffusion for *Klebseilla pneumonia* isolates and show resistant to all essential oil

Table (6): Minimum inhibitory concentration of essential oils that effect on bacterial isolate :

Antibiotic Sensitivity	Cinnamon oil (Concentration)			Thyme oil (Concentration)			Tea tree oil (Concentration)		
	100%	88%	66%	100%	88%	66%	100%	88%	66%
Staph. Areus	1	1	0	1	1	0	3	1	1
Pseudomonas aeruginosa	1	1	1	2	2	2	1	1	1
FET	1.23			1.13			1.04		
P	1.0			1.0			1.0		

Tea tree oil is the most effective oil against *Staph. aureus* with conc 66% and thyme oil is the most effective oil against *Pseudomonas aeruginosa* with conc 66%. There were no significance value.

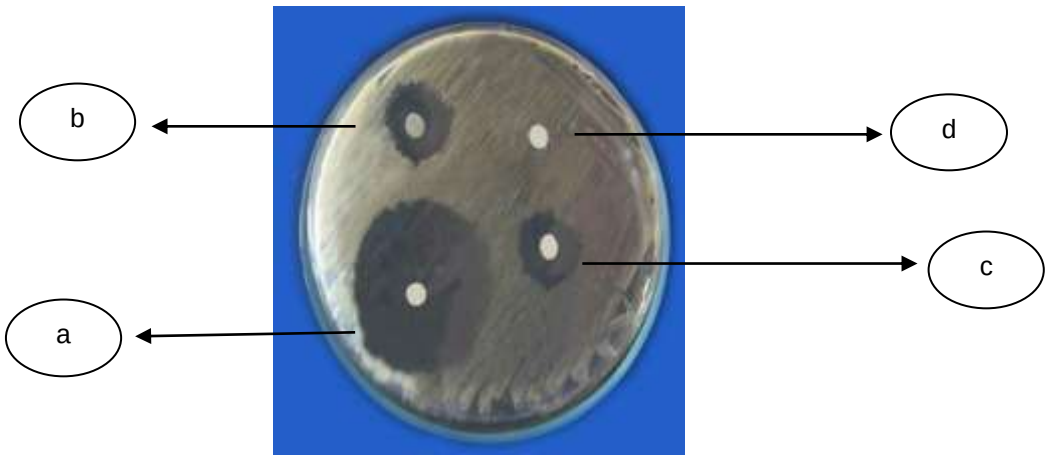

Figure (5) :(a) 100% conc , (b) 88% conc , (c) 66% conc and (d) 44% conc and show the minimum inhibitory concentration for *Pseudomonas aeruginosa* were the third dilution (c) 66% conc

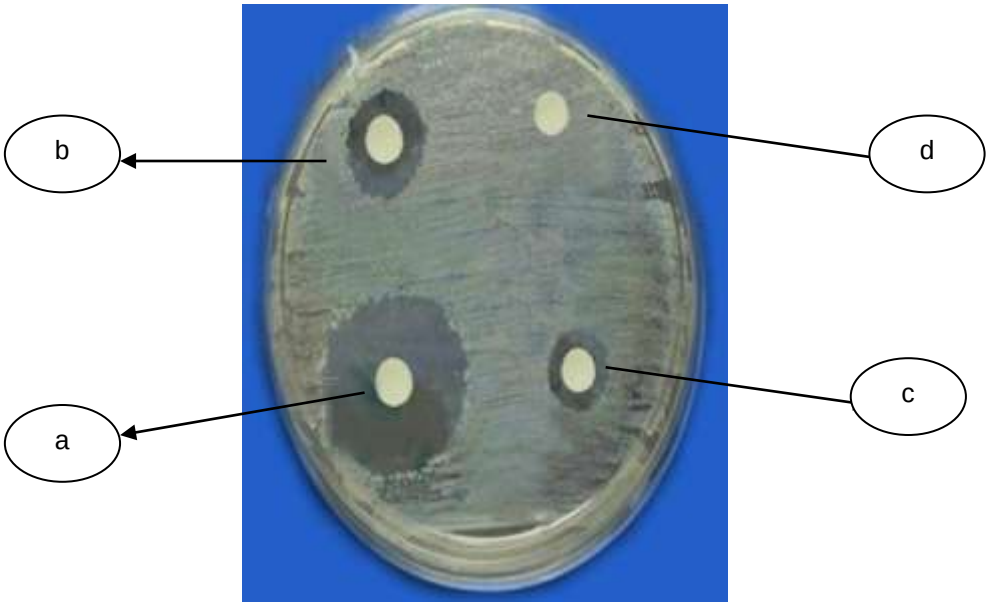

Figure 6 :(a) 100% conc , (b) 88% conc , (c) 66% conc and (d) 44% conc and show the minimum inhibitory concentration for *Staph. aureus* were the third dilution (c) 66% conc .

Table 7: The effect of antibiotic combination with the tested essential oils on *K. pneumoniae* isolates in terms of inhibition zone diameter in milimeters.

Antibiotic / Oil	Ceftazidime (30ug)	Cefotaxime (30 ug)	Amoxicillin clavulanate (30ug)	Amikacin (30 ug)	Ciprofloxacin (5ug)	Gentamycin (10ug)	Levofloxacin (5ug)	Piperacillin (100ug)	Imipenem (10ug)
Cinnamon oil	0	0	0	2	0	0	0	0	0
Thyme oil	0	0	0	0	0	0	0	0	0
Tea tree oil	0	0	0	3	0	0	0	0	0
Pepprmint oil	0	0	0	4	0	0	0	0	0
Chamomile oil	0	0	0	0	0	0	0	0	0
Basil oil	0	0	0	0	0	0	0	0	0
Cumin oil	0	0	0	0	0	0	0	0	0
Origanum oil	0	0	0	0	0	0	0	0	0
Olive oil	0	0	0	0	0	0	0	0	0

Table(7): shows that all *K. pneumoniae* isolates resist all synergistic combination between antibiotics and essential oils according to National Committee for Clinical Laboratory Standars (NCCLS ,2012) .

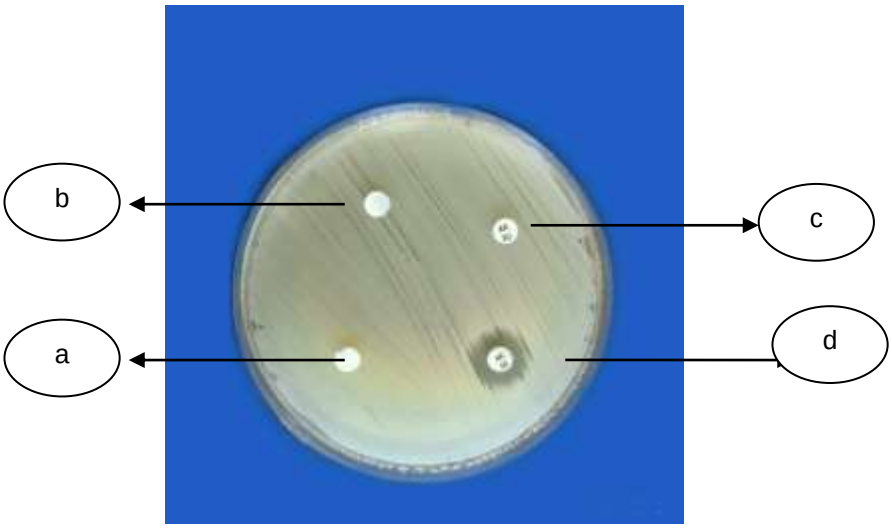

Figure 7: the effect of Amikacin and Pepprmint oil combination on *K. pneumoniae* (a) control , (b) oil, (c) antibiotic and(d) combination.

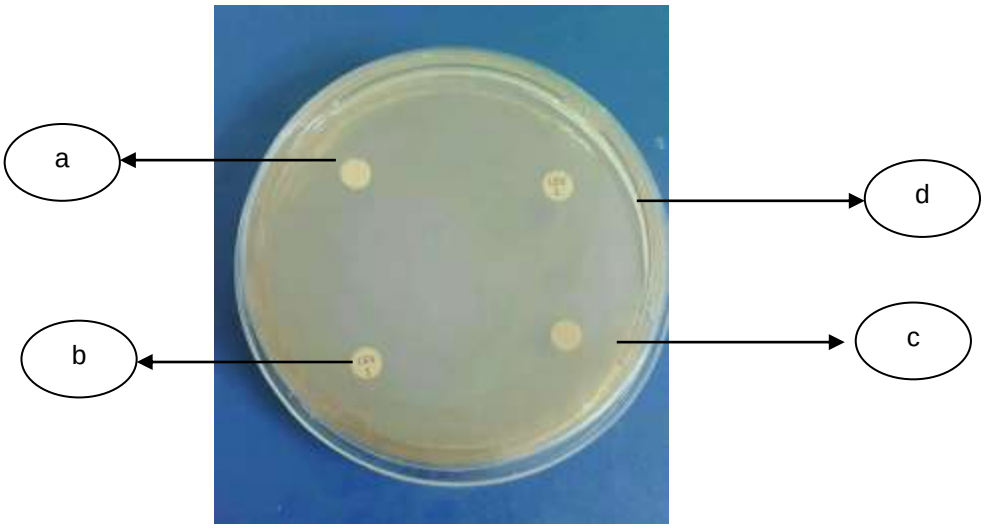

Figure 8 : the effect of Levofloxacin and Cinnamon oil combination on *K. pneumoniae* (a) control, (b) antibiotic, (c) oil and (d) Combination.

Table 8: The effect of antibiotic combination with the tested essential oils on *P.*

***aeruginosa*isolates in terms of inhibition diameter in milimeter**

Antibitic Oil	Ceftazidime (30ug)	Cefotaxime (30 ug)	Amoxicillin clavulanate (30ug)	Amikacin (30 ug)	Ciprofloxacin (5ug)	Gentamycin (10ug)	Levofloxacin (5ug)	Piperacillin (100ug)	Imipenem (10ug)
Cinnamon oil	0	**0**	0	**0**	**0**	0	**0**	**0**	0
Thyme oil	0	**0**	0	0	0	**0**	**0**	21	23
Tea tree oil	0	**0**	**0**	**0**	**0**	0	**0**	**0**	0
Pepprmint oil	**0**	0	0	**0**	**0**	0	**0**	**0**	0
Chamomile oil	**0**	**0**	0	**0**	**0**	0	**0**	**0**	0
Basil oil	**0**	**0**	0	**0**	**0**	0	**0**	**0**	0
Cumin oil	**0**	**0**	0	**0**	**0**	0	**0**	**0**	0
Origanum oil	**0**	0	0	**0**	0	0	**0**	**0**	0
Olive oil	**0**	**0**	0	**0**	**0**	0	**0**	**0**	0

Table(8): shows that *P. aeruginosa* isolate is sensitive to synergistic combination between Imipenem and Thyme oil (with diameter 25 mm) , and to combination between Piperacillin and Thyme oil (with diameter 25 mm) according to National Committee for Clinical Laboratory Standars (NCCLS ,2012) .

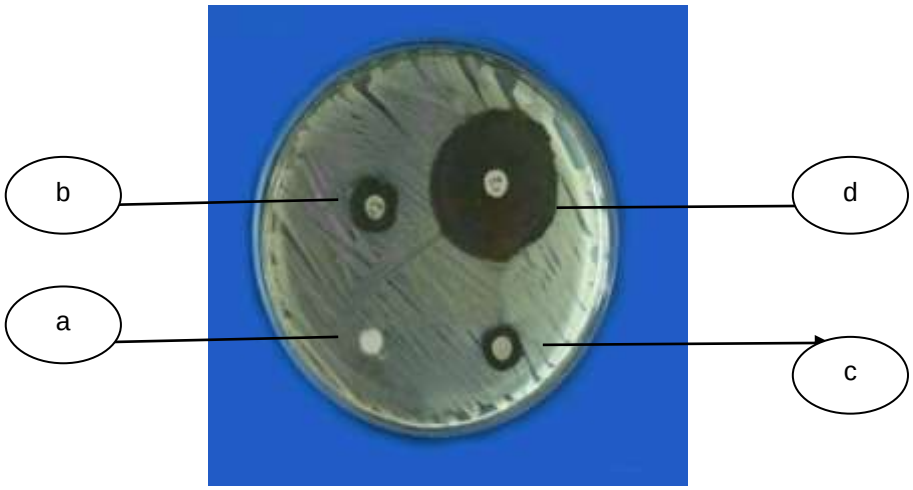

Figure 9: the effect of Imipenem and Thyme oil combination on *P. aeruginosa*(a) control, (b) antibiotic, (c) oil and(d) combination.

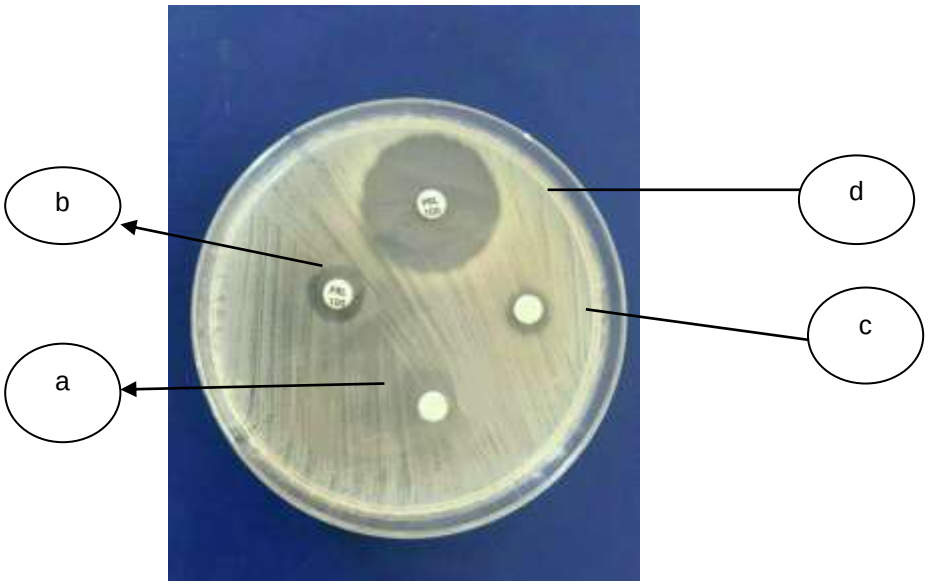

Figure 10 : the effect of Piperacillin and Thyme oil combination on *P. aeruginosa*(a) control, (b) antibiotic, (c) oil and (d) combination.

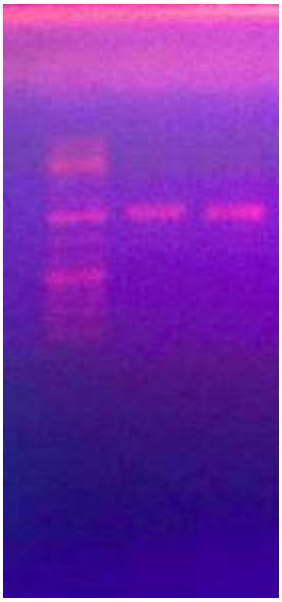

Figure 11: Sample 1200bp PCR product DNA Ladder 100bp, 250bp, 750bp and 1000bp.

Molecular identification of the test isolate using 16sr RNA technique:

two isolates are resistant to antibiotics and sensitive to some essential oil.

DNA was extracted and isolated using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene. The extracted DNA was used as template for amplification of 16S rRNA gene. The universal primers 27F and 1429R were used for the amplification and sequencing of the 16S rRNA gene fragment. The optimum annealing temperature was found to be 55ºC. An intense single band was visible on 1% agarose gel stained with ethidium bromide for the two tested isolates (*pseudomonas aeruginosa* and s*taph aureus*). (Fig.11)

Molecular identification of the most potent *Pseudomonas aeruginosa:*

The 16S rRNA gene sequence of *Pseudomonas spp*. was compared to that in GeneBank, and the phylogenetic tree was constructed. The obtained sequence proved that *Pseudomonas spp.* (isolate no. 4) was *Pseudomonas aeruginosa*. strain DSM 50071.

Pseudomonas aeruginosa strain DSM 50071 16S ribosomal RNA gene, partial sequence

Query 6 CGGGAACGTATTCACCGTGACATTCTGATTCATGATTA-AAACGATTCCCACTTCACGCA 64
Sbjct 1370 CGGGAACGTATTCACCGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCA 1311

Query 65 GTCGAGTTGCATACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGC 124
Sbjct 1310 GTCGAGTTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGC 1251

Query 125 GGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCC 184
Sbjct 1250 GGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCC 1191

Query 185 ATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGT 244
Sbjct 1190 ATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGT 1131

Query 245 GCCCACCCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCA 304
Sbjct 1130 GCCCACCCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCA 1071

Query 305 ACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGC 364
Sbjct 1070 ACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGC 1011

Query 365 ACCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGC 424
Sbjct 1010 ACCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGC 951

Query 425 TTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTT 484
Sbjct 950 TTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTT 891

Query 485 AACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATC 544
Sbjct 890 AACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATC 831

Query 545 TCAAGGATCCCAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATC 604
Sbjct 830 TCAAGGATCCCAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATC 771

Query 605 CTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCA-TCCAGGTGGTCGCCTTCG 663
Sbjct 770 CTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCG 711

Query 664 CCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAAGAAATTCCACCACCC 723
Sbjct 710 CCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCC 651

Query 724 TCTACCGT 731
Sbjct 650 TCTACCGT 643

Figure 12; Nucleotide sequence of the 16S rRNA gene of *Pseudomonas aeruginosa* isolate 4.

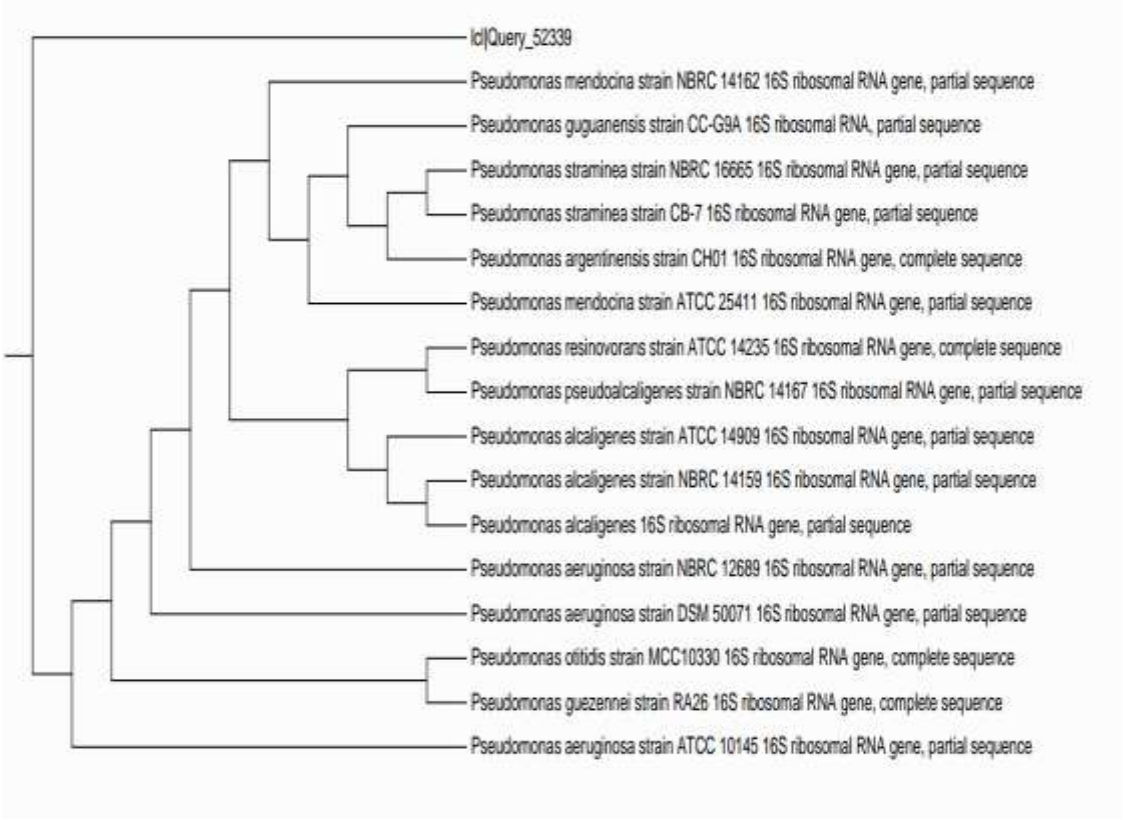

Figure 13: Phylogenetic tree of the *Pseudomonas aeruginosa*(Accession number : DSM 50071)

2-Molecular identification of the most potent Staph aureus :

The 16S rRNA gene sequence of Staph. spp. was compared to that in Gene Bank, and the phylogenetic tree was constructed. The obtained sequence proved that Staph. *spp. (isolate no. 5) was Staph. aureus. strain NBRC 100910.*

Staphylococcus aureus strain NBRC 100910 16S ribosomal RNA gene, partial sequence

Query 8 GGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCAC 67
Sbjct 1412 GGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCAC 1353

Query 68 CGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGACT 127
Sbjct 1352 CGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGACT 1293

Query 128 ACAATCCGAACTGAGAACAACTTTATGGGATTTGCTTGACCTCGCGGTTTCGCTGCCCTT 187
Sbjct 1292 ACAATCCGAACTGAGAACAACTTTATGGGATTTGCTTGACCTCGCGGTTTCGCTGCCCTT 1233

Query 188 TGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGACGT 247
Sbjct 1232 TGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGACGT 1173

Query 248 CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCAACTTAGAGTGCCCAACTTAATGAT 307
Sbjct 1172 CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCAACTTAGAGTGCCCAACTTAATGAT 1113

Query 308 GGCAACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA 367
Sbjct 1112 GGCAACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA 1053

```
Query  368   GCTGACGACAACCATGCACCACCTGTCACTTTGTCCCCCGAAGGGGAAGGCTCTATCTCT  427
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1052  GCTGACGACAACCATGCACCACCTGTCACTTTGTCCCCCGAAGGGGAAGGCTCTATCTCT  993

Query  428   AGAGTTGTCAAAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCA  487
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  992   AGAGTTGTCAAAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCA  933

Query  488   CATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGT  547
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  932   CATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGT  873

Query  548   ACTCCCCAGGCGGAGTGGTTAATGCGTTAGCTGCAGCA-TAAGGGGCGGAAACCCCCTAA  606
              ||||||||||||||| |||||||||||||||| ||||||||||||||||||||||||||
Sbjct  872   ACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAA  813

Query  607   AA-TTAGCACTCATCGTTT-CGGGGTGGAC-ACCAGGGTA-CTAATCCGGTT-GATCCC-  660
              | |||||||||||||||| ||| |||||| |||||||| ||||||| ||| ||||||
Sbjct  812   CACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCCCC  753

Query  661   ACGCTTTCGCA-ATCA  675
              |||||||||| ||||
Sbjct  752   ACGCTTTCGCACATCA  737
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Figure 14; Nucleotide sequence of the 16S rRNA gene of Staph.aureus isolate 5.

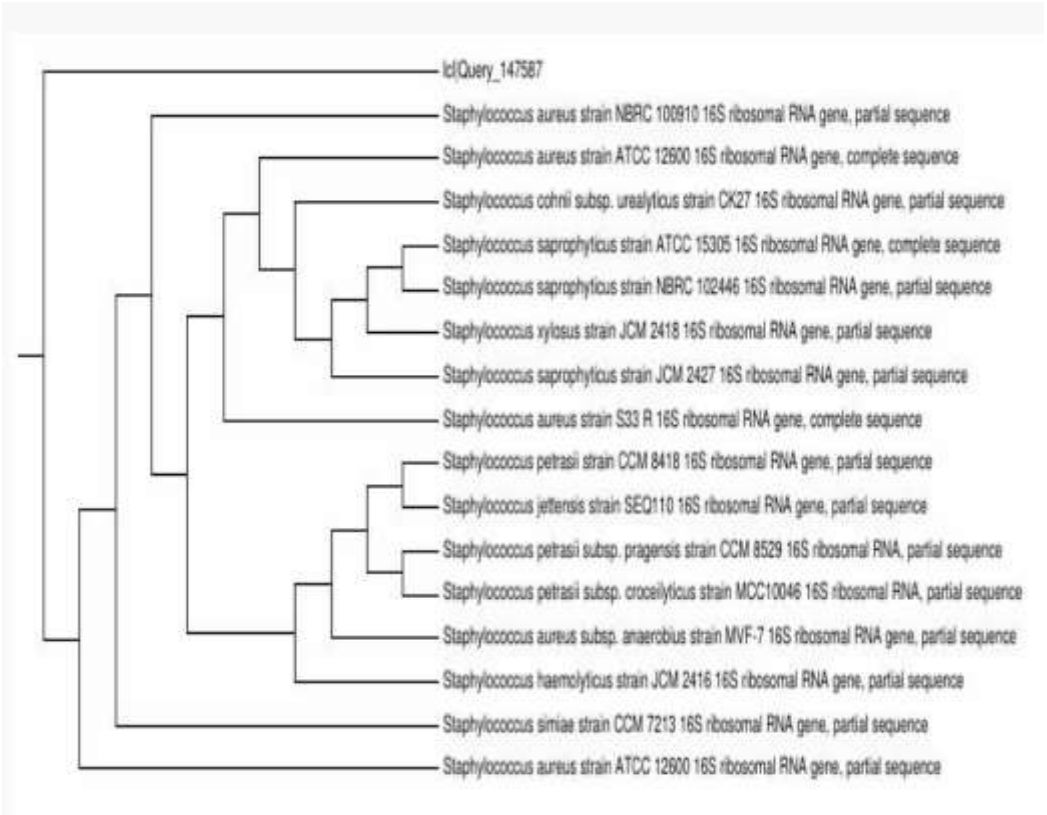


Figure 15: Phylogenetic tree of the *Staph. aureus*(Accession number : NBRC 100910)

The effect of essential oils on bacterial cells by scanning and transmission electron microscope

The most effective essential oil (thyme oil) on the most effected bacteria (*Pseudomonas aeruginosa) was* examined under electron microscope

Scanning electron microscope

It show alternation in the structure of the cell envelope and cell membrane and generally alternation in the external structure of the cell.

Transmission electron microscope

It show alternation in the morphology of the cell, coagulation of the cytoplasmic content, vaculation in the cell, leavage of intracellular constitutant , and generally alternation in the internal structure of the cell.

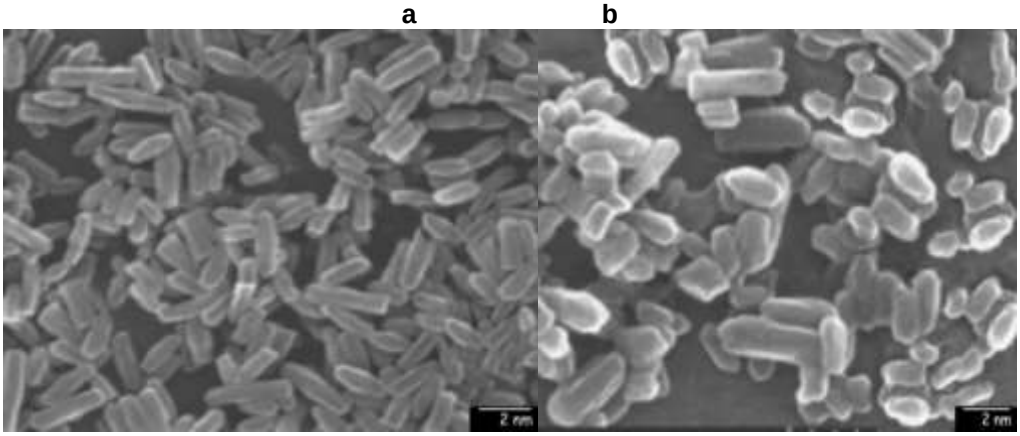
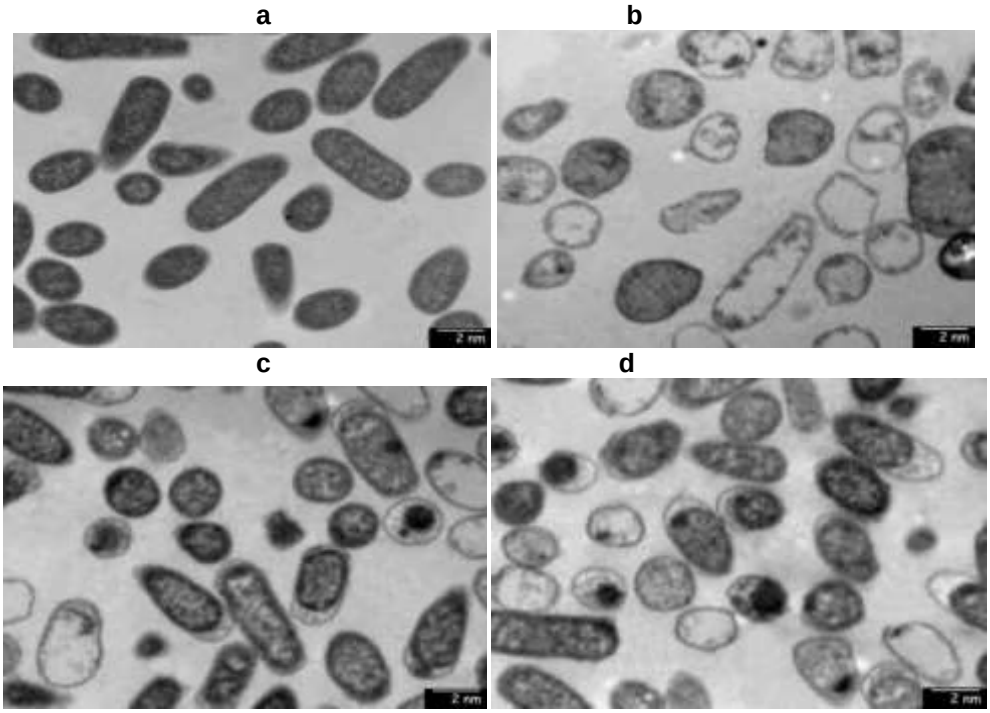


Figure 16.Photographs of scanning electron microscope reveal the effect of antibacterial substances on *Pseudomonas aeruginosa* (a) represents control; (b) represent effect of thyme oil.



e

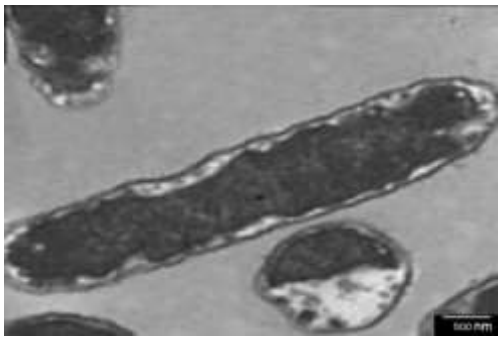


Figure 17;Photographs of transmission electron microscope reveal the effect of antibacterial substances on *Pseudomonas aeruginosa* ;(a)represents control; (b-c-d- e) represents effect of thyme oil.

DISCUSSION

Burns are damage to the skin caused by variety of non-mechanical sources including chemicals, electricity, heat, sunlight or nuclear radiation.The most dominant bacteria in burn wounds is *Pseudomonas aeruginosa*, followed by *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacte rbaumanii , Escherichia coli and Proteus vulgaris* isolates were also detected (Mona et al., 2016).

Essential oils (EOs) are known for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties .They are widely used in Folk medicine and food industry for these purposes (Silva et al.,2010).

Sometimes the use of single antibiotic does not produce the desired effective inhibitory effects and to overcome this, a combination of essential oils (EO's) and antibiotics together are more strongly antimicrobial than their major components individually (Nanis et al.,2015) The research aims to isolation and identification of bacteria that are prevalent in burn wounds and to study their antibiotic resistance pattern and the possibility of the use of some essential oils in inhibition of the growth of these antibiotic resistant bacteria.

The clinical data of all studied cases (100 cases) showed that the age of the patients ranged from 1 to70 years. They were 60 females (60%) and 40 males (40%). 30 cases (30%) were outpatients while 70 cases (70%) were admitted in Burn and Plastic Surgery department in Benha teaching Hospital.

In the present study, Bacteria isolated from only 81 burn wound swabs from the total 100 swab indicated that 81% of examined patients had invasive burn wound infections, and only 19 samples (19%) were negative in bacterial growth. This idea supported the investigation of(Raja and Singh, 2007) who explained that the burn wound infections are one of the most important and potentially serious complications that occur in the acute period following injury.

Also, the overall bacterial isolation rate of 81%is consistent with the rate reported similar studies in Nigeria (83%)(Motayo et al ., 2013) and elsewhere (79%) (Mohammed and Tsegaye, 2014)

There were significant differences between bacterial positive and negative patients regarding to age in all age group ($p < 0.001$) this mean that in each age group most patients were been infected . According to age distribution of our cases, the majority of burn infection cases(49.38%) were between 16:50 years . This could be due to the fact that this group is the most active group, and most involved in outdoor activities. Our study is in agreement with (Forson et al.,2017) who found that the 21-40 age group being the most affected age group.

According to mechanism of burn there were significant differences in the proportion of Mechanism of burn groups ($P=0.018$).The highest proportion of burn infection due to heat (67.9). This may be due to equipments and products were more responsible for flame burn diverse, but the most important of them were home equipment's used for cooking or heating. Another factor contributing to the risk of these is that cylinders usually places it near the cooker equipment's inside the kitchen, especially in old buildings. Our study is in agreement with Forson et al.,(2017)who reported that (66%) of the studied patients had flame injury caused by Gas ,

also with (kamal et al., 2017) who found that the flame burns were the second cause of burn injury 39.1%.

According to Degree of burn This study revealed that majority of burn injuries with second degree of burn (54.32) followed by first degree (37.04%) may be occur by boiling water, chemicals or extended contact with hot surfaces, hot liquids, or flames .the reason may be due to longer period of burn exposure results in a potential longer time of contact with body surface, resulting in degree (depth) of burn..

This was similar to the results reported by (Sabetha et al.,2017) who showed highest distribution of burn wound infection in burn patients who had sustained second-degree burn (52%) followed by first degree (34%) .

In our study we found that *Pseudomonas aeruginosa* was the most common isolated organism (44.74%) of cases, as it was considered as a major factor in the etiology of burn wound infection, followed by *Staph aureus* (23.46%) of cases, which is in agreement with (Neda et al., 2017) who found *Pseudomonas aeruginosa* to be the cause of burn wound infection in 42.1% of patients, followed by *Staphylococcus areus* 22.1%.

While other studies Imran et al.,(2007)[28]reported that *S. aureus* was the most common isolate in burn wound infection .

Our study show that, In case of *Pseudomonas aeruginosa* the most sensitive antibiotic is Imipenem (75.76%) while the most resistance antibiotic is Cefotaxime (78.79%).

Our study is consistent with (Anushka and *Solabannavar.,* 2017) who reported that the most sensitive antibiotic to *Pseudomonas aeruginosa* is Imipenem (98%) and with (Sabetha et al.,2017) who found that (80%) were Cefotaxime resistant .

In case of *Staph aureus* the most sensitive antibiotic is clindamycin (73.68%) while the most resistance antibiotic is Methicillin (78.95%).

Our study is consistent with (Sabetha et al., 2017) who reported that (81%) of *S. aureus* isolate show sensitivity to clindamycin and with (Anushka *and Solabannavar.,* 2017) who found Methicillin is the most resistance antibiotic to *Staphylococcus aureus.*

In our study there were 8 isolates resistant to all antibiotics .these are *staph areus* (3 isolates), *Pseudomonas aeruginosa* (3 isolates) and *klebseilla pneumonia* (2 isolates). When use essential oils with these isolates, only 5 isolates (3 *Staph. aureus* and 2 *Pseudomonas aeruginosa)* show sensitivity to some of oils.

When used essential oils this study has shown that only two strain of gram negative bacterial isolates (*Pseudomonas aeruginosa*) show sensitivity to essential oils .Many researches work confirm that Gram negative bacteria are more resistant against essential oils because of the cell wall structure. Gram-negative bacteria have an outer lipopolysaccharide wall that can work as a barrier against toxic agents this agree with (Fisher and Phillips, 2009).

The recent study of these oils was found to be more effective on Gram positive (3 *strain of staph aureus bacterial isolate*) than Gram negative bacteria, It may be due to absence of lipopolysaccharide layer in gram positive bacteria that may have acted as a barrier against any incoming bimolecular, this agree with (Fisher and Phillips, 2009) and (Heba et al., 2015).

In case of *Staph.aureus* the most sensitive essential oil is Tea tree oil (%100) with low MIC (66%) as Tea tree oil and its major compounds including terpinen-4-ol has strong antimicrobial effects on *Staph aureus* this is in agreement with (Cuaron et al.,2013) who found that tea tree oil has high antimicrobial activity against *Staph. aureus* with lowest MIC.

While (Mona et al., 2016) reported that thyme oil is the most effective followed by cinnamon oil against *Staph. aureus* isolates.

In case of *Pseudomonas aeruginosa* the most sensitive essential oil is Thyme oil (66.7%) with low MIC (66%) , The antibacterial activity of thyme oil may be due to the presence of p-cymene that has a high affinity for membranes and causes membrane expansion and affect the membrane potential of intact cells and Thymol which are able to disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP, this is in agreement with (Mona et al., 2016) and (Dahiya and Purkayasth, 2012) who found that thyme oil was found to be the most active oils against *P.aeruginosa* isolates with lowest MIC.

As regard to synergistic combination between essential oils and Antibiotics *K.pneumoniae* isolates resist all synergistic combination between antibiotics and essential oils according to National Committee for Clinical Laboratory Standars but *P. aeruginosa*isolate is sensitive to synergistic combination between Imipenem and Thyme oil (with diameter 23 mm) and this agreement with (Fadila and Tajelmolk., 2016), who reported that combination between Imipenem and Thyme oil

has a high sensitivity effect than everyone alone with diameter 25 mm ,and to combination between Piperacillin and Thyme oil (with diameter 21 mm) and this agreement with (*Lobna* et al., 2014), who reported that combination between Piperacillin and Thyme oil was more effective than everyone alone with diameter 20 mm.

Our electron microscopy study has demonstrated that the impact of antimicrobial peptides p-cymene on the bacterial cell passes through several stages, depending on the cell type, the peptide, and its concentration as it has a high affinity for membranes and causes membrane expansion and affect the membrane potential of intact cells then Thymol disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP, this is in . After treatment of representative strains of the Gram-negative p. aeruginosa with MIC of thyme oil , several distinct signs of damage to the cell envelope were clearly observed in the SEM and TEM micrographs, such as blisters, protruding bubbles, membrane stacks, mesosomes, deep craters, and burst cells (Mareike et al., 2010) .

Scanning electron microscope show alternation in the structure of the cell envelope and cell membrane and generally alternation in the external structure of the cell.

Transmission electron microscop show alternation in the morphology of the cell, coagulation of the cytoplasmic content, vaculation in the cell, leavage of intracellular constitutant , and generally alternation in the internal structure of the cell.

CONCLUSION

1- Burn patients were most commonly infected with *P. aeruginosa*and *S. aureus.*

2-The most effective antibiotic to Gram negative bacteria were Imipinem and Amikacin while the most resistance antibiotic were Cefotaxime and Ceftazidime, and the most sensitive antibiotic to Gram positive bacteria was Clindamycin while the most resistance antibiotic was Methicillin.

3- 16S RNA-based PCR assays provide rapid, simple and reliable identification of P.aeruginosaand *S.aureus*, and its differentiation from other phylogenetically closely related species.

4- Essential oils possess antibacterial activity against Gram positive bacterial than Gram negative bacterial isolates.

5- Thyme oil considered one of the most important oils in the antimicrobial activity.

6- Combination between antibiotics and essential oils were more effective against different bacterial isolates than using both individually.

7- Both SEM and TEM are needed as complementary techniques to gain insight into AMP action, by revealing not only cell surface but also intracellular alterations.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

This work was financially supported by Ph. D. scholarship for Mrs. Dina S. Ramadan from Faculty of science, Menofia University, Egypt.

AUTHOR CONTRIBUTIONS

MTS supervised the microbiology experiments. DSR executed all of the experiments and wrote the article. SME supervised the molecular Biology part. DSR carried out the statistical analysis. All authors read and approved the final version.

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