International Journal of Pharmacology

ISSN 1811-7775

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2017.313.322

Research Article
Molecular Characterization of β-lactam Antibiotic Resistant *Pseudomonas aeruginosa* Isolated from Egyptian Food

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Abstract

Background and Objective: β-lactam resistant bacteria are emerging highly drug-resistant causing infections accompanied with significant importance. This study was undertaken to detect the β-lactam antibiotic resistant *P. aeruginosa* obtained from edible foods in El-Giza governorate, Cairo, Egypt and role of the plant oils in reducing β-lactam resistance for *P. aeruginosa*. Fifty samples from edible food were collected from supermarkets. There are multi drug resistant bacteria presented in the tested edible food. **Materials and Methods:** Isolation were carried out using classical method by using selective medium then the isolates were identified using modern techniques by PCR and DNA sequencing techniques. **Results:** Results showed identified isolates were 50 pathogenic bacteria as *Staphylococcus* spp., 10 isolates (40%), *Micrococcus* spp., 1 isolate (4%), *E. coli* 11 isolates (44%), *Citrobacter freundii* 1 isolate (4%), *Enterobacter* species 1 isolate (4%), *Enterobacter cloacae* 1 isolate (4%) and *P. aeruginosa* 1 isolate (4%). The *P. aeruginosa* strain was resistance to piperacillin/tazobactam and meropenem, intermediate to gentamicin while sensitive to amikacin, levofloxacin and ciprofloxacin. Moreover, *P. aeruginosa* strain was sensitive to thyme oil but resistant to fennel, caraway and peppermint oils. **Conclusions:** Results concluded that treatment β-lactam resistant *P. aeruginosa* by aminoglycosides or fluoroquinolones antibiotics has significant value. Also, thyme oil considered one of the most important oils in the antimicrobial aspects. Finally treatment of resistant strain of *P. aeruginosa* by combination of antibiotic and medicenal plants even if resistant to these oils has significant potentiating effect.

Key words: *Pseudomonas aeruginosa*, 16S rRNA gene sequence, phylogenetic relationship, NCBI, BLAST, β-lactam antibiotic resistance

Received: December 18, 2016 Accepted: January 18, 2017 Published: March 15, 2017

Citation: Shehab E. Talat, Mahmoud Abd El-Mongy, Mona I. Mabrouk, A.B. Abeer Mohammed and Hanafy A. Hamza, 2017. Molecular characterization of β-lactam antibiotic resistant *Pseudomonas aeruginosa* isolated from Egyptian food. Int. J. Pharmacol., 13: 313-322.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Food-borne bacterial microorganisms are common worldwide and cause public health concern. In particular, microorganisms belonging to the Pseudomonadaceae are important causative agents of food infection[1]. Prolonged uses of antibiotics lead to bacterial adaptation especially the use of antibiotic under therapeutic doses resulting in the development of multidrug resistance bacteria[2]. For many years, the β-lactam antibiotics have been widely used for treatment of *P. aeruginosa* infections, the resistance to β-lactams can be acquired either by cephalosporinase produced by chromosomal genes or by poor permeability of the antibiotic in the bacteria[1]. Essential oils are valuable natural products used as raw materials in many fields, including cosmetics, perfumes, phyto-therapy, spices, aromatherapy and nutrition. So, many scientists screened plants and studied the biological activities of their oils from and pharmacological application and therapeutic aspects[3]. Also, natural compounds of plant origin such as the reserpine, kaempferol rhamnoside, silybin, capsaicin, piperine, methoxylated flavones, isoflavone, porphyrin pheophorbide, etc., may be considered as efflux pump inhibitors[4]. On the other hand, plant which has antimicrobials effect such as carvacrol, thymol and catechins act by disruption of cell membrane, followed by release of intracellular contents leading to loss of ATP[5]. Also, carvacrol, thymol and eugenol cause increase the sensitivity of bacteria to antibiotics[6]. Since the mechanism of antimicrobial resistance in bacteria is mainly mediated by interaction between specific transporters of antibiotics and efflux pump. So, the plant compounds could act through modulation of these efflux pumps which increase the antibiotic sensitivity of the bacteria[7]. Moreover, Garvey *et al.*[8] stated that some medicinal plants have efflux inhibitory activity against bacteria. Also, multi-drug efflux pumps were considered a major cause of multi-drug resistance where the multi-drug effluxes pump systems represented in the cell membrane of the bacteria actively eliminate antimicrobial agents from bacterial cells[1]. Finally, Stavri *et al.*[9] stated that using *Thymus vulgaris* oil has a strong synergic activity when used in combination with the β-lactam antibiotics against some tested bacteria. Thus the objective of this study is focusing on the role of the plant oil in reducing β-lactam antibiotic resistance for *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial isolation: Fifty samples from edible food were collected from supermarkets in El-Giza governorate, Cairo, Egypt. From January till December, 2015. According to the method of El-Jakee *et al.*[10].

Growth media: Nutrient broth, nutrient agar, Mueller-Hinton agar and Pseudomonas cetrimide agar[11].

Identification of bacteria using API: The API 20E test strips (BioMerieux, France) were used as a biochemical identification system for identification of the isolated genera to the species level.

Molecular identification of *P. aeruginosa* using PCR

DNA extraction: Genomic DNA was extracted by the method of Menassa *et al.*[12].

Primers: The *P. aeruginosa* relevant 16S rRNA primers based on GenBank database were used. Primers were F-(GGGGGATCTTCGGACCTCA) and R-(TCCTTAGAGTGCCCAC CCG).

Polymerase Chain Reaction (PCR) procedure: Conducted in a micro-amplification tube (PCR tubes) of 25 µL volume containing 2 µL of genomic DNA from the *P. aeruginosa*, 6.5 µL of deionized water, 12.5 µL master mix, 2 µL of each primer specific for 16S rRNA gene of *P. aeruginosa*. The PCR cycles were initial-denaturation for 2 min at 95°C, 25 cycles were completed, each cycle consisting of 20 sec at 94°C, 20 sec at the appropriate annealing temperature (58°C) and 40 sec at 72°C. A final extension of 1 min at 72°C was applied. Before 30 min electrophoresis at 70 V, the 1% agarose gel was stained with 2 µL ethidium bromide and photographed under trans illuminator ultraviolet (UV) light. A 100 bp DNA ladder (MBI Fermentas USA) was used as marker for the molecular weight of PCR products. Sequencing was performed by MacroGen, MD, USA in Animal Health Research Institute. Sequences were submitted to NCBI GenBank using BankIt (http://www.ncbi.nlm.nih.gov/WebSub/?tool=gene bank).

Oil extraction and analysis: The four medicinal plants oils (thyme, fennel, caraway and peppermint) were obtained from Phytochemistry Department, Applied Research Center for Medicinal Plants, National Organization for Drug Control and Research (NODCAR), Giza, Egypt. The oil extraction of 100 g of each explant powder were covered with sufficient water in the flask and subjected to steam distillation[13] for 4 h to obtain essential oil. The oils were dehydrated by anhydrous sodium sulfate and stored in black vials at 5°C. All the tested oils were complied with[13] specifications. Then oil analysis was done by gas chromatography.

Gas chromatography: Essential oils were analyzed using an agilent technologies 6890 gas chromatograph, equipped

with a Flame Ionization Detection (FID) detector and HP5 column (30 m×0.25 mm×0.25 μm film thickness). Injector and detector temperatures were set at 225 and 275°C, respectively. Oven temperature gradually raised from 60-280°C at an initial rate of 10°C min^{-1}. Nitrogen (purity 99.9%) was the carrier gas, at a flow rate of 1 mL min^{-1}. Diluted sample (1/100 in n-hexane, v/v) of 1.0 μL injected in the split mode (ratio 1:10). Quantitative data were obtained electronically from FID area present data without the use of correction factors. Peak integration and quantification were performed automatically with HP chemistation software. A checking of the integration of peak is carried out and corrected manually if necessary.

Antibacterial activity of medicinal plant oils: Antibacterial activity of medicinal plants oil against various tested clinical bacterial isolates was studied by agar well diffusion method[14].

Detection of synergetic interaction between plant oils and antibiotics: According to Moussaoui and Alaoui[15] with some modification, the plates inoculated with 0.5 mL oil/50 mL Mueller-Hinton Agar (MHA) then allowed drying before placing the diffusion antibiotic disks. Susceptibility of the tested isolate to various tested antibiotics was performed by disk diffusion method as described by CLSI[16]. Using commercially available antibiotic disks purchased from Oxoid Ltd., Co., containing piperacillin/tazobactam (110 μg), meropenem (10 μg), gentamicin (10 μg), amikacin (30 μg), levofloxacin (5 μg) and ciprofloxacin (5 μg) were placed on the surface of the inoculated MHA plates with *P. aeruginosa*. The inoculated plates were then incubated at 37°C for 24 h. Inhibition zone diameters were measured inclusive of the diameter of the disks. Results were expressed as sensitive, intermediate and resistant according to CLSI[16].

RESULTS AND DISCUSSION

Test of microbes isolated from 50 edible food samples: The results indicated that there are 50 pathogenic bacteria like *Staphylococcus* spp., 10 isolates (40%), *Micrococcus* spp., 1 isolate (4%), *E. coli* 11 isolates (44%), *Citrobacter freundii* 1 isolate (4%), *Enterobacter* species 1 isolate (4%), *Enterobacter cloacae* 1 isolate (4%) and *P. aeruginosa* 1 isolate (4%).

***Pseudomonas aeruginosa* isolation and characterization:** There is only one β-lactam resistant *P. aeruginosa* isolated from food (kareesh cheese) (Table 1). The *P. aeruginosa* was Gram-negative, which shows positive result on catalase, oxidase, indole, citrate and nitrate test with green pigment production.

***Pseudomonas aeruginosa* fluorescence under UV illumination:** The *P. aeruginosa* give fluorescents under UV lamp due to pyocyanin pigment production are presented in Fig. 1.

Molecular identification: The PCR assay for 16S rRNA gene was done to identify the isolate are presented in Fig. 2. The PCR size of the amplicon obtained from the tested isolate corresponded to the predicted size of about 956 bp.

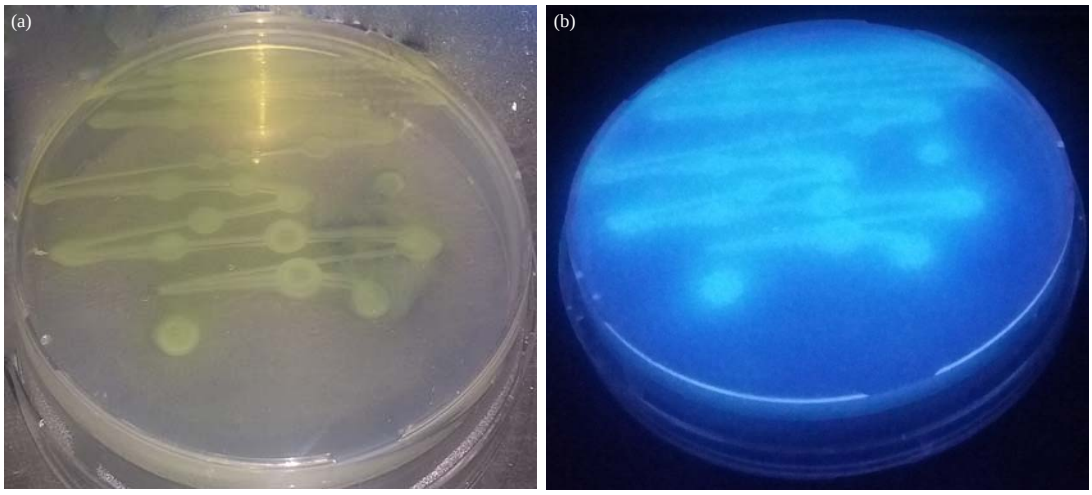


Fig. 1(a-b): *Pseudomonas aeruginosa* on Pseudomonas cetrimide agar, (a) *P. aeruginosa* under light lamp showing green pyocyanin pigment and (b) *P. aeruginosa* give fluorescence under UV lamp

The 16S rRNA sequence similarity for *P. aeruginosa* strain with some isolate presented in NCBI database is presented in Fig. 3. The PCR product was sequenced and the sequence was blasted against available sequences in the NCBI GenBank to confirm the identification of the different *P. aeruginosa*. Partial sequence was submitted to the NCBI GenBank with accession No. KX452946 (*P. aeruginosa*) gene partial sequence is presented in Fig. 4. The identity percent was 96% for *P. aeruginosa* strain BHWSL6 which isolated from soil and water environments in Algeria.

The tree was constructed by using the neighbor-joining method with online Clustal Omega. Result showed 96% similarity to *P. aeruginosa* strain BHWSL6 that was carbapenemase resistance bacteria isolated from soil and water environments in Algeria, where our susceptibility results showed the same resistance pattern to that isolate presented in Table 2.

Table 1: Morphologial and biochemical identification of *P. aeruginosa*

Parameters	Characteristics of *P. aeruginosa*
Morphological identification	
Gram staining	Gram-negative, rods
Colony	Small, pigmented, circular
Biochemical identification	
Catalase test	+
Oxidase test	+
Methyl red test	-
Indole test	+
Citrate test	+
Nitrate test	+
Pyocyanin production	+
H_2S production	-
Urease test	-
Citrate utilization	+
Vogues-Proskauer test	-
Methyl red test	-
Ornithine decarboxylase test	-
Lysine decarboxylase test	-
Arginine utilization test	-
Gelatin liquefaction test	+

+: Positive, -: Negative

Antimicrobial susceptibility and resistance profile: The *P. aeruginosa* strain was tested for susceptibility to 6 antibiotics (Piperacillin/tazobactam, meropenem, gentamicin, amikacin, levofloxacin and ciprofloxacin which are belonging to β-lactamase inhibitor combination, β-lactam, aminoglycosides and fluoroquinolones and 4 different medicinal oils thyme, fennel, caraway and peppermint. The antibiotic resistance profiles of the tested isolate against the antibiotics are presented in Table 2. Results showed resistance to piperacillin/tazobactam and meropenem, intermediate to gentamicin, while sensitive to amikacin, levofloxacin and ciprofloxacin.

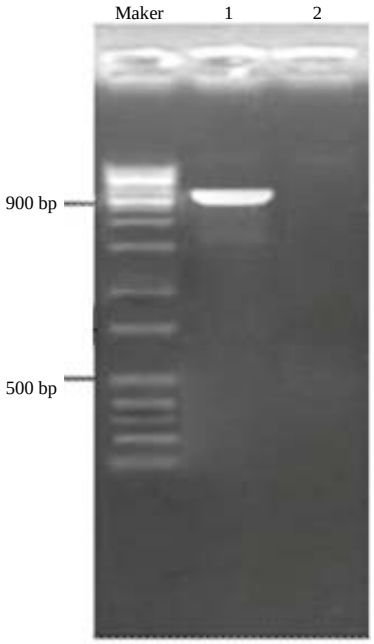


Fig. 2: PCR product electrophoresis on agarose gel 1% (30 min/70 V), Lane marker: 100 bp DNA ladder, Lane 1: PCR product of *P. aeruginosa* (956 bp) and Lane 2: Negative control

Description	Maximum score	Total score	Query cover (%)	E-value	Identification (%)
Pseudomonas aeruginosa strain Giza 2016 16S ribisomal RNA gene, partial sequence	1960	1960	100	0.0	100
Pseudomonas aeruginosa strain BHWSL6 16S ribisomal RNA gene, partial sequence	1944	1944	96	0.0	96
Pseudomonas sp., Mexd310 16S ribisomal RNA gene, partial sequence	1572	1572	96	0.0	95
Pseudomonas aeruginosa strain KVD-HS45 16S ribisomal RNA gene, partial sequence	1550	1550	96	0.0	94

Fig. 3: 16S rRNA sequence similarity for *P. aeruginosa* strain

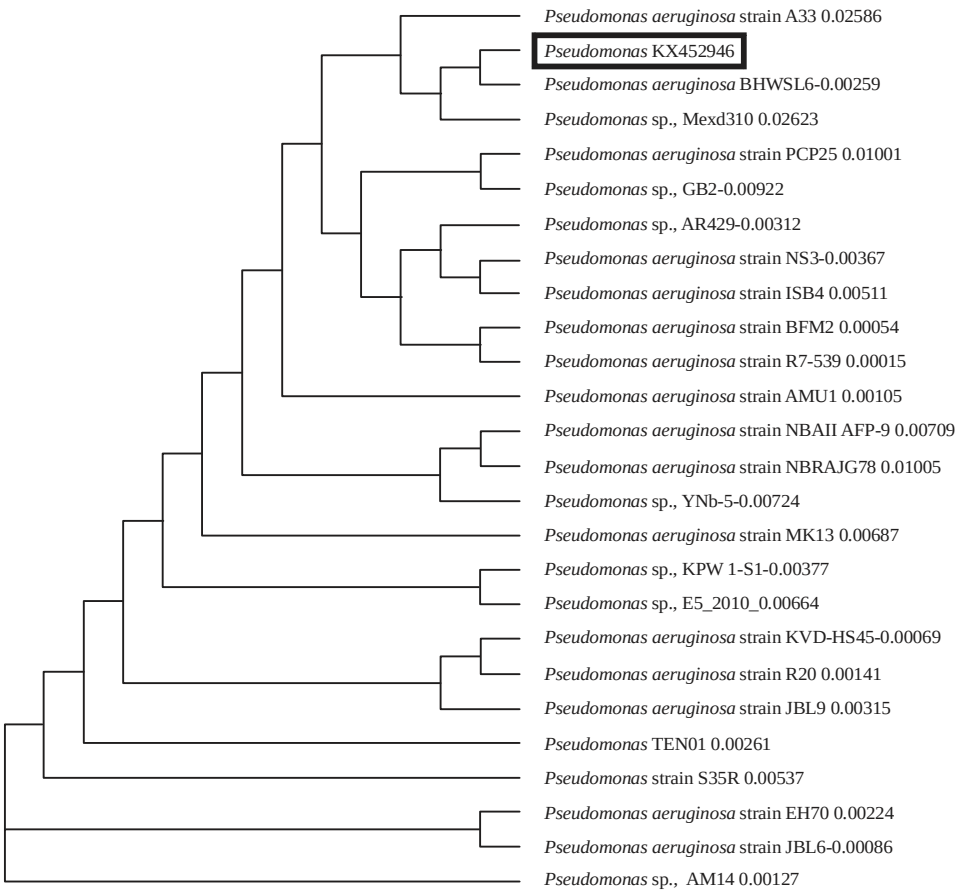


Fig. 4: Dendrogram and phylogenetic relationships for *P. aeruginosa*

Table 2: Antibiotic susceptibility using disk diffusion method against *P. aeruginosa*

Antibiotic classes	Antibiotic disks (Concentration/disk)	Inhibition zone (mm)
β-lactam (β-lactamase inhibitor combination)	Piperacillin/tazobactam (110 µg)	12 (R)
Carbapenem (β-lactam)	Meropenem (10 µg)	0 (R)
Aminoglycosides	Gentamicin (10 µg)	14 (I)
	Amikacin (30 µg)	20 (S)
Fluoroquinolones	Levofloxacin (5 µg)	18 (S)
	Ciprofloxacin (5 µg)	28 (S)

R: Resistant, S: Sensitive, I: Intermediate

Results indicated that this *P. aeruginosa* strain is β-lactam antibiotics resistant. In this respect, Ang *et al.*[17] isolate *P. aeruginosa* resistant to meropenem from e a pediatric cystic fibrosis patient who developed a pulmonary exacerbation in USA. Also, Radan *et al.*[18] stated that piperacillin/tazobactam broad spectrum antibiotic has broad antimicrobial activity against many but not all organisms containing plasmid mediated lactamases.

Antimicrobial activities of the four oils against the *P. aeruginosa* strain are presented in Table 3 and Fig. 5 indicated that *P. aeruginosa* strain was sensitive to thyme oil but resistant to fennel, caraway and peppermint oils. Thyme has phenolic structures, such as carvacrol and thymol which have the greatest bactericidal activities, followed by aldehydes, ketones, alcohols, ethers and hydrocarbons[19]. On the other hand, the cell wall of Gram-negative bacteria is more resistant to the toxic effects of essential oils than Gram-positive bacteria[20]. Also, Mahmoudi *et al.*[21] reported that *P. aeruginosa* was resistant to fennel. Moreover, Becerril *et al.*[22] reported that there is tendency of the bacterial strains to develop resistance to the essential oils. In fact, bacterial resistance mechanism to the essential oils including efflux pumps mechanism[23].

Tarek *et al.*[24] revealed that the Gram-negative bacteria have high resistant to many essential oils. Finally, Da Costa *et al.*[25] stated that *P. aeruginosa* was more resistant to the essential oil.

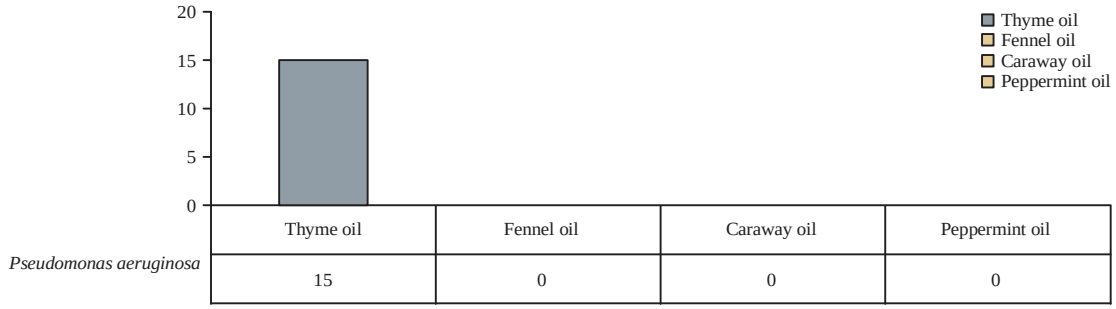
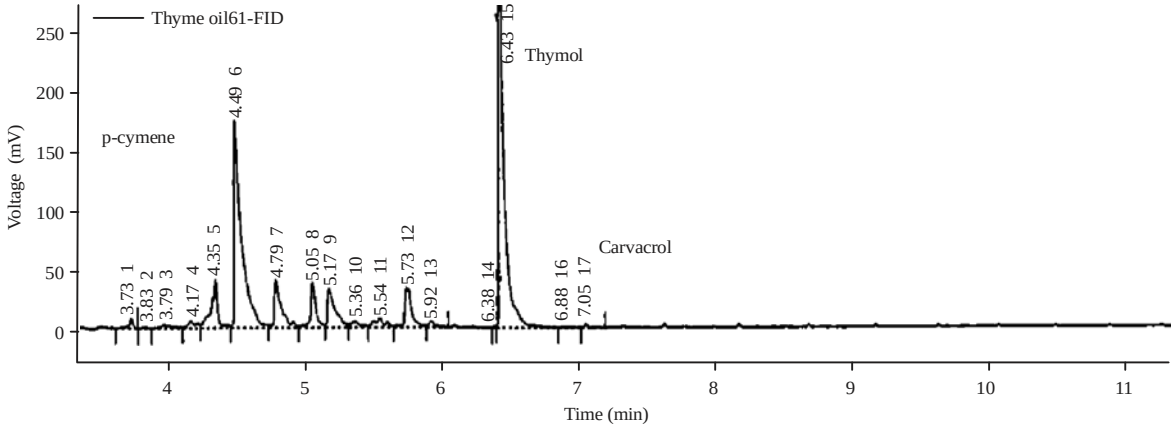


Fig. 5: Antibacterial effectiveness diagram of some medicinal plants oils against *Pseudomonas aeruginosa*



Result table (Uncal-thyme oil61-FID)

	Reten time (min)	Start time (min)	End time (min)	Start value (mV)	End value (mV)	Area (mV sec)	Height (mV)	Area (%)	Height (%)
1	3.733	3.617	3.780	2.858	2.588	17.862	8.044	0.7	1.0
2	2.930	3.783	3.880	2.589	2.614	2.389	0.668	0.1	0.1
3	3.967	3.880	4.107	2.614	2.672	18.855	3.200	0.8	0.4
4	4.167	4.107	4.237	2.672	2.705	25.640	6.147	1.0	0.7
5	4.347	4.237	4.457	2.705	2.761	134.485	40.096	5.5	4.8
6	4.493	4.457	4.733	2.761	2.832	655.830	173.726	26.8	21.0
7	4.787	4.733	4.950	2.832	2.888	145.259	39.960	5.9	4.8
8	5.050	4.950	5.143	2.888	2.937	103.728	37.879	4.2	4.6
9	5.170	5.143	5.313	2.937	2.981	129.249	32.251	5.3	3.9
10	5.360	5.313	5.457	2.981	3.017	24.113	4.942	1.0	0.6
11	5.540	5.457	5.643	3.017	3.065	39.603	7.928	1.6	1.0
12	5.733	5.643	5.883	3.065	3.127	117.971	33.300	4.8	4.0
13	5.971	5.883	6.043	3.127	3.167	14.792	4.951	0.6	0.6
14	6.380	6.363	6.393	2.312	2.316	1.844	2.110	0.1	0.3
15	6.433	6.393	6.847	2.316	2.372	1007.076	430.441	41.2	51.9
16	6.880	6.847	7.013	2.372	3.393	3.331	0.540	0.1	0.1
17	7.047	7.013	7.193	2.393	2.415	5.180	3.045	0.2	0.4
	Total					2447.206	829.227	100.0	100.0

Fig. 6: Assay of p-cymene, thymol and carvacrol in the thyme oil using GC analysis

Table 3: Antibacterial effectiveness of some medicinal plants oils using well diffusion method against *P. aeruginosa*

Oil	Inhibition zone (mm)
Thyme	15 (S)
Fennel	0 (R)
Caraway	0 (R)
Peppermint	0 (R)

R: Resistant, S: Sensitive

Determination of active component percentage in the thyme oil: The data obtained from thyme oil GC analysis were presented in Fig. 6 and indicated that it comply with 13 specifications. Where the p-cymene was 26.8% (must be 15-28%) and thymol was 41.2% (must be 36-55%). In this respect Bouhdid *et al.*[26] stated that p-cymene which

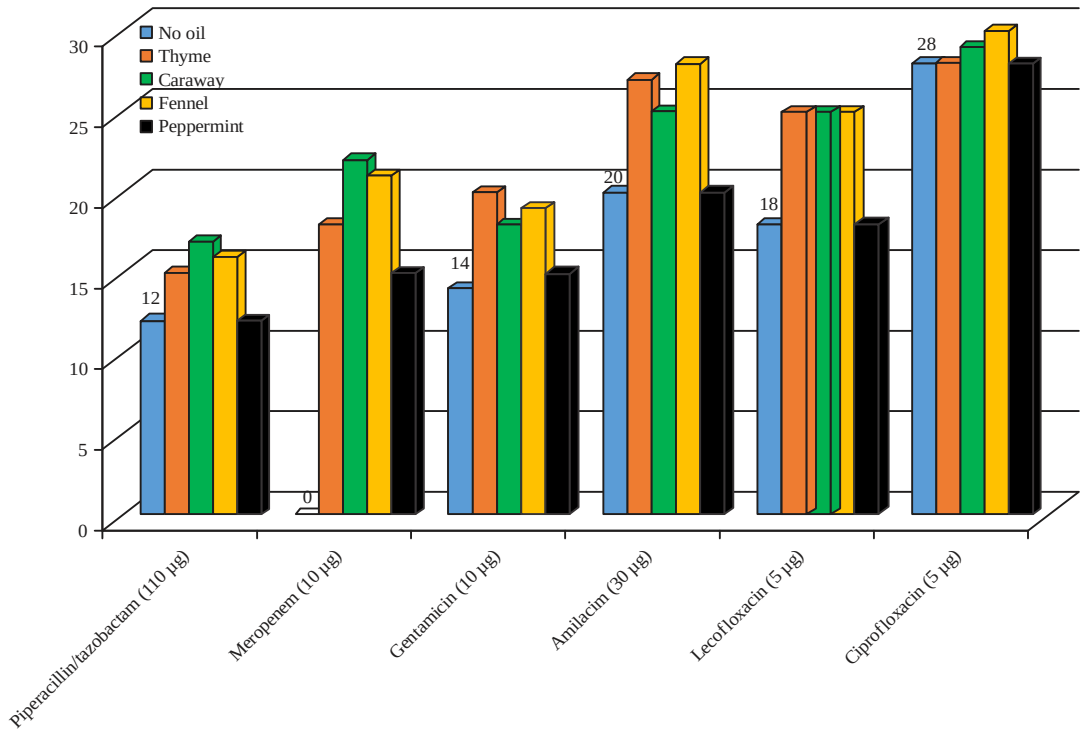


Fig. 7: Synergistic action of medicinal plant oils against *P. aeruginosa*

considered as relatively weak antibacterial was responsible for the expansion of the bacterial cell membranes. This agrees with Lambert *et al.*[27], who stated that thymol able to disintegrate the outer membrane of *P. aeruginosa*. Moreover, the hydrophobicity of the aromatic ring is an important characteristic of this phenolic component, which leads them to expand and divided the lipids of bacterial cell membrane causing leakage of critical component, molecules and ions that causing cell death. Also, thymol and carvacrol in combination show synergistic effects against *P. aeruginosa*. This agree with Du *et al.*[28], who stated that strong antibacterial effects of thymol and carvacrol were observed against pathogenic bacteria but weak activity towards beneficial *Lactobacillus* strains *in vitro*. These results make advice to treatment of *P. aeruginosa* infection with thyme systemically.

Detection of synergistic interaction between some oils and antibiotics: The antibiotic susceptibility profiles of the identified isolate against the 6 antibiotics in agar medium contain 1 mL thyme oil/100 mL media (v/v) is presented in Table 4 and Fig. 7 showing significant increasing of antibiotic sensitivity for all tested antibiotics except piperacillin/tazobactam and ciprofloxacin there is no significant change. Also, the antibiotic susceptibility profiles of the identified isolate against 6 antibiotics in the agar medium contain 1 mL caraway oil/100 mL media (v/v) showing significant increasing sensitivity of all antibiotics in agar medium contained caraway oil.

The antibiotic susceptibility pro les of the identified isolate against 6 antibiotics in the agar medium contain 1 mL fennel oil/100 mL media (v/v) showing showing significant increasing sensitivity of all antibiotics in agar medium contained fennel oil. Also, the antibiotic susceptibility profiles of the identified isolate against the 6 antibiotics in the agar medium contain 1 mL peppermint oil/100 mL media (v/v) showing increase sensitivity of all antibiotics in agar medium contain peppermint oil except piperacillin/tazobactam, amikacin, levofloxacin and ciprofloxacin had not significantly changed in agar medium contained peppermint oil.

Previous results agree with Lorenzi *et al.*[29], who reported that when an essential oil is combined with antibacterial agents, there is a synergistic effect against multi-drug resistant bacteria. Also, synergistic interactions of antibiotics with essential oils due to efflux pump inhibitory activity[30]. Moreover, essential oils enhance the antimicrobial effect of antibiotics against resistant Gram-negative bacteria as most volatile compounds disrupt the microbial cell membrane, thus facilitating antibiotic penetration to bacterial targets, enzymatic alteration, reduction of ATP and DNA synthesis and inhibiting efflux pumps[31].

From all above mentioned results, it could be summarized that the sensitivity of tested identified isolate to various

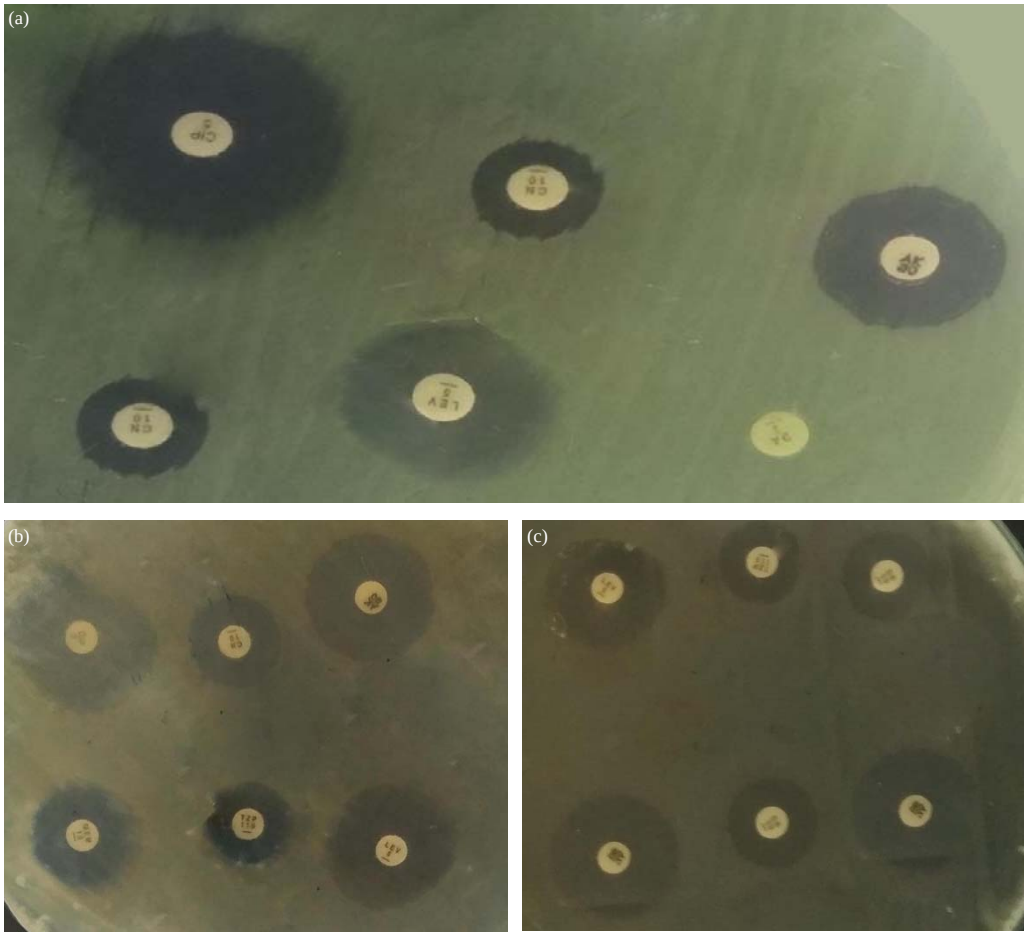


Fig. 8(a-c): Antibiotics susceptibility to some antibiotics using disk diffusion method against *P. aeruginosa* (a) Without oil, (b) With fennel oil and (c) With caraway oil

Table 4: Antibiotic susceptibility using disk diffusion method in combination with 1 mL medicinal plant oil/100 mL media (v/v) against *P. aeruginosa*

		Thyme		Caraway		Fennel		Peppermint	
Antibiotic classes	Antibiotic disks (Concentration/disk)	Inhibition zone (mm)	Action	Inhibition zone (mm)	Action	Inhibition zone (mm)	Action	Inhibition zone (mm)	Action
β-lactam (β-lactamase inhibitor combination)	Piperacillin/tazobactam (110 µg)	15 (I)	N	17 (I)	A	16 (I)	A	12 (R)	N
Carbapenem (β-lactam)	Meropenem (10 µg)	18 (I)	A	22 (S)	A	21 (S)	A	15 (R)	A
Aminoglycosides	Gentamicin (10 µg)	20 (S)	A	18 (S)	A	19 (S)	A	15 (S)	A
	Amikacin (30 µg)	27 (S)	A	25 (S)	A	28 (S)	A	20 (S)	N
Fluoroquinolones	Levofloxacin (5 µg)	25 (S)	A	25 (S)	A	25 (S)	A	18 (S)	N
	Ciprofloxacin (5 µg)	28 (S)	N	29 (S)	A	30 (S)	A	28 (S)	N

R: Resistant, S: Sensitive, I: Intermediate, N: No significant change, A: Sensitivity of antibiotic increased

tested antibiotics might be changed in agar medium contained medicinal plant oil depending on type of antibiotic and oil.

The results obtained from Fig. 8 revealed that significantly improvement the sensitivity of tested *P. aeruginosa* strain to piperacillin/tazobactam, meropenem, gentamicin, amikacin, levofloxacin and ciprofloxacin when agar medium contained fennel or caraway oils 1.0% (v/v) even if *P. aeruginosa* strain was resistant to these oils.

CONCLUSION

In conclusion, 16S rRNA-based PCR assays provide rapid, simple and reliable identification of *P. aeruginosa* and it's differentiation from other phylogenetically closely related *P. aeruginosa* species and resistant pattern. Comparing with API, the PCR assay was more accurate and faster. Treatment β-lactam resistant *P. aeruginosa* by aminoglycosides or fluoroquinolones antibiotics has significant value. Thyme oil

considered one of the most important oils in the antimicrobial aspects. Finally treatment of resistant *P. aeruginosa* by combination of antibiotic and medicinal plant oils even if resistant to these oils has significant value but the mechanisms of synergistic interaction between medicinal plant oils and conventional antibiotics are very complex and not completely understood as they involve multiple interactions.

ACKNOWLEDGMENTS

The corresponding author would like to thank all scientists who have assisted in this manuscript. We must thank especially Assistant Professor Wael Abu El-Wafa, Assistant Professor Hayam M. Hamouda, Dr. Neveen Mohamed Saleh, Dr. Walaa Abd El-All and Dr. Ekbal Abd EL hafez from Microbiology Department, National Organization of Drug Control and Research (NODCAR), Dokki, Giza, Egypt and member of Microbial Biotechnology Research Institute, Sadat City University for helpful support and advice.

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