Investigate *Pseudomonas aeruginosa* resistant to antibiotics and studying the antibacterial effect of Rosemary oils at different concentrations

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Abstract: In this work, 110 clinical samples were collected, including samples of wounds, burns, otitis and urinary tract infection, from some hospitals in Baghdad Governorate, from November 2019 to January 2020. All samples were cultured on differential culture media, as 53 *P. aeruginosa* bacterial isolates were identified using microscopic examination and biochemical test. (33) Isolates belonging to *Pseudomonas aeruginosa* were confirmed by VITEK_2 system. The polymerase chain reaction technique was used to detect the (16s rRNA) and (toxin A) genes. The results of the electrophoresis of the results of the polymerase chain reaction to diagnose the specific gene (16S rRNA) showed the expected gene bundles with the size of (956) base pair for 33 isolates. Addition, The electrophoresis results for the gene detection (toxin A) showed bundles with the size of (352) base pair of 81% of these isolates. The sensitivity of these bacterial isolates to some antibiotics was studied. Most of them were resistant to Piperacillin, Amikacin, Gentamicin and Meropenem by 71%, 63% and 60%, respectively. Whereas were resistant to Imipenem by 27% and Ceftazidime by 33%.The chemical analysis of the oily extract for the rosemary plant (Rosmarinus officinalis) was carried out using gas chromatography-mass spectrometry (GC-MS), and the essential compounds were β_ trance ocimene 33.27%, Camphor 12.77%, Camphene 12.37%, Benzene methanol 7.22% and linalool ester 2.46%. The antibacterial activity of volatile oil extract for *Rosemarinus Officinalis* plant was evaluated and reached to (6.25 $mg.ml^{-1}$), therefore, we concluded from this study that rosemary plant oils have anti-bacterial activity against *P.aerugginosa.*

Keywords: *Pseudomonas aeruginosa, PCR, 16S rRNA, Rosmarinus* officinalis, antibiotic

التحري عن بكتريا *Pseudomonas aeruginosa* المقاومة للمضادات الحياتية ودراسة تأثير الضد البكتيري لبعض تراكيز زيوت نبات اكليل الجبل

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الخلاصة:

في هذا العمل، جمعت 110 عينة سريرية ضمت عينات الجروح والحروق والتهاب الاذن والتهاب المسالك البولية من بعض مستشفيات محافظة بغداد، من تشرين الثاني من عام 2019 إلى كانون الثاني من عام 2020. زرعت جميع العينات على اوساط زرعية تفريقية، أذ تم تحديد 53 عزلة بكتيرية *P. aeruginosa* باستخدام الفحص المجهري و الاختبار الكيموحيوي. تم تأكيد (33) عزلة للبكتيريا*P. aeruginosa* من خلال نظام VITEK_2. استخدمت تقنية تفاعل البلمرة المتسلسل للكشف عن جينات 16s rRNA و *toxin A*، أذ أظهرت نتائج الترحيل الكهربائي لنواتج تفاعل البلمرة المتسلسل للكشف عن الجين المحدد (*16S rRNA*) حزماً للجين المتوقع بحجم (956) زوج قاعدي لـ 33 عزلة. بالأضافة الى ذلك، اوضحت نتائج الترحيل الكهربائي للكشف عن الجين(*toxin A*) ظهور حزم بحجم (352) زوج قاعدي لـ 81% من هذه العزلات. درست حساسية هذه العزلات لبعض المضادات الحيوية فكانت اغلبها مقاومة للمضادات Piperacillin وAmikacin وCiproflaxicine وللمضادين Gentamycine و Meropenem بنسبة 71% و 63%و51% و60% على التوالي، في حين كانت مقاومتها للمضاد Imipenem بنسبة 27 % وللمضاد Ceftazidime بنسبة 33%. تم إجراء التحليل الكيميائي للمستخلص الزيتي لنبات اكليل الجبل (*Rosmarinus officinalis*) باستخدام جهاز قياس الطيف الكتلي للغاز (GC-MS) وكانت المركبات الأساسية هي β_ trance ocimene 33.2٪، Camphor 12.7 ٪، Camphene 12.3 ٪،Benzene methanol 7.2٪ , لينالول استر 2.46٪.. قيمت الفعالية التثبيطية لمستخلص الزيوت الطيارة لنبات اكليل الجبل *Rosmarinus officinalis* وبلغت (5.12 mg.ml-1) ويستنتج من هذه الدراسة ان لزيوت نبات اكليل الجبل فعالية مضادة ضد بكتريا *P.aerugginosa* .

الكلمات المفتاحية: الزائفة الزنجارية، تفاعل البلمرة المتسلسل،*16S rRNA* ، اكليل الجبل، المضاد الحيوي

Introduction

Pseudomnas aeruginosa is an important opportunistic pathogen primarily causing nosocomial infections in immunocompromised patients and is responsible for high mortality rates in burn centers (1,2). It causes infections in canser, burn, urinary tract, eye , blood,surgical wound , cystice fibrosis(3)Because of this it is an extremely abundant organism and found mainly in soil, water , plants, humans , animals and hospitals (4), *P. aeruginosa's* pathogenicity is largely due to multiple bacterial virulence factors and genetic versatility which helps it to survive in a varied environment. A number of these factors help colonization while others allow invasion by bacteria(5). In nosocomial health care, antibiotic resistance in bacteria has reached a near-crisis point (6).

Rosemary (*Rosmarinus officinelis L.)* has been widely used.in foodstuffs in traditional medicine, cos metics and flavoring.Rosemary essential oil has antibacterial properties on both Gram negative and positive bacteria, antifungal, antibiotic, antimutagenic (8). The major compounds of essential oil (EO) determine their biological effect that can cause the damage of wall and membranes, inhibition of protein synthesis, interference with metabolism or interference with DNA/RNA synthesis and function(9). Therefore, the aims of this study were to determine the antibacterial effect of Rosemary essential oil.

Materials and Methods

Bacterial Isolate: A total of 110 different clinical isolate of *P.aeruginosa* were collected from several hospital in Baghdad between November 2019 and January 2020 , we got 53 isolates of *P. aeruginosa* was performed by incubating these clinical isolates on different agar media (Nutrient agar ,Blood agar ,Maconcky agar, and Cetrimide agar which are a selective media for *Pseudomonas*) and the incubation at 37˚C for 24 hrs. and identified using VITEK 2 system and PCR.

Antibiotic susceptibility

were tested by using Kirby-Bauer disk diffusion method following CLSI(10) guidelines , using commercially available 6mm discs (Bioanalyse Turkey) The susceptibility of the isolate was determined against 7 antibacterial agents , They include: Ceftazidim , Amikacin, Impinem, Ciprofloxacin, Piperacillin, ,Gentamycine,and Meropenem, on muller hinton agar plate, using overnight culture at a 0.5 McFarland standard followed by incubation at 37 for 18 to 24 h.

DNA Extraction

Genomic DNA was extracted from bacterial growth according to the protocol of ABIOpure Extraction, DNA template of all isolates was prepared by boiling method (30 min in 100°C). The DNA of isolates was targeted for the 16s rRNA and *toxin A* using the primers listed in Table (1).

Preparation of primer and PCR analysis

These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl , The PCR products were analyzed using gel electrophoresis (1% agarose) and stained with safe dye and visualized by Gel Doc apparatus (Biobure).

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Table 1: show primer pairs sequence and amplicon size

Primer	Sequence		Size(bp)
Tox A gen	Forward	GGTAACCAGCTCAGCCACAT	352bp
	Reverse	TGATGTCCAGGTCATGCTTC	
16S rRNA gen	Forward	GGGGGATCTTCGGACCTCA	956bp
	Reverse	TCCTTAGAGTGCCCACCCG	

Table (2) Condition of PCR Reaction for s16 r RNA and *toxin A* genes of *P.aeruginosa*

Gene	Initial.Denaturation	Denaturation	Annealing.	Extension.
S 16 gen	95 C. 5 min.	95 C 30 sec	55 or 58 30 sec	72 C 1 min.
tox A gen	95 C. 5 min	95 C 30 sec	55 or 58 30 sec	72 C 1 min.

Plant extracts:

The leaves of plant were collected from the local markets during November (2019), which had been identified previously the leaves were left at room tempreture by whom to dry, and after dryness, they .were powdered with a coffee grinder

Preparation of essential oils

The volatile oil was removed using a Clevenger and distillation, 100gm moist sample was put in a one liter flask attached to the unit, and 1000 ml of distilled water was applied to the sample ,then distilled at 100 ° C (27) for three hour ,the samples were placed in lined tubes and stored in a dark freezer . the samples were then processed at a temperature of 4 °C in the laboratory for qualitative analyzes(11)

The antimicrobial activity

Antimicrobial assay was designed to determine the activity of essential oil of rosemary as a potential against the tested microorganisms. MIC determination was conducted by well diffusion and agar dilution methods, using Mueller-Hinton (MH) agar for growth of bacteria ,Dimethyl sulfoxide (DMSO) was used to facilitate mixing of the oils with the broth , Different concentrations of essential oils (2.56, 5.12,10.25, 20.5) µg / ml, were added to sterile petri-dishes. Then melted MH agar for growth of bacterial isolates were, poured into the plates and to mix the components well. It left to solidify, then 100 µl of suspension containing the tested microorganisms were has been published using sterile glass rod and then incubated at 37 ˚C for 24 hrs for growth of bacterial isolates. Colonies were then counted and the count multiplied by reciprocal of sample dilutions which represented the count of the microorganism (12) . The MIC was taken as the lowest concentration of oil at which the tested organism did not show visible growth . On other hand, wells were made in the solidified medium using cork borer. Aliquots of 100 µl of inoculums were applied to the wells, then incubated as mentioned above. Diameters of zones of inhibition were measured in milli meter All the tests were performed in triplicate.

Statistical analysis :

Was performed , the data presented are an average of three replicates. Least significant deffirence (LSD) were calculated. Statical Analysis system _SAS was used to analysis.

Analysis of essential oil components by Gas Chromatography-MSpectrometry:

Gas chromatography Mass (SHIMADZi) GC MS-2010 ULTRA at Ministry of science and technology of Baghdad . the capillary column (30x0.25mm) internal Diameter,film thickness (0.25µm) at a flow rate 1.53 ml.min, helium carrier gas was used.Injection mode was split and injection temperature was split and injection mode was split and injection temperature was 240 °C , the oven temperature was programmed at 70 °C for 3 min, then raised to 150 °C with hold time 2 min and raised to 240° C, the ionization mode was electronic impact mode (SEI) at 70e.

RESULT AND DISCUSSION

Bacterial Isolates:

In this study 53 of *aeruginosa* were collected frome hospitalized patients in Baghdad . depending on phenotypical and biochemical tests, , 53 isolates grow and shows , the colonies were pale colorless and non-fermenting lactose sugar (14) , most of these colonies produce a greenish yellow pigment (Pyoverdin) in addition to the pyocyanin pigment, which is greenish blue (15),Microscopically examination showed that the bacterial cells negative for gram stain reaction , non motile.

The identification was performed using the automated VITEK -2 system using the GN-ID cards containing 64 biochemical tests, from (53) P. aeruginosa isolate,(33) P. aeruginosa positive result shown positive result of the P. aeruginosa demonstrated.

Origin of samples	**Numbers of sample with Percentage**
burn swab	33(30%)
wound swab	11(10%)
Ear swap	3(2.73%)
Urin	6(5.46%)
Total	53(48.2%)

Table (3) Distribution of *Pseudomonas aeruginosa* isolates in clinical samples

Antimicrobial susceptibility of *P.aeruginosa* isolates

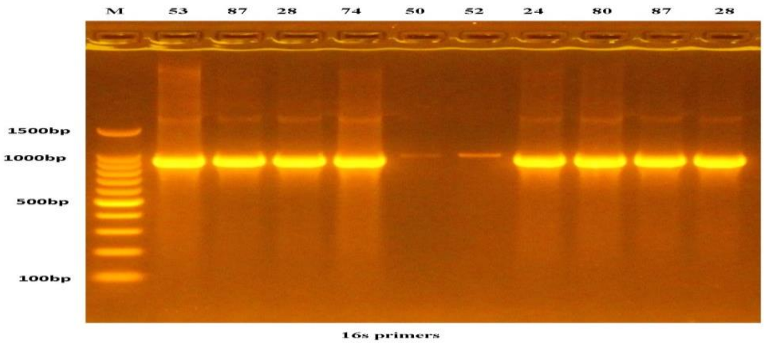
The isolates were tested for the sensitivity of *Pseudomonas aeruginosa* to seven antibiotics. The isolates showed a clear variation in their resistance, It showed 63% anti-Amikacin, 27% anti-Impenim, 33% anti-Ceftazidime, Gentamycine and Meropenem at 60%, and Piperacillin at 72%, and anti-Ciprofloxacin at 51%. We note that bacterial isolates recorded high resistance against the Pencillin-β-lactam group, including Piperacillin, One of the main reasons for the resistance of P. aeruginosa to beta-lactam Oantibacterial agents is due to the production of lactamase_βPencillinas and Cephalosporinase enzymes that attack beta-lactam rings that are found in the penicillin nucleus, making them ineffective antagonists in addition to having efflux pump pumps that change the permeability of the membrane. Due to the loss of the outer membrane openings (16). the isolates revealed resistance to gentamicin,ciprofloxacin,the result are in agreement with those obtained by AL-Margani and Khadam(2016) (17). In fact, they isolated P. aeruginosa multi- drug resistant from different infection sites. This high multi resistance could be due to the production of Hydrolytic enzymes and the acquisition of resistancemechanisms by P.aeruginosa strains (18) showed that 94.7 % of isolates were imipenem .

Molecular detection

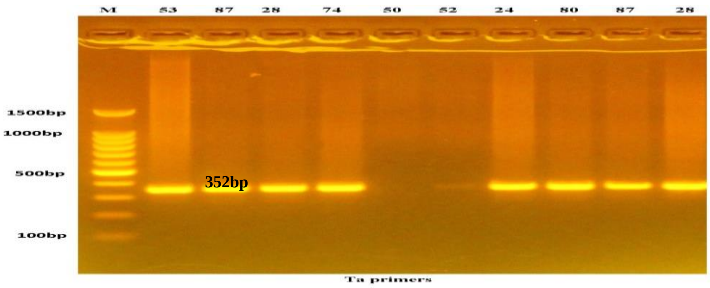
Genetic detection of the diagnotic 16s rRNA gen in *P.aeruginosa*, This gene contains a fixed sequence for each type of bacteria to stabilize the sequence of this gene. This gene plays an important role in the accurate diagnosis of *P. aeruginosa*.. Avery important role in classification of primitive biology and is considered as alternative compared to the phenotypic methods that were used previously and widely on Laboratory range (19). The results of the molecular diagnosis of P. aeruginosa showed that all isolates possess this gene within their genetic structure by 100%, as shown in the figure , This finding was consistent with what was stated by (20)

The results of screening for the toxin encoding the exotoxin A(ETA) y PCR technique showed that 81% of the clinical isolates possess the tox A gene within their genetic structure, as in the figure , This finding was consistent with what (21) recorded, as they found that 81.5 of the clinical isolates.

isolated from wounds infections had a toxin gene.



Figur1:Electrophoresis of PCR of the 16 s gene RNA(956) base pair of *P. aeruginosa* isolates with a difference of 100 V for 75 minutes



figur 2:Electrophoresis of PCR of the *tox A* (352) gene base pair of *P. aeruginosa* isolates with a difference of 100 V for a period of 75 minuts.

Compounds in rosemary essential oil analyzed by GC MS.

Chemical analysis of rosemary EO extract revealed the presence of 29 active compounds at different quantities as shown in table 4 and figure 3.

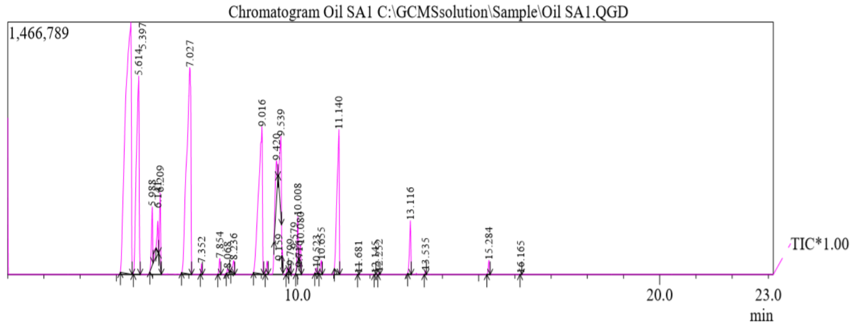
Table (4) Retention time and pa (%) of the active compounds found in rosemary callus extract analyzed by GC-MS

Compound	**RT**	**Peak area%**
B-trance-ocimen	5.379	33.27
camphen	5.614	12.31

منسّق:الخط: (افتراضي) +برنامج نصي معقد لعناوين (Times New Roman)، 14 نقطة، خط اللغة العربية وغيرها: +برنامج نصي معقد لعناوين (Times New Roman)، 14 نقطة

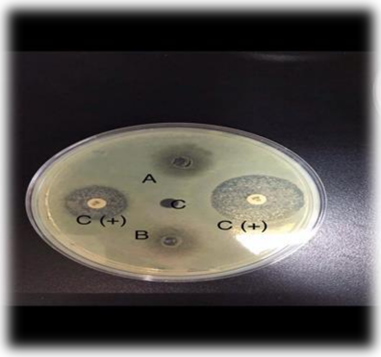
B-penen	5.988	1.60
Propanoic acid	6.141	0.71
B-myrcene	6.209	1.72
Terpineol	7.027	19.55
Gamma terpinene	7.352	0.21
Octane,2,6,dimethyl	7.854	0.41
Cyclo pentane	8.068	0.11
Linalool ester	8.236	0.45
Camphor	9.016	12.77
Tri fluoro acetat(ester)	9.159	0.35
Isobomyl alcohol	9.420	2.21
Acetic acid	9.539	2.46
Lilac aldehyde	9.579	0.13
Alpha terpineol	9.717	0.11
7-octadin -2-01	9.800	0.15
Cyclo penta-1-ethanol	10.008	1.15
Verbenon	10.083	0.15
Heptan-2-methanol-6,6	10.525	0.50
4-Isopropenyl methanol	10.658	0.16
Bomyl acetat	11.142	0.42
Benzene methanol	11.683	7.22
Propane,2-bromo	12.142	0.01
5,9-undicadien	12.250	0.01
Trans-alpha- bergamotene	13.117	0.04
Ocimene	13.533	1.56
Patchoulane	15.283	0.06
Furanon	16.167	0.34

Data showed that the most abundant compounds were B-trans ocimen at 33.27%, Terpineol at 19.55%, Camphor Camphor 12.77%, Caffeine Caphen at 12.37%, Benzen methanol 7.22%, Linalool ester 2.46% as main components and other secondary compenent. There is a great diversity in the chemical composition of the essential oils, and these differences in the chemical composition may be attributed to the climatic effects on plants in addition to the genetic differences, the time of extracting the plant sample and the extraction technique used to obtain the essential oils.



figur(3):the chemical composition of the essential oils extracted from the leaves of rosemary plant using GC_MS technique

The MIC value of Rosemary EO agints *Pseudomonas aeruginosa* was determined by broth microdilution method it was releaved of EO was (6.25 mg.ml-1), the lowest lethal consentarion is (10.25 mg.ml-1, The inhibitory efficacy of volatile oils of the rosemary plant was tested against the resistance of *Pseudomonas aeruginosa* resistance and the results were compared with DMSO as a negative control and the Amikacin antibiotic as a positive control, The results of the statistical analysis showed that there were significant differences between the different concentrations of volotiles oil for rosemary at a probability level less than 0.05. The oil extract at a concentration of 20.5 µg / ml has better inhibitory efficacy than other concentrations, as it showed the average inhibition zone diameter (11,11.5, 12, 12.5, 13, 13.5, 14, 15,17 mm), The results were consistent with what Al Hussain and his group (2010) found a good effect of rosemary oil inhibition of Pseudomonas sp. It gave an inhibition diameter of (17 mm), while the result was different for the study Petrova and his colleagues (2013)(22) and (Gur, 2017) (23) where the effect of oil was minimal on Pseudomonas sp Moral limit not reached, the strength of antimicrobial essential oils depends on a number of turpines and phenolic compounds that disturb the cytoplasmic membrane and disrupt the driving force of the proton (Disrupting the proton motive force). One of the most important characteristics of essential oils is that they are hydrophobic, allowing them to interact with the fats of the bacterial cell membrane and mitochondria, which occurs An imbalance in the structure and structure of the bacterial cell, and this makes the membrane more permeable and the loss of important molecules and ions from the bacterial cells, which causes their death. , Camphor, Terpineol And Isobornyl aceat (24) and this was confirmed by the results of conducting a chemical analysis of the pilot oil of the rosemary plant using the GC_MS technique.



A: 20.5 µg / ml

B: 10.25 µg / ml

F: 5.12 µg / ml

D: 2.56 µg / ml

C: Negative control

C (+) : Positive control

Figur (4) The effect of serial concentrations of volatile rosemary oils on the growth of *P.aeruginosa.*

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