

ANTIOXIDANT, ANTIBACTERIAL AND ANTIBIOFILM POTENTIALS OF ANISE (*Pimpinella anisum*) SEEDS EXTRACTED ESSENTIAL OILS

SH. A. Al- wendawi
Assist Prof.

L. A. Gharb
Lecturer

R. S. Al ghrery
Biologist

Dept. of Biology, College of Science, University of Baghdad, Baghdad/ Iraq

Shadan59alwendawi@gmail.com

lamyagharb@yahoo.com

ABSTRACT

Anise (*Pimpinella anisum*) is an annual herbaceous flowering plant, its widely used as spices, flavoring agent and fragrance. Anise seeds and its essential oils (EOs) has been used widely in the recipes of folk medicine in the Arab region, including Iraq. Therefore, the goal of this work was to extract and define the chemical compositions of aniseeds EOs, and study its antioxidant, antibacterial, and antibiofilm potentials. Aniseed EOs were extracted by hydro-distillation technique using Clevenger apparatus. Twenty-five distinct compounds have been recognized by Gas chromatographic–Mass spectral (GC-Mas) analysis, the major constituents were anisole (87.99%) followed by estragole (3.01%), and all other components were presented in less than 2%. The aniseed EOs showed potent antioxidant activity for scavenging of 2,2 diphenyl - 1-picrylhydrazyl (DPPH) in concentrations-dependent manner, the highest antioxidant (84.9±0.267%) was recorded at 10000 ppm. Antibacterial activity regards to minimum inhibitory concentration (MIC) against burn wounds isolated *Pseudomonas aeruginosa* were of the rang 4000-7000ppm. Aniseed EOs revealed excellent antibiofilm activity against *P. aeruginosa* biofilms formation with reduction percentage ranged from 26 - 86.41% in co-treatment assay, while the inhibition potentials for pre-developed biofilm decreased significantly (P<0.05) by 100% for the majority of *P. aeruginosa* candidate isolates.

Key words: Medical plants, plants aromatic compounds, burn wounds, *Pseudomonas aeruginosa*, antimicrobial agents and minimum inhibitory concentration (MIC).

الوندائي وآخرون

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الفعالية ضد تأكسدية وال ضد بكتيرية و ضد تكوين الاغشية الحيوية للزيوت العطرية المستخلصة من بذور نبات الينسون (*Pimpinella anisum*)

رفل سلام الغريري

لمياء عبد الزهرة غرب

شادان عباس الوندائي

بايولوجي

مدرس

أستاذ مساعد

قسم علوم الحياة - كلية العلوم - جامعة بغداد / العراق

المستخلص

نبات الينسون (*Pimpinella anisum*) من النباتات العشبية الحولية المزهرة ويستخدم كتوابل وكمواد منهكة وعطرية واستخدمت مستخلصات بذور وزيوت الينسون منذ الازل في وصفات الطب الشعبي في البلاد العربية وبضمنها العراق. لذلك هدفت الدراسة الحالية الى استخلاص الزيوت العطرية من بذور الينسون وتشخيص مكوناته الكيميائية ودراسة بعض صفاته ومنها الفعالية ضد تأكسدية وال ضد بكتيرية و ضد تكوين الاغشية الحيوية البكتيرية. استخلصت الزيوت العطرية من بذور الينسون بالتقطير المائي و باستخدام جهاز الكلافنجر و تم تشخيص خمسة وعشرون مركب بالتحليل الكروماتوغرافيا الغازية (GC-Mas) و كانت نسبة الانيسول (anisole) هي الأعلى (87.99 %) وتلاها مركب الإستراكلول (estragol) وبنسبة 3.01 % اما بقية المكونات فتواجدت بنسب اقل من 2 % . و أظهرت الزيوت العطرية فعالية ضد تأكسدية عالية في اختزال محلول المركب DPPH واعتمادا على التراكيز المستخدمة حيث ان اعلى نسبة اختزال اقترنت بأعلى تركيز مستخدم (10000ppm) و كانت 84.9±0.267 % . انحصرت الفعالية ضد بكتيرية وبدلالة التراكيز المثبطة الدنيا (MIC) ضد عزلات بكتريا *Pseudomonas aeruginosa* المعزولة من جروح الحروق بين 4000-7000 ppm. وأشارت النتائج الى فعالية اختزالية عالية للزيوت العطرية المستخلصة على قابلية تكوين الاغشية الحيوية في بكتريا *P. aeruginosa* حيث انخفضت قابلية تكوين الاغشية الحيوية و بنسب 26 - 86.41% في حين ان الفعالية التثبيطية للأغشية الحيوية المكونة مسبقا انخفضت معنويا (P<0.05) وبنسبة 100 % ولأغلب العزلات المختارة.

الكلمات المفتاحية: النباتات الطبية، المركبات العطرية النباتية، جروح الحروق، بكتريا الزوائف الزنجارية، العوامل المضادة للمكروبات، التراكيز المثبطة الدنيا

INTRODUCTION

Burns are considered to be devastating forms of trauma in patients with serious thermal injury. In most developing countries burning wound remains a major clinical challenge, and burning patients are very susceptible to infection due to the loss of skin barrier. The source of infection in most cases is the patient's normal flora or exogenous contamination, from contaminated wound dressing equipment's or from the hospital setting. Different groups of microorganisms have been reported to be associated with burns wound infections (27). *Pseudomonas aeruginosa* is Gram-negative opportunistic pathogen and its one of the most common bacteria in nosocomial infections (3). The bacterium is one of the leading microorganisms associated with burning wounds infections. *P. aeruginosa* is included in the category of community and hospital acquired pathogens in burn units, and therefore is considered as the most important factor in burning wound infections (18). The dramatical antimicrobial resistance of *P. aeruginosa* has led to serious restriction in the treatment of different pseudomonal infections. The speedy acquisition of multidrug resistance (MDR) in recent years has increased dramatically in *P. aeruginosa* (30). It became the main MDR bacteria, and has resulted in a strong demand for new therapy by consumers. *P. aeruginosa* is a pathogen presenting a large genome that can develop a large number of factors associated with MDR mechanisms, including, reduces the outer membrane permeability, expression of efflux pump, production antibiotic degrading enzymes, alginate production and transfer of resistance genes (3). Biofilm formation is one of the protecting strategies of *P. aeruginosa* makes the bacterium more difficult to treat than their planktonic counterparts, consequently leads to persistent, sometimes deadly, infections. Therefore, biofilm formation is one of the indirect modes of action by which bacteria are resistant to therapeutic agents. The physical barrier created by enclosed exopolymeric substances may account for enhanced antibiotic resistance in biofilms (16), a proportion of dormant bacteria that are inert toward antibiotics, and resistance genes that

are unique to biofilms (25). Together, these bacterial characteristics that generate resistance to antibiotics, drive the need for a new approach that will kill bacterial biofilms efficiently. Globally plants are potential sources of novel antimicrobial compounds can be provided vast array of complex and structurally divers compounds. The aromatic volatile, essential oils (EOs) extracts from plants was approved to possess characteristics antimicrobial properties (28). The EOs are secondary metabolites that are synthesized in deferent parts of plants, they play a crucial ecological role in protecting producing plants against microorganisms, herbivores and other stresses, but also in attracting insect pollinators and seeds. EOs have a potential antiseptic, antibacterial, antiviral, antioxidant, anti-parasitic, antifungal, and insecticide, and can therefore be a useful tool for reducing microbial resistance (23). The biological properties of EOs are generally characterized by their two or three major distinct components, which are present at relatively high concentrations (20–70%) compared to other trace components (8). Plant EOs have been used for hundreds of years as natural medicines to combat a multitude of pathogens, including bacteria, fungi, and viruses. Several essential oils confer antimicrobial activity by damaging the cell wall and membrane, leading to cell lysis, leakage of cell contents, and inhibition of proton motive force (31). Among the plant's producers of aromatic compounds, is Anise (*Pimpinella anisum*), is herbal flowering plant which grow in several region all over the world, belonging to the Apiaceae family, one of the oldest medicinal plants in Iraq, the member of this family is well known for its distinctive flavors, which come from EOs and typical aromatic compounds. In fact, there is evidence that effectively kill microorganisms without promoting the development of resistance, with low toxicity to mammalian cells, and are relatively easy to obtain. They degrade quickly in water and soil, making them relatively environmentally friendly substances (5). The objective of this study was identification of hydro-distilled aniseed EOs and evaluate its antibacterial and antibiofilm potentials against MDR burns wound isolated *P. aeruginosa*, as well as

investigation the antioxidative activity of the extracted EOs.

MATERIALS AND METHODS

Extraction of aniseed essential oil (EOs)

Anise (*Pimpinella anisum*) seeds used for extraction of aromatic EOs. Seeds were purchased from local market, and extraction was performed by using hydro-distillation method by Clevenger apparatus. 100 g of anise seeds was gently crushed and mixed with 600ml of distilled water. Then introduced to Clevenger apparatus which was previously prepared for distillation. The distillation carried out for 3hr, the distillate was transferred to separate funnel, and allowed to settle down for 24hr, until two phases of different density become visible, the upper layer representing EOs layer was withdrawn, and held in tightened vials at 4°C for further laboratory analysis.

Gas chromatographic–mass spectral (GC-MS) analysis of aniseed Eos

The aniseed EOs components were identified and quantified by means of GC-MS (Shimadzu GC-MS-QP2010 Plus). The carrier gas was helium at flow rate of 13ml.min⁻¹, analyzer was used to identify the main chemical compounds in aniseeds oil. The mass spectrometer was set to scan 12.00-500.00 m/z. Samples were injected with a splitting ratio of 5.0 and the injector temperature was set to 280°C. The total flow-rate was 13.1ml.min⁻¹ and the column flow were 1.69 ml.min⁻¹. The column oven temperature was 50°C and the hold time was 2-5 min. The total run time was approximately 30 min.

Antioxidant potential of aniseed EOs

The antioxidant activity of aniseed EOs was investigated in terms of free radical scavenging activity, using the radical solution, 2,2-Diphenyl-1-picrylhydrazyl (DPPH). The efficiency for scavenging DPPH was quantitatively evaluated by EOs ability to reduce and decolorize the purple-colored of DPPH solution to yellow (26). In brief, 100µL of different serial concentrations (1000 up to 10000 ppm) of aniseed EOs were mixed with equal volume of freshly prepared DPPH (60µM, in absolute methanol), the mixtures were vortexed and allowed to stand for 30 min in dark at 25°C. The absorbance of resulting solution was measured at 517nm (OD₅₁₇)

against blank (methanol). L-ascorbic acid (0.1%) was used as reference antioxidant (positive control), and control was set up by mixing 100µL of distilled water with 100µl of DPPH. Assay were performed in triplicate. The percentage of scavenging activity against DPPH was calculated, based on control reading by following formula:

$$\text{Reduction\%} = [A_0 - A_1 / A_0] \times 100$$

Where, A₀ is the absorbance of control, A₁ is the absorbance of sample.

Antibacterial activity of aniseed EOs

Bacteria and growth conditions

Forty identified *P. aeruginosa* isolates were used in the study, the bacterial isolates were obtained from severe burn wounds infection from patients attended to the burn's unit of Al wastey teaching Hospital / Baghdad. The identification of isolates was confirmed using Vitek 2 compact system. *P. aeruginosa* isolates were preserved on brain heart infusion agar (BHIA, Himedia/ India) at 4°C, and refreshed every two weeks using same medium.

In vitro antibacterial activity

Antibacterial activity of aniseed EOs against *P. aeruginosa* planktonic forms was investigated by agar-well diffusion assay, using Mueller-Hinton agar (MHA, Himedia/India). The MHA plates were individually swab inoculated with 100 µL of *P. aeruginosa* standardized inoculum (10⁸ CFU/ml, adjusted to MacFarland's standard No. 0.5). Wells of 5 mm size were made with sterile cork borer, 80 µL of aniseed EOs added to wells, plates were incubated at 37°C for 24 hr. Antibacterial activity was evaluated by measuring the inhibition zone diameter (IZD), and sensitivity to EOs was classified to three categories. IZD ≤ 8 mm not sensitive, IZD > 9–14 mm sensitive, and IZD >15mm highly sensitive (4).

Minimal inhibitory concentration (MIC)

The *P. aeruginosa* isolates that were exhibited high sensitivity to EOs action was included for MIC assay. The assay was carried out in triplicate by microdilution technique in 96-well microtiter plates, 100 µl of aniseed EOs was added to the first well of plate and serially diluted 1:1(1000 up to 10000ppm). 100 µL of standardized bacterial inoculum (10⁸CFU/ml) were individually added to each well. The

control was set up as MH broth and bacterial inoculum, plates incubated at 37°C for 24hr. The MIC was determined visually as the lowest concentration that inhibited bacterial growth (14).

Quantification on of biofilm formation

The biofilm formation capacity of *P. aeruginosa* isolates was quantitatively assessed using microtiter plates. *P. aeruginosa* isolates were individually refreshed in BHI broth supplemented with 1% glucose. Aliquots 200 µl (10⁸ CFU/ml) of each culture were dispensed to the wells, plates incubated at 37°C for 24hr. Contents of wells were gently aspirated, wells washed 3-times with phosphate buffer saline (PBS), the attached bacteria were fixed using 2% sodium acetate, subsequently wells were stained with 0.1% crystal violet (CV) solution for 15 min, the excess stain was removed by washing with PBS three times, and 200 µl of 95% ethanol was added to each well for 15 min. Control was set up with CV binding to the wells exposed only to the culture medium without bacteria. Biofilm formation was measured at 620nm (OD₆₂₀) by ELISA microplate spectrometer (Asays, Belgium), assay was performed in triplicate and the mean biofilm absorbance was applied for calculation of biofilm generation degree as follow:

$$\text{Biofilm} = \text{ODs} - \text{ODc}$$

Where, ODs, donated the absorbance of samples, ODc, donated the absorbance of control.

According to the mean of OD, the isolates were classified into three categories, based on the following criteria; ODs ≤ 2× ODc weak biofilm producer, ODs ≤ 4× ODc moderate biofilm producer, and 4×ODc < OD strong biofilm producer (8).

Antibiofilm potentials of aniseed Eos

Eight *P. aeruginosa* isolates that exhibited strong biofilm formation capacity were included to antibiofilm potentials assay. The assay was conducted in 96-well microtiter plates, and performed by two independent methods;

Inhibition of biofilm formation: it was evaluated by co-treatment assay (15). In each well of microtiter plate, 100 µL of bacterial suspension (10⁸ CFU/ml) was added, and 100 µL of aniseed EOs equivalent to 0.5×MIC,

1×MIC and 2× MIC values were individually added to bacterial loaded wells. The microplates were incubated at 37°C for 24 hr. Well without addition of EOs was considered as a control, and blank well was set up as well with only EOs. Thereafter the biofilms formation ability was carried out by CV assay as previously described, and the assay was performed in triplicate. The biofilm inhibition efficacy of EOs, was expressed as percentage of biofilm reduction, based on control reading by the following formula:

$$\text{Biofilm reduction\%} = [(C - B) - (T - B) / (C - B)] \times 100$$

Where; B, denotes the average absorbance per well for blank (no biofilm, no treatment). C, denotes the average absorbance for control wells (biofilm, no treatment); T, denotes the average absorbance per wells for treated wells (biofilm and treatment).

Inhibition of pre-developed biofilm. Biofilm formation was established as previously mentioned. After 6hr of incubation plates were removed from the incubator and 100 µl of aniseed EOs at concentrations of 0.5×MIC, 1×MIC, and 2× MIC values were individually added to loaded wells, Plates were re-incubated for 24 hr at 37°C. Well without addition of EOs was considered as control, and well with only EOs was set up as a blank well. Following incubation, plates were emptied and washed thrice with sterile PBS. the adherent cells were fixed with 2% sodium acetate, and biofilms detection was carried out by CV assay. Biofilm inhibition percentage was calculated using the same followed formula of co-treatment assay.

Statistical analysis

The Statistical Analysis System- SAS, program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test was used to significant compare between means in this study.

RESULTS AND DISCUSSION

The yield of aniseed (*Pimpinella anisum*) EOs after 3hr of hydro-distillation was 2%. The oil was pale-yellow in color, with a mild aromatic odor. Several techniques had been involved for the extraction of EOs, like, hydro distillation, turbo distillation, super critical fluid extraction, solvent extraction, use of

superheated and supercritical water and other such as microwave assisted processes, out of these techniques the hydro distillation is one of the ancient and official approved techniques for extraction of oil, and the superiority of aqueous extraction was indicated over other extraction techniques (11). Twenty-five distinct compounds have been recognized by GC-MS analysis, the main compounds are listed in table 1. and chromatogram is in fig.1, in order of their elution. The identification of GC peaks was made by compared their retention times (RT) with those of the original samples and by matching the MS data with those held in the Mass Spectra Library. The major constituents were anisole or *trans*-anethol (87.99%) followed by estragole (3.01%), and all other components were present in less than 2%. It is clear that monoterpenes represent the main groups (90%), and it is of interest to note the presence *trans*-anethol (bioactive compound), which is distinctive of anise and usually was detected in very high percentage (up to 93%) in most of analyzed anise EOs. This finding is in accordance with previous articles related with anise derived EOs (22). The recorded percentage of estragole (the flavoring agent) was relatively high, as in most published experimental data is varied from 0.38 to 0.61%. Moreover, high content (1.54%) of the

Phyto marker phenylpropane [Phenol, 2-methoxy-4-(1-propenyl)-, acetate] was detected. The increase in the accumulation of phenylpropane is consequence of environmental stress such as, pathogen attack, UV-irradiation, wounding, nutrient deficiency, and herbicide treatment (29), the ripening process was also demonstrated to affects the biosynthesis and the contents of Phyto markers (30). In general, the components of EOs vary between species, between portions of same plants, and among the geographical areas. The discrepancy in the composition of anise EOs grown in Iraq and elsewhere may be assigned to different factors, such as climate, season, high light impact, age of the plant, humidity of the harvested plant material, extraction technique and the existence of chemotypes (24). The composition and yield of EOs are impacted by numerous factors, in most cases it is difficult to isolate these factors from each other as they are interrelated and influence each other. These parameters include seasonal variations, the organ of the plant used, and the maturity of the plant, as well as its geographical origin and genetics. Generally, the biological properties of the EOs are determined by their major components including two groups of distinct biosynthetic origin (9)

Table 1. Main chemical compounds in anise seeds essential oils extracted by hydro-distillation

Peak No.	Retention time	Composition (%)	Compound name
3	11.379	3.01	estragole
5	13.854	87.99	anisole
13	15.024	1.50	3,5,5-trimethyl-9-methylene 1H- Benzocycloheptene
17	15.647	0.74	3,5,5,9-tetramethyl 1H-Benzocycloheptene
23	19.505	1.54	Phenol, 2-methoxy-4-(1-propenyl)-, acetate

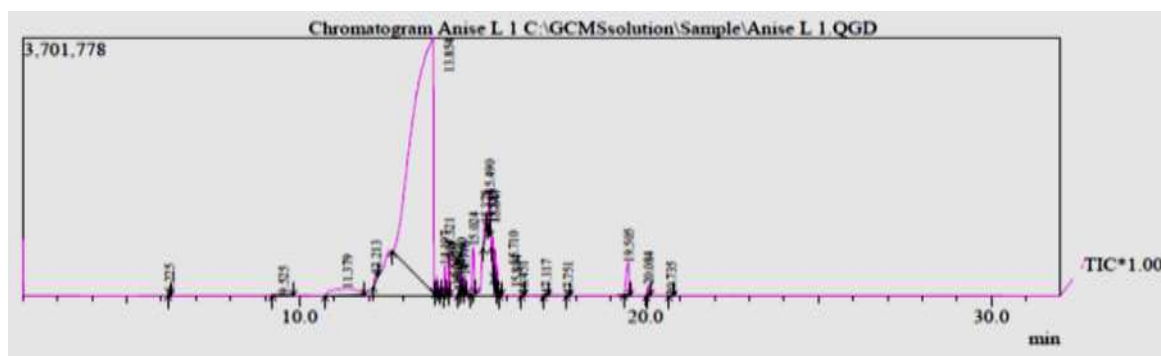


Figure 1. Gas chromatography spectra of aniseed essential oils extracted by hydro-distillation

Anti-oxidant activity

The potential antioxidant activity of aniseed EOs was evaluated in respect of scavenging of DPPH solution. In concentration-dependent

manner, EOs had potent antioxidant activity, the highest percentage scavenging of DPPH was 84.9%, recorded for the highest used concentration (10000ppm), compared with

ascorbic acid as a reference antioxidant (Fig.2).The experimental data in this work revealed that aniseed EOs exhibited reducing ability against the used free radicals,

represented by discoloration of purple-colored DPPH, and this serves as a significant indicator of its potential antioxidant capacity (26)

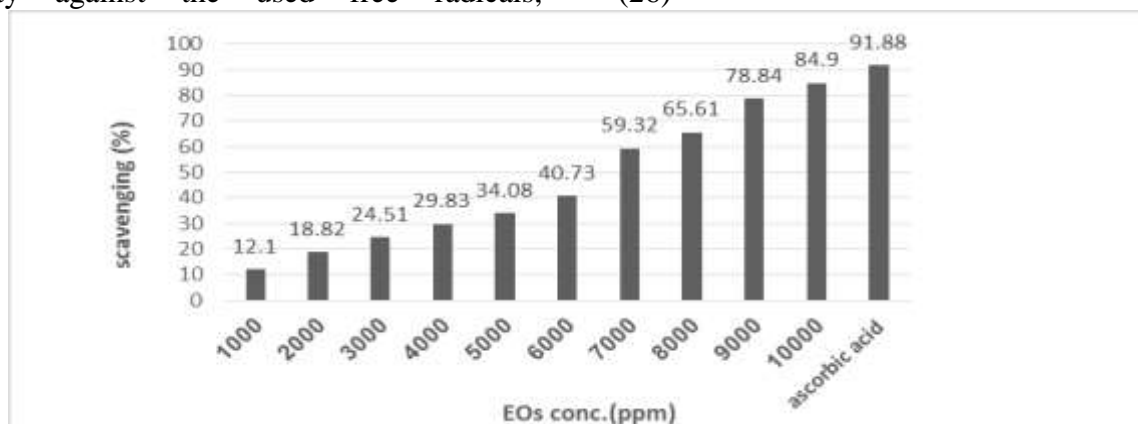


Figure 2. DPPH radical scavenging activity of aniseed EOs, each value represents means \pm SD, (n=3).

The aniseed EOs reducing capacity of DPPH have been confirmed in other studies. In both *in vivo* and *in vitro* experiments evidence have demonstrated significant antioxidant abilities of EOs from different plants against different free radicals' models (20). Antioxidant interact with free radicals and transfer either electrons or hydrogen atom, thus neutralized its free radical characteristic feature. Phenolic compounds are the main components that can donate hydrogen or electrons to free radicals and thus block the chain reaction of lipid peroxidation at the initiation stage. The high potency of phenolic compounds to scavenge radicals, owing to their phenolic hydroxyl groups (21). Indeed, redox properties of phenolic compounds allow them to fulfill multiple biological tasks, including, redox agents, hydrogen and electron donators, singlet oxygen quenchers as well as metal ions chelator (12). The major reason of antioxidant properties of aniseed EOs related to their high contents of total phenolic compounds. It has been reported that polyphenols contribute significantly to the total antioxidant potentials in fruits and vegetables. In current study, monoterpenes (phenolic compounds) were the major identified components of aniseed EOs, these have been considered as natural high antioxidants agents, which could be used as additives in food supplements to prevent the oxidative stress, which is the leading etiological cause of degenerative diseases in human. However, the effect of minor constituents of EOs should be considered as

well, and the potential synergistic effects between these compounds might account for the antioxidant activity (9). In a study carried out in Iran, the antioxidative potentials of some umbelliferae belonged plants have been reported. Among the included plants, *P. animus* exhibited the strongest reducing activity against DPPH ($IC_{50}=109.80$), and the study was confirmed positive correlation between antioxidant potency and phenolic contents of the studied fractions (6). The ethanolic extracted of aniseed EOs in concentration-dependent manner showed reducing scavenging activity against nitric oxide, superoxide and DPPH radicals. The alcoholic and water extract of aniseeds EOs displayed a marked antioxidative activity, in both linoleic acid and liposome model systems, and the water extract showed higher antioxidant activity than the corresponding alcohol fractions (9).

Antimicrobial activity

in vitro antibacterial activities of aniseed EOs against *P. aeruginosa* planktonic forms were assessed using agar-well diffusion method. EOs had certain antibacterial activity on majority of the experiment included pathogens. The candidate isolates exhibited different susceptibility profiles, high percentage of isolates were sensitive to introduced EOs. Hence, the susceptibility of bacteria was confined among three categories, 8 isolates were non-sensitive (20%), 25 isolates were sensitive (62%), while, 7 isolates exhibited high sensitivity (18%). Meanwhile,

the MIC values of aniseed EOs against selective isolates (highly sensitive *P. aeruginosa* isolates), were between 4000-7000ppm. The isolate RS40 with a mean of MIC of 4000 ppm was the most susceptible one to EOs action (Table.2 and Fig.3). *P. aeruginosa* is frequent cause of burns wound infections, and is one of the causes of delay in burn wounds healing (18). Recently *P. aeruginosa* was included to a comprehensive list of priority pathogens was released by the World Health Organization (WHO), these pathogens have high levels of resistance to most existing antibiotics such as carbapenem, vancomycin, penicillin, ampicillin, and the third-generation antibiotic cephalosporin (2). So, the toxicity of aniseed EOs against *P. aeruginosa* is of great relevance. On the other hand, *P. aeruginosa* is Gram-negative organism and Gram-negative bacteria generally had a higher resistance to the plant extracts and other antibacterial agents than Gram-positive bacteria. This attributed to the distinct feature of Gram-negative cell wall morphology, in contrast to those of Gram-positive bacteria. Gram negative bacteria enclosed inside hydrophilic lipopolysaccharide outer layer, which is resistant to the

penetration of antibacterial agents, as well as the presence of some lytic enzymes in the periplasmic space which attack and breaking down antibacterial molecules (4). Anise is one of many spices that contain bioactive constituents, phenolic and flavonoid compounds. The antibacterial activity of aniseed EOs can be assigned to their active components, such as anethole. Owing to existence of different bioactive components, there was not a specific mechanism for antibacterial effects of aniseed EOs, therefore referred to a cascade of damaging mechanisms, involving the entire bacterial cell. The proposed bactericidal mode of actions, including cell walls integrity disruption, loss of cell contents, destroying of cytoplasmic membrane and membranous proteins, coagulation of cytoplasmic fragile proteins, and dysfunction of the system activated proton transfer. Membrane damages are the most confirmed inhibitory effect of EOs (17). On the other hand, the MIC values of aniseed EOs was ranged from 4000 to 7000 ppm, that showing high values to resistant strains. These results agree with other studies that evaluated the MIC of EOs from different plants against *P. aeruginosa* (15).

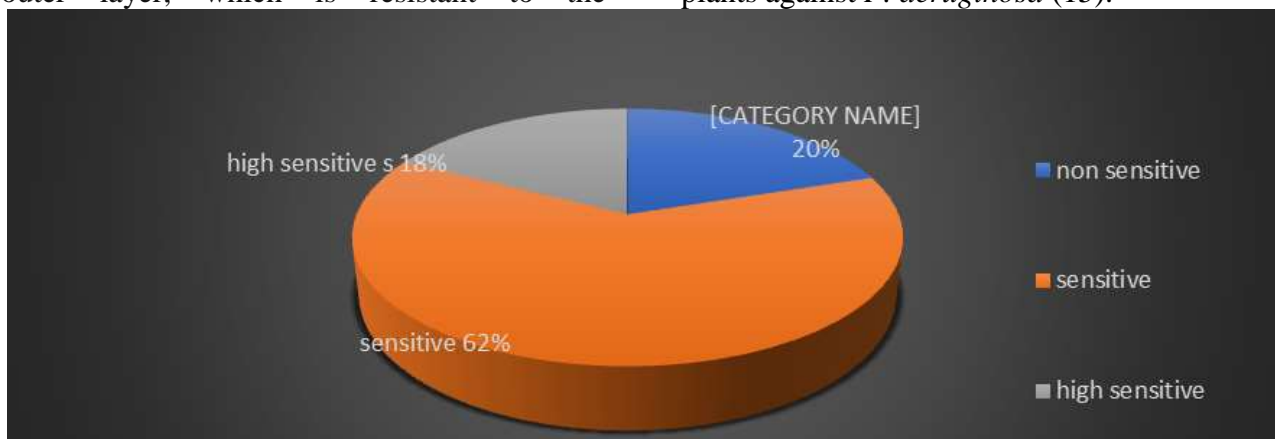


Figure 3. Susceptibility profile of burns wound isolated *P. aeruginosa* isolates to aniseed EOs

Table 2. Antibacterial activity of aniseed EOs against *P. aeruginosa* isolates, in respect of presented inhibition zones (mm) and MIC values(ppm)

<i>P. aeruginosa</i> isolates	Antibacterial activity of aniseed EOs	
	Inhibition Zone(mm)	MIC (ppm)
RS 4	12.6	5000
RE10	11.1	6000
RS 12	10.7	5000
RS23	10.2	7000
RS 28	9.5	7000
RS 37	9.6	6000
RS40	13.7	4000
LSD value	2..884*	246.07*
	* (P≤0.05)	

Biofilm formation capacity

The majority of burn wounds isolated *P. aeruginosa* were biofilm producers on polystyrene surface, in that 34 isolates (85%) were able to form biofilm. Whereas, 8 (23.5%) isolates were high biofilm producers with optical density (OD₆₂₀) values ranging from 0.95 to 0.467, and 21(61.76%) isolates considered as moderate biofilm producers, while, 5 (14.70%) isolates were weak biofilm producers. The other isolates (15%) did not show any biofilm formation capacity (Fig.4). The *P. aeruginosa* tested isolates exhibited high productivity for biofilm generation

compared to documented studies (19). *P. aeruginosa* is a gram-negative opportunistic pathogen known for its propensity to form biofilms (2). Biofilms are extracellular coating matrices, composed mainly of bacteria cells derived exopolysaccharides, extracellular DNA, proteins, and bacterial cells themselves. Several copies of the biofilm matrix, exopolysaccharides (alginate, Psl, and Pel) were recognized in the biofilms of *P. aeruginosa* (4). Majority of burns wound infection bacterial isolates are biofilm producers and are MDR strains.

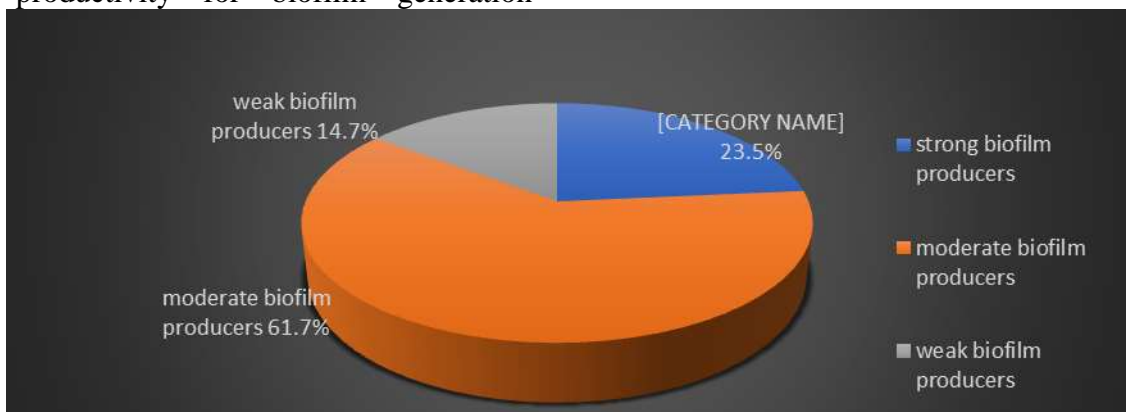


Figure 4. Biofilm formation capacity pattern of burns wounds isolated *P. aeruginosa* isolates

Antibiofilm potentials detection

Inhibition of biofilm formation: Antibiofilm potentials of aniseed EOs was evaluated against selected high biofilm producer *P. aeruginosa* isolates (8 isolates), using various concentrations ranged from sub inhibitory concentration (0.5×MIC) to double inhibitory concentration (2×MIC), The CV assay showed that the biofilm formation was significantly inhibited ($p < 0.05$) by all used MIC values. Whereas, in dose-dependent pattern the EOs consistently demonstrated inhibitory effect on biofilm mass, as the EOs concentrations increased the biofilm inhibition percentage also increased, with inhibition percentage ranged from 26 to 86.41 %. The maximum antibiofilm activity was recorded against the isolate *P. aeruginosa* RS28 at 2×MIC concentration (Table .3).

Inhibition pre-developed biofilms: The aniseed EOs was extremely less potent against previously generated biofilm of *P. aeruginosa*, the inhibition rates decreased by 100% for the majority of tested isolates. However, less scoring was recorded at highest used concentration (2×MIC) on the sensitive isolate

(*P. aeruginosa* RS28), the inhibition decreased by 3.4 folds, as 25.12% biofilm mass was reduced (Table .4). A key afforded feature by biofilm lifestyle is the inherent drug resistance brought to bacterial cells within the matrix. Bacterial cells residence within the biofilm architecture require nearly 1000× the antimicrobial concentration needed to kill their planktonic individuals (26). Therefore, finding out of alternative therapy for biofilm infections has become one of the attractive areas for researching in last few years. Plant derived EOs are well known for its antimicrobial activity against planktonic forms of bacteria (23). However, there is no enough information concerning the antibiofilm properties of plants EOs (1). Even though a few studies have shown that the bacterial biofilm could effectively inhibited by EOs such as cinnamon oil, eucalyptus and tea tree oil (10). Moreover, to best of our knowledge, this is the first study to provide data on aniseed EOs that play a role in the inhibition of biofilm generated by *P. aeruginosa*. In co-treatment assay the EOs achieved excellent biofilm removing potency, and this could be referring to interaction with

bacterial surface proteins, that masking the phase of initial connection on surfaces. The success in inhibiting cellular link can be explained as the cellular connection, which is the initial stage in the formation of the biofilm. As well as the interfere of EOs with quorum sensing systems, considered one of worthy biofilms inhibition strategies, and this can be explained by the presence of phenolic compounds (monoterpenes), identified in this study, which are capable of reducing biofilm synthesis owing to their suppressive activity of the autoinducer-2 responsible for cell-to-cell communication in biofilm building steps (14). Unlike the excellent inhibitory activity against biofilms generation the EOs presented little or no inhibin activity on pre-developed biofilms.

This can be explained by the fact that the exopolysaccharide overproduction, which play a key role in protecting the metabolically active bacteria embedded in biofilm community, that the EOs could only attack the cells closest to biofilm interface (13).

Conclusions

Aniseed EOs could be considered a good source of compounds with significant antioxidant, antimicrobial, and antibiofilm potentials, which can be assigned to the high percentage of the main constituents (monoterpenes) or to synergistic effects among different constituents of EOs, which induced excellent biocidal effect against burns wounds opportunistic *P. aeruginosa* isolates.

Table 3. The antibiofilm potentials of aniseed EOs against *P. aeruginosa* biofilms, results are expressed as percentage of inhibition

Isolates	Biofilm Inhibition rate				LSD value
	0.5 ×MIC	1 ×MIC	2 ×MIC		
RS5	30.3%	74.0%	56.0%		7.82*
RS12	41.2%	54.7%	57.6%		6.59*
RS22	45.6%	48.0%	73.3%		4.97*
RS25	26.0%	63.2%	65.2%		8.25*
RS28	61.02%	68.1%	86.41%		8.97*
RS31	50.8%	85.7%	83.11%		7.55*
RS36	53.5%	69.2%	80.7%		8.62*
RS40	42.8%	77.12%	86.41%		8.96*

* (P≤0.05).

Results are mean ±SD, (n=3)

Table 4. The antibiofilm potentials of aniseed EOs against *P. aeruginosa* generated biofilms. Results are expressed as percentage of inhibition

<i>P. aeruginosa</i>	Biofilm Inhibition rate				LSD value
	0.5 ×MIC	1 ×MIC	2 ×MIC		
RS5	0%	0% ±	0%		0.00NS
RS12	0%	2% ±	14%		2.71*
RS22	0%	0%±	0%		0.00NS
RS25	0%	7.0%±	0%		2.18*
RS28	0%	0% ±	25.12%		5.96*
RS31	0%	0% ±	0%		0.00NS
RS36	0%	0% ±	2.5%		1.78*
RS40	0%	1.8% ±	4.26%		1.96*

* (P≤0.05).

Results are mean ±SD, (n=3).

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