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# Antimicrobial activity of black seed oil & water extracts On multidrug resistant *Pseudomonas aeroginosa*

**Muna S. Al-Delaimi**

University of Duhok - Faculty of Medical Sciences.

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**Keywords:**
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**ABSTRACT**

*Pseudomonas aeruginosa* is most common cause of nosocomial infections in burn centers. This opportunistic and multidrug resistant bacterium causes severe problems for hospitalized burn patients. Samples were collected from patients attending Burn& Plastic Surgery Hospital in Duhok / Iraq, between September 2011 and March 2012, for preliminary identification of *P. aeroginosa* using conventional methods, then confirmed with the use of BD -Phoenix™ Automated Microbiology System. Isolates were considered Multi Drug Resistant *Pseudomonas aeroginosa* (MDRPa) if they showed resistance to the three or more classes of antipseudomonal agents. Accordingly thirty isolates were found MDRPa. The pattern of resistance revealed that the highest resistance was for Gentamicin 96.7%, Cefepime 96.7%, Ceftazidime 90% and Aztreonam 83.3%,while lowest resistance was detected against Amikacin 36.7% and Colistin 3.3% .The results of disc diffusion assay demonstrated that antibacterial activities of black seed oil acetone extract at 1:1,1:10,1:25 concentrations were found effective against all tested MDRPa isolates, which were statistically significant ($P < 0.05$),while at highest dilution 1:75 . All MDRPa isolates showed lowest activity of oil extract . The antimicrobial effects of water extract showed lowest and poor activity against all tested bacteria.

## Introduction:

Burn injury is a major public health problem throughout the world.[1] These injuries still produce significant morbidity and mortality in developing countries.[2] Infection in burn patients is difficult to control due to the presence of dead and denatured burn eschar, and moist environment , that act as a good growth medium for microbes.[3] Due to prolonged hospital stay these patients are at high risk of nosocomial infection. In this situation topical antimicrobial agents play a limited role that reduces the incidence of septic complication but the incidence of bacterial colonization had not decreased .[4,5] *Pseudomonas aeruginosa* is one of the most commonly isolated pathogens, and is the most frequently isolated non fermentative bacillus in clinical specimens.[6] It is also a part of normal skin flora of humans but can cause life threatening opportunistic infections specially in immunocompromised hosts .

This organism is a significant cause of burn and nosocomial infections. The ability of *Pseudomonas aeruginosa* to destroy tissue may be related to the production of various extracellular enzymes.[7]Complicating the empiric selection of adequate therapy is the increasing prevalence of antimicrobial drug resistance among *P. aeruginosa* , it is estimated that as many as 75% of all deaths following burn injury are related to infection.[8]

An alarming increase in bacterial strains resistant to existing antimicrobial agents demands a renewed effort to seek agents effective against pathogenic bacteria resistant to current antimicrobials.[9] The seeds of Nigella sativa L. (Ranunculaceae), commonly known as black seed or black cumin, are used in folk (herbal) medicine all over the world for the treatment and prevention of a number of diseases .Its seeds have a great medicinal importance and have been reported to exhibit many pharmacological effects that include anti-parasitic, antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory activities.[10]

* Corresponding author at: University of Duhok - Faculty of Medical Sciences.E-mail address:

However only a limited data is available for its efficacy against multi drug resistant *P. aeruginosa* ,which isolated from different clinical specimens.[11,12,13]

The present study was therefore designed to determine the antimicrobial activity of different concentrations of black seed extracts against MDRPa isolated from burn patients, it was the first trial in our country

**Materials & methods:**

**Isolation, characterisation and identification**

The study was performed at Burn& Plastic Surgery Hospital in Duhok / Iraq, between September 2011 and March 2012. Burned patients were enrolled in this study and from each one a cotton swabs collected to preliminary identification of *Pseudomonas aeroginosa* using colony structure and colony morphology, gram stain, oxidase positive reaction, typical smell, and development of pyocyanin pigments.[14] Then confirmed with the use of BD - Phoenix™ Automated Microbiology System (BD Diagnostics System, Sparks, MD, USA) / Bacteriological Laboratory / Azadi teaching Hospital, which is designed for the rapid identification (ID)(Table 1) and quantitative determination of antimicrobial susceptibility testing (AST)( Table 2) by Minimal Inhibitory Concentration (MIC) of Gram Negative aerobic and facultative anaerobic bacteria from pure culture .[15] Isolates were considered Multi Drug Resistant Pseudomonas aeroginosa (MDRPa) if they showed resistant to the three or more classes of antipseudomonal agents.16,17,18. Accordingly thirty isolates were found as MDRPa .

**Preparation of black seed extracts**

Plant seed were purchased from a local market in the city of Duhok. The extraction was conducted at Chemistry Department\ Faculty of Science –University of Duhok. To obtain the oil extract, the seed were powdered mechanically using as blender for five minutes. A total of 80 grams of the powder was dissolved in100 ml cold acetone; the mixture was then filtered using Whatman filter paper number 41. The filtrate was left to dry in an open Petridish at room temperature for 24 hours to allow the solvent to evaporate, then transferred into sterile vials (1ml of each) and stored at -200C till used. The following concentration (1:1, 1:10, 1:25, 1:50, 1:75) of oil extract were obtained by diluting the pure oil in absolute alcohol.

Black seed water extract, was done by the modified method of Ibraheem. 19 : 80 gm powder of black seed was placed in sterile beaker containing 100ml of distilled water. A magnetic stirrer was used to continuously mix the suspension for 18 hours. The mixture was then filtered using Whatman filter paper No. 41. The filtrate was left to dry in an open dish for 24 hours , and 0.5 g was dissolved in 100 ml distilled water to obtain a stock solution of 0.5% w/v of the extract. Concentrations of 0.05% w/v, 0.025% w/v and 0.005% w/v were prepared from the stock solution by adding distilled water.

***Preparation of the drug impregnated filter paper disc***

Whatman Filter paper No. 1 was used to prepare discs (6 mm). The discs were then sterilized by autoclaving. During sensitivity testing, 4µl of both oil extract in pure or diluted form and aqueous extract was kept on filter paper disc, placed on Mueller Hinton Agar plate inoculated with bacteria (Fig.3). Prepared discs were stored at 4°C in the refrigerator till used.
Disc containing absolute alcohol (dilutent of the extract) was used as negative control.

***Inoculation of plates:***

This was done by the modified method of Pelczar *et al* [20] using flood-inoculation technique. Bacterial suspension in Nutrient Broth having turbidity equivalent 0.5 McFarland was freshly prepared and 2 ml of this was transferred onto the Mueller Hinton (MH) Agar plate and distributed gently over the surface of medium with gentle rocking. The excess fluid was removed from the plate and the plate was kept in incubator at 37ºC for 30 minutes for drying before application of discs. A fully sensitive strain of *Pseudomonas aeroginosa* was used as standard for the black seed oil and water extract discs of anti *Pseudomonas* susceptibility test.

***Antimicrobial susceptibility testing***

The antibiotic susceptibility pattern of the *Pseudomonas aeruginosa* isolates was determined using the disk diffusion method according to the modified Kirby-Bauer technique, by placing discs impregnated with test material on surface of inoculated Muller Hinton agar plates. The plates were then kept in incubator at 37ºC for 18 hours and diameters of

zones of inhibition were measured. The experiment was performed in triplicate.

Statistical Analysis:

Done with SPSS ver.18 for ANOVA one way.

**Results:**

During the study period thirty MDRPa were isolated which showed different resistance to various antibacterial drugs as follows : four were resistant to four antibiotics, eight to five antibiotics, five to six antibiotics , four to eight antibiotics, four to nine antibiotics and five to ten antibiotics (Table 1).

The overall pattern of resistance revealed that the highest resistance was for Gentamicin 96.7% (29 /30), Cefepime 96.7% (29/30), Ceftazidime 90% (27/30) and Aztreonam 83.3% ( 25/30) .Ciprofloxacin 63.3%(19/30), Levofloxacin 60% (18/30) , Piperacillin 60%(18/30), while lowest resistance was detected against Imipenem 46.7% (14/30) , Meropenem 43.3% (13/30), Amikacin 36.7%(11/30) and Colistin 3.3% (1/30) ( Fig. 4).

The present investigation revealed that the acetone extract yield 20-30% of black seed oil, is being reported for the first time, it was reddish brown liquid , oily in nature**.**

Antibacterial activities of black seed oil extract at it's different concentrations against a total of thirty MDRPa strains were shown in (Table-2) .Five different concentrations 1:1, 1:10 , 1:125 ,1:50 and 1:75 per disc were used , among them 1:1,1:10 and 1:125 were found more effective against all isolates (mean of diameter of inhibition zone were 23.08 ±1.401mm , 19.482 ±2.014 mm and 14.553 ±3.421 mm respectively ) which was found statistically significant ($P < 0.05$).

Table-3 revealed that the oil extract inhibited MDRPa isolates in dose dependent manner. In conclusion this table indicate that the three concentrations (1:1, 1:10,1:25) of oil extract had resulted in highest antibacterial activity (16 - >25mm) and remaining 7 strains showed lowest activity pattern (≤ 10mm) at dilution 1:25. Out of thirty resistant isolates tested with dilution (1:50) showed activity against 9 isolates (11-15mm), and the remaining 21 isolates showed lowest activity pattern (≤ 10mm). While at highest dilution 1:75 all MDRPa isolates showed lowest activity of oil extract (≤ 10 mm).

Disc containing absolute alcohol (dilutent of the extract) produce no zone of inhibition. It was observed that controlled strain was sensitive against black seed oil extract concentrations.

Aqueous extract of black seed at concentrations of 0.5% w/v, 0.05% w/v, 0.025% w/v and 0.005% w/v produced narrow diameter of inhibition zone (2-10 mm) which were not reproducible.

**Discussion:**

*P. aeruginosa* remains the leading pathogen causing burn wound infection.[21] It survives well in the hospital environment. Once it is established, it can persist for months within a unit, posing as Multi drug resistant nosocomial infection risk for patients being treated there.[22,23] Pseudomonas is very resistant to most antibiotics and the resistance in this organism develops very rapidly. The rate of development of resistance to new antibiotics is much faster than the rate of invention and development of new antibiotics.[24]

Present study showed that the highest resistance was for Gentamicin 96.7%, this finding is similar to other studies conducted in Tohid burn centre Tehran Iran, and in Jinnah Postgraduate Medical Centre Karachi, where more than 95% and 93.2 strains of *Pseudomonas aeruginosa* were resistant to Gentamicin respectively.[3, 21] Gentamicin is a cheap and easily available drug that is used extensively in general and hospital practice in clinically suspected Gram-negative infections. This may be the main reason for the development of resistance in bacteria against this drug. Also the MDRPa isolates were highly resistant to the fourth generation Cephalosporins (Cefepime) 96.7% ,this result agree with Satti study which found that 71% of MDRPa isolated from various clinical specimens were resistant to Cefepime.[25] In the study of Mizuta *et al*., *P. aeruginosa* isolates were most often susceptible to Ceftazidime (87% of isolates), Amikacin (84%),[26]which disagree partially to our results which showed high resistance to Ceftazidime (90%) but accepted for Amikacin (36.3%) resistance. Aztreonam is a monobactam β-lactam drug, it has excellent activity against *Pseudomonas* species but has a limited treatment option against MDR strains of *Pseudomonas aeruginosa* ,[22] the findings in the present study are in accordance with Douglas study, with about 83.3% resistance in MDRPa against this drug.[22] *P. aeruginosa* isolates showed resistance to Ciprofloxacin (63.3%), this might be due to the widespread prescribing of Fluoroquinolones in empirical therapy for *Pseudomonas* infections, which

may be associated with delays in administering effective therapy resulting in adverse outcomes. Similar results were noted in other studies.[27] Present study showed resistance to Levofloxacin (60% ), which agree with other study done by Kaye.[28] Piperacillin showed resistant ( 60%) to Pseudomonas *aeruginosa* isolates, this finding disagree to other study in which *Pseudomonas aeruginosa* remained 90% susceptible to Piperacillin.[29] Carbepenems (Imipenem, Meropenem) are useful in treatment of some cases of multi-drug resistant strains of *P. aeruginosa* ,[22] in this study the resistance of *P. aeruginosa* against Imipenem and Meropenem were 46.7% and43.3% respectively. Similar results have been reported in other studies.[1,17] The highest sensitivity of *P. aeruginosa* was found to Colistin, this might be due to the less frequent use of this drug in the general practice because of the un sustained availability in hospitals and local markets.

In present study acetone extract yielded 20-30% of black seed oil .The ether extract made by Hanafy and Hatem, [30] was also dark brown and oily in nature but it was a clear liquid and yielded 11% v/w, while ether & methanolic extract made by Salman *et.al*.[31] yielded 16.7%, 32.5% respectively .These differences might be due to difference in the black seed sample, duration of soaking and / or temperature at which extraction was done.[31]

Our observation indicated that the antibacterial activity of oil extract against MDRPa isolates was dose dependent manner. This result is in accordance with earlier study.[31] The results of different concentration of black seed oil in present study showed direct relation with the diameter of inhibition zone and was found that acetone extract has clear effectiveness against MDRPa . However, all tested MDRPa isolates showed clear inhibitory effects of acetone extract at concentration 1:1, 1:10, 1:25 with inhibition zone of 23.08±1.401, 19.482±2.014, 14.553±3.421 respectively. Another study done by Salman tested oil and methalonic extract against *P. aeruginosa* isolated from different sources showed less significant effect at 1:1,1:10, the average size of inhibition zones were 11±1and10±1respectively.[11] On contrary, the study was unable to achieve any degree of susceptibility to *P. aeruginosa* , *E.coli* and *Candida albicans* even with double or four times increase in volumes of oil or whole seed added.[32]This variation in antimicrobial activities might be due to that black seed oil obtained from different commercial sources or isolated by different methods from the same seeds have been shown to vary significantly in their content of Thymoquinone, which has antibacterial activity and various storage conditions are expected to make a difference in the amounts of the quinone constituents of the oil, especially if the seed oil samples are exposed to heat and light.[3 3,34] Data presented in Table 3 indicated that 1:1,1:10,1:25 concentrations of oil extract resulted in highest antimicrobial activity produced zone of inhibition ranging from 16 - >25mm.Out of thirty MDRPa isolates tested with dilution 1:50 showed activity against nine isolates produce zone of inhibition 11-15mm.These results disagree with Salman results who mentioned that four and one strain inhibited by oil at concentration 1:1 produced 12-16mm and 17-21mmzone inhibition respectively.[11] Out of twenty one strains of *P. aeruginosa* tested , one was inhibited by oil at concentration 1:10 showed 12-16mm.The reason might be the differences in tested isolates. The antimicrobial effects of water extract showed the lowest and poor activity against all tested bacteria ,this result is in agreement with Salman et al,[31] and it could be explained by the fact that the active ingredient, thymoquinone, is present in the oil extract, such an active substance may be missing or present in small amounts in the water extract .[35]

***Conclusion and recommendation*:**

The black seed oil extract using acetone is very effective as antibacterial agent against MDRPa and it gave better results than the oil extracted by other solvents. The water extract of black seed showed very little antibacterial activity against MDRPa. Although this study was done in vitro, the significant results obtained should direct us toward next step in using the oil extract in vivo, including topical application and / or systematic medication.

**Knowledgments:**

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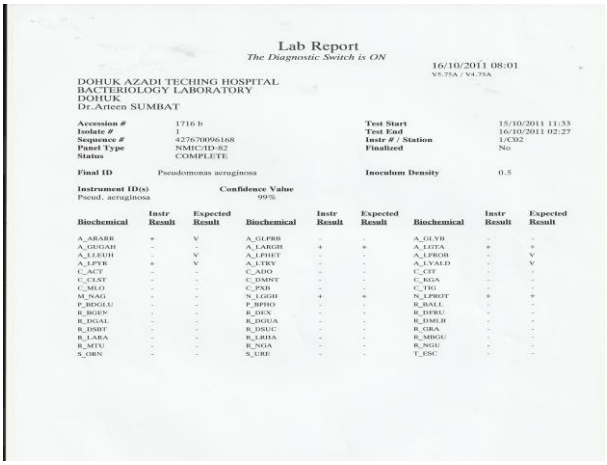


Lab Report
*The Diagnostic Switch is ON*

16/10/2011 08:01
V5.75A / V4.75A

DOHUK AZADI TECHING HOSPITAL
BACTERIOLOGY LABORATORY
DOHUK
Dr.Arteen SUMBAT

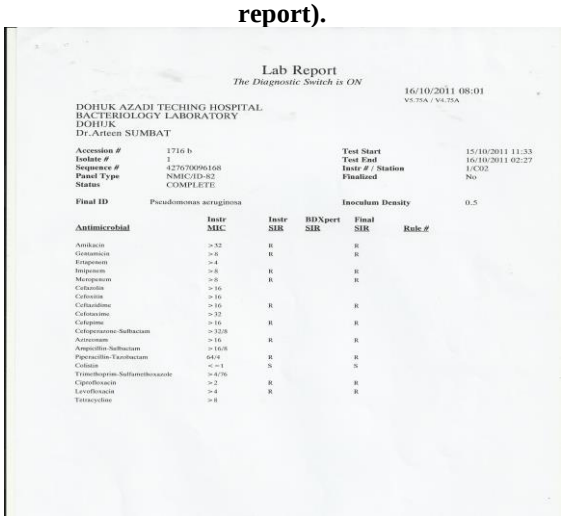
| | | | |
|---|---|---|---|
| **Accession #** | 1716 b | **Test Start** | 15/10/2011 11:33 |
| **Isolate #** | 1 | **Test End** | 16/10/2011 02:27 |
| **Sequence #** | 427670096168 | **Instr # / Station** | 1/C02 |
| **Panel Type** | NMIC/ID-82 | **Finalized** | No |
| **Status** | COMPLETE | | |

**Final ID** Pseudomonas aeruginosa **Inoculum Density** 0.5

**Instrument ID(s)** Pseud. aeruginosa **Confidence Value** 99%

| Biochemical | Instr Result | Expected Result | Biochemical | Instr Result | Expected Result | Biochemical | Instr Result | Expected Result |
|---|---|---|---|---|---|---|---|---|
| A_ARABR | + | V | A_GLPRB | - | - | A_GLYB | - | - |
| A_GSGAR | - | - | A_LARGB | + | + | A_LGTA | + | + |
| A_LLEUH | - | V | A_LPHET | - | - | A_LPROB | - | V |
| A_LPYR | + | V | A_LTRY | - | - | A_LYALD | - | V |
| C_ACT | - | - | C_ADO | - | - | C_CIT | - | - |
| C_CLST | - | - | C_DMNT | - | - | C_KGA | - | - |
| C_MLO | - | - | C_PXB | - | - | C_TIG | - | - |
| M_NAG | - | - | N_LGGH | + | + | N_LPROT | + | + |
| P_BNGLU | - | - | P_BPHO | - | - | R_BALI | - | - |
| R_BGEN | - | - | R_DEN | - | - | R_DFRU | - | - |
| R_DGAL | - | - | R_DGUA | - | - | R_DMLB | - | - |
| R_DSBT | - | - | R_DSUC | - | - | R_GKA | - | - |
| R_LARA | - | - | R_LRHA | - | - | R_MBGU | - | - |
| R_MTU | - | - | R_NGA | - | - | R_NGU | - | - |
| S_ORN | - | - | S_URE | - | - | T_ESC | - | - |

**Table .1: Identification of P. aeruginosa by BD - PhoenixTM Automated Microbiology System (original report).**



Lab Report
*The Diagnostic Switch is ON*

16/10/2011 08:01
V5.75A / V4.75A

DOHUK AZADI TECHING HOSPITAL
BACTERIOLOGY LABORATORY
DOHUK
Dr.Arteen SUMBAT

| | | | |
|---|---|---|---|
| **Accession #** | 1716 b | **Test Start** | 15/10/2011 11:33 |
| **Isolate #** | 1 | **Test End** | 16/10/2011 02:27 |
| **Sequence #** | 427670096168 | **Instr # / Station** | 1/C02 |
| **Panel Type** | NMIC/ID-82 | **Finalized** | No |
| **Status** | COMPLETE | | |

**Final ID** Pseudomonas aeruginosa **Inoculum Density** 0.5

| Antimicrobial | Instr MIC | Instr SIR | BDXpert SIR | Final SIR | Rule # |
|---|---|---|---|---|---|
| Amikacin | > 32 | R | | R | |
| Gentamicin | > 8 | R | | R | |
| Ertapenem | > 4 | | | | |
| Imipenem | > 8 | R | | R | |
| Meropenem | > 8 | R | | R | |
| Cefazolin | > 16 | | | | |
| Cefoxitin | > 16 | | | | |
| Ceftazidime | > 16 | R | | R | |
| Cefotaxime | > 32 | | | | |
| Cefepime | > 16 | R | | R | |
| Cefoperazone-Sulbactam | > 32/8 | | | | |
| Aztreonam | > 16 | R | | R | |
| Ampicillin-Sulbactam | > 16/8 | | | | |
| Piperacillin-Tazobactam | 64/4 | R | | R | |
| Colistin | < =1 | S | | S | |
| Trimethoprim-Sulfamethoxazole | > 4/76 | | | | |
| Ciprofloxacin | > 2 | R | | R | |
| Levofloxacin | > 4 | R | | R | |
| Tetracycline | > 8 | | | | |

**Table .2: Antibiotic sensitivity of P. aeruginosa by BD - PhoenixTM Automated Microbiology System (original report).**

**Fig.3**: Revealed to susceptibility of *P. aeruginosa* to different
concentrations of black seed oil impregnated filter paper on MuellerHinton Agar Plate.

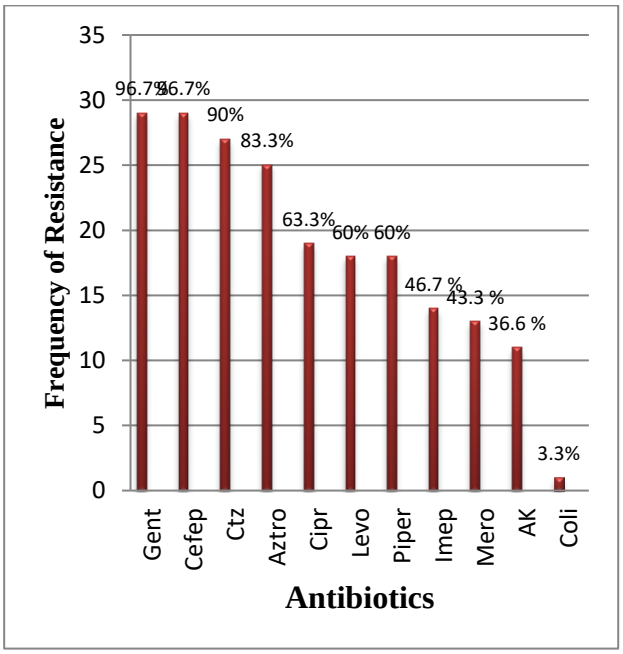



Fig. 4 : Resistant pattern of Pseudomonas aeruginosa isolates .
( Gent- Gentamicin , Cefep- Cefepime ,Ctz- Ceftazidime , Aztro- Aztreonam, Cipr-Ciprofloxacin ,Levo- Levofloxacin , Piper- Piperacilli, Imep-Imipenem , Mero- Meropenem ,Ak- Amikacin , Coli- Colistin).

Table 1: Distribution of resistance among 30 P. aeruginosa isolates against 11 antimicrobial drugs.

| Resistant to 4 | Resistant to 5 | Resistant to 6 | Resistant to 8 | Resistant to 9 | Resistant to 10 |
|---|---|---|---|---|---|
| 04 (13.3%) | 08 (26.6%) | 05 (16.6%) | 04 (13.3%) | 04 (13.3%) | 05 (16.6%) |

Table 2: Inhibition zones (measured as mm) of different concentrations of black seed oil extract against MDRPa.

| MDRPa | Black seed oil extract (zones in mm) | | | | |
|---|---|---|---|---|---|
| | 1:1 | 1:10 | 1:25 | 1:50 | 1:175 |
| n= 30 | Mean ±SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM |
| | 23.08*±1.401 | 19.482*±2.014 | 14.553*±3.421 | 7.882 ±4.818 | 4.077 ±3.471 |

* P < 0.05

Table 3: Sensitivity pattern of MDRPa to different concentrations of black seed oil extract

| Zone of inhibition (mm) | Black seed oil concentrations | | | | |
|---|---|---|---|---|---|
| | 1:1 | 1:10 | 1:25 | 1:50 | 1:75 |
| >25 | 7 | 1 | - | - | - |
| 21-25 | 19 | 11 | - | - | - |
| 16-20 | 4 | 16 | 17 | - | - |
| 11-15 | - | 2 | 6 | 9 | - |
| ≤ 10 | - | - | 7 | 21 | 30 |
| Total No. of isolates | 30 | 30 | 30 | 30 | 30 |

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# تاثير الفعالية المقاومة للميكروبات لمستخلص الحبة السوداء المائي والزيتي على بكتيريا الزائفة الزنجارية المتعددة المقاومة للمضادات

**منى سلمان الدليمي**

**الخلاصة**

تعد جرثومة الزائفة الزنجارية احدى عدوى المستشفيات الاكثر شيوعا في مراكز الحروق . وتشكل هذه الجرثومة احدى المخاطر التي تهدد حياة مرضى الحروق اذ كونها انتهازية ومعروفة لمقاومتها المضادات الحيوية. جمعت عينات الدراسة للمرضى الراقدين في مستشفى الحروق والجراحة التجميلية في دهوك\ العراق ,للفترة من ايلول2011 ولغاية اذار2012، وتم استخدام الطرائق التقليدية للتشخيص التمهيدي لهذه الجرثومة وعززت باستحدام جهاز BD – PhoenixTM، واعتبرت هذه العزلات من الجراثيم متعددة المقاومة للمضادات ,اذا اظهرت مقاومة لثلاث اواكثر من المضادات التي تستخدم في علاج الخمج الناجم عن الاصابة بهذه الجراثيم ووفقا لذلك عزلت ثلاثون عزلة متعددة المقاومة للمضادات خلال الدراسة .واظهرت العزلات اعلى نسبة مقاومة للمضادات جنتامايسين وسيفيبيم و سيفتازديم وازترونام (96.7%، 96.7% و 90% و83.3%) على التوالي وكان المضاد اميكسين وكوليستين الاقل مقاومة (36.7% 3.3%). وقد اختبرت الفعالية المضادة للجراثيم لمستخلص الحبة السوداء المائي والزيتي على جراثيم الزائفة الزنجارية المتعددة المقاومة للمضادات .واظهر زيت الحبة السوداء تركيز 1:1 ,1:10 , 1:25فعالية مضادة للجراثيم اكثر معنوية من زيت الحبة السوداء بتركيز 1:75وكانت الفعالية المضادة للجراثيم للمستخلص المائي منخفضة وقليلة على جميع العزلات المدروسة.