ISSN: 0067-2904

The Antimicrobial Effects of Alcoholic Leaves Extract of *Salvia Officinalis* Against Multidrug Resistant *Pseudomonas Aeruginosa*

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Received: 3/4/2020 Accepted: 5/7/2020

Abstract

Two isolates of *Pseudomonas aeruginosa* were isolated from patients with Urinary Tract Infection (UTI). The aim of this study was to determine the antimicrobial effect of alcoholic leaves extract of *Salvia officinalis* on Multidrug resistant (MDR) *P. aeruginosa*. Using the well diffusion test, the alcoholic leaves extract at 100mg/ml and 200 mg /ml was shown to possess antimicrobial activity against the tested microorganism. The inhibition zones of *S. officinalis* at 200 mg/ml, and 100 mg/ml of the extract showed diameters of 23mm and 20mm, respectively. But the diameters of the inhibition zones caused by treatment with the antibiotics Ciprofloxacin, Ticarcillin + Clavulanic acid, and Cefotaxime were 28mm, 27mm, and 25mm) for both isolates, respectively. The results indicate that *P. aeruginosa* was resistant to most antibiotics of different groups used in this study. It was found that the isolates were Multidrug Resistant (MDR) by sensitivity test. The results of minimum inhibitory concentration (MIC), by using E-test strips, showed that MIC of Cefoxitin was 4µg/ml for *P. aeruginosa* 1, but *P. aeruginosa* 2 was resistant. MIC value for Cefoperazone was 8µg/ml against *P. aeruginosa* 1, whereas *P. aeruginosa* 2 was resistant. MIC value was also determined for the alcoholic leaves extract against *P. aeruginosa*, showing a value of 100 mg/ml, while the plant extract also had synergistic effects with Ciprofloxacin, Cefotaxime, and Ticarcillin+ Clavulanic Acid. It can be concluded that the alcoholic leaves extract of *S. officinalis* had considerable antimicrobial effects on MDR *P. aeruginosa*. Thus, it can be used instead of antibiotics for the treatment of UTI caused by MDR *P. aeruginosa* to reduce the side effects of antibiotics. On the contrary, its use with antibiotics enhances their action without interfere with them.

Keywords: *Pseudomonas aeruginosa*, antimicrobial effects, *Salvia officinalis*, alcoholic leaves extract.

التأثير الضد مايكروبي للمستخلص الكحولي لأوراق *Salvia officinalis* ضد *Pseudomonas aeruginosa* المتعددة المقاومة للمضادات الحيوية

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الخلاصة

تم الحصول على عزلتين من بكتريا الزائفة الزنجارية من المرضى المصابين بالتهابات المجاري البولية. الهدف من الدراسة تحديد التأثير المضاد للمايكروبات للمستخلص الكحولي لأوراق المرمية ضد بكتريا الزائفة الزنجارية وتم اختبار مستخلص الإيثانول الكحولي لمستخلص باستخدام اختبار انتشار الحفر ،حيث امتلك

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المستخلص الكحولي للأوراق عند تركيز 200 ملغم / مل وتركيز 100 ملغم / مل نشاطا مضادا للبكتريا ،حيث كانت منطقة التثبيط 23 , 20على التوالي تجاه العزلتين .و منطقة التثبيط للسيبروفلوكساسين و 28ملم و للتيكارسيلين+ حامض الكلافيولانك كان 27 مل, بينما للسيفوتاكسيم كانت منطقة التثبيط 25ملم لكلا العزلتين على التوالي .لكن كانت الزائفة الزنجارية مقاومة لمعظم المضادات الحيوية ولمجاميع مختلفة المستخدمة في هذه الدراسة . ووجد ان العزلات كانت متعددة المقاومة للمضادات الحيوية باستخدام اختبار الحساسية. وكانت نتائج اختبار التركيز المثبط الادنى للمضادات الحياتية باستخدام اختبار E بالاشرطة . حيث كانت قيمة التركيز المثبط الادنى للسيفوكزيتين 4مايكروغرام/مل, بينما السيفبيرازون 8 مايكروغرام/مل للعزلة الاولى الاولى ومقاومة للعزلة الثانية .تم تحديد التركيز المثبط الادنى للمستخلص الكحولي ضد بكتريا الزائفة الزنجارية المتعددة للمضادات الحيوية و ايضا كان للمستخلص تأثير تآزري مع المضادات الحيوية مثل سيبروفلوكساسين ، سيفوتاكسيم ، تيكارسيلين + حامض الكلافولانيك. المستخلص يمكن استخدامه بدلاً من المضادات الحيوية لعلاج التهاب المسالك البولية الناتج عن الزائفة الزنجارية المتعددة المقاومة للمضادات الحيوية, لتقليل الآثار الجانبية للمضادات المستخدمة بل على العكس استخدامه مع المضادات الحيوية يعزز من عمل هذه المضادات المستخدمة في علاج التهابات المسالك البولية دون ان يتداخل معها.

Introduction:

The effects of *Pseudomonas aeruginosa* are often associated with many of virulence factors secreted by this bacterium, including Pyocyanin [1]. Pyocyanin has antimicrobial activity against wide variety of micro-organisms, which may benefit *P. aeruginosa* by elimination of competing microorganisms; pyocyanin is used as an antimicrobial agent, selectively inhibiting Gram-positive and Gram-negative bacteria other than *Pseudomonas* spp. [2]. *P. aeruginosa* is resistant to high concentrations of salts and dyes, weak antiseptics, and commonly used antibiotics [3].

Pseudomonas aeruginosa is an opportunistic pathogen for humans [4]. It is not the main causing agent of UTI, but many cases may be caused by this pathogen. *Pseudomonas aeruginosa* is resistant to most of the antibiotics used in the treatment of UTI, especially those associated with a nosocomial infection in patients with weak immunity [5]. Plants contain many active components like alkaloids, flavonoids, steroids, and saponins such as *Salvia officinalis*. The pharmacological findings for *Salvia officinalis* include anticancer, anti-inflammatory, antinociceptive, antioxidant, antimicrobial, antimutagenic, antidementia, hypoglycemic, and hypolipidemic effects [6].

Salvia officinalis was reported to have antibacterial and antifungal effects. Phenolic acids, such as salvin and salvin monomethyl ether, excreted by this plant have antimicrobial activities, especially against *Staphylococcus aureus, Pseudomonas aeruginosa* and other bacteria [7]. The Aim of this study investigated the sensitivity of *P. aeruginosa* to antibiotics and determine MIC values to some antibiotics by using E-test strips. We also aimed at determining the antimicrobial activity of alcoholic leaves extract of *Salvia officinalis* against MDR *Pseudomonas aeruginosa* isolated from UTIs and estimating the synergic effects of alcoholic leaves extract of *Salvia officinalis* with antibiotics against MDR *P. aeruginosa*.

Materials and Methods:

Isolation of *Pseudomonas aeruginosa:*

The bacteria were collected from Al- Yarmouk hospital in Baghdad city. Two isolates of bacteria *Pseudomonas aeruginosa* were detected from patients with UTIs, by using standard methods [8]. Urine samples were cultured onto plates of MaCconkey agar, blood agar and Cetramide agar.

Identification of *Pseudomonas aeruginosa:*

Pseudomonas aeruginosa was identified depending on the morphological features on culture media as well as the biochemical tests described elsewhere [9], while the isolates were confirmed with VITEK 2 compact system.

Preparation of alcoholic leaves extract of *Salvia officinalis*:

Salavia officinalis leaves were acquired from plant stores in Baghdad. To clear dust, the leaves were washed with water and then dried in an air oven at 60 C for 3 days. To obtain the plant powders, the air-dried plant materials were ground in a blender with a suitable size. 10 g of each plant powder was extracted by Soxhlet 8 hrs. with 200 ml of 80 % (v / v) aqueous ethanol. The extracts were dried in a rotary evaporator at 60℃ and the resultant powder was kept in a closed glass container until use in refrigerator [10].

The sensitivity test of *P. aeruginosa* to antibiotics:

The discs method was used to conduct *in vitro* sensitivity test according to published methodology [11]. Fifteen antibiotics discs from (Bioanalysis / Turkey) were used: Erythromycin, Clindamycin, Cefoxitin, Cephalothin, Trimethoprim, Ciprofloxacin, Imipenem/Cilactin, Cefotaxime, Ticarcillin + Clavulanic acid, Norfloxacin, Amoxicillin + Clavulanic acid, Ticarcillin, Cephalexin, Amikacin, and Imipenem. Bacteria were grown for 18 hours in Mueller Hinton broth at 37^{o}C and then inoculum was placed on the media by sterile swabs after dilution to no. 0.5 McFarland criteria (1x10^{8} cell / ml). The cultures and antibiotic discs were incubated to overnight incubation at 37^{o}C. The findings were compared with the CLIS (Clinical and Laboratory Standards Institute) data [12].

MIC determination of *Pseudomonas aeruginosa* by E-test:

Five antibiotic strips of E-test were used, namely those for Cefoperazone (CFP), Cefoxitin (FOX), Cefotaxime/ Cefotaxime+Clavulanic acid (CTX/CTX+), Gentamicin (HLG), and Levofloxacillin (LEV), which were obtained from (Bioanalysis/Turkey). The antibiotic sensitivity test of the clinical isolates was achieved as previously reported [10]. The bacteria were grown in Mueller Hinton broth for 18 hrs. at 37ºC, and then inoculums were placed on Mueller Hinton agar by sterile swabs after dilution to McFarland no.0.5 tube. Then, the antibiotic strips were placed on media, pressed, and then incubated at 37oC overnight. The results were compared with CLSI data [12].

Antibacterial activity of alcoholic leaves extract of *Salavia officinalis*:

The antimicrobial activity of *Salavia officinalis* ethanol leave extracts was tested by previously described methods [13, 14] by using Mueller Hinton Agar with wells method. Previously prepared, *P. aeruginosa* bacterial suspensions (with turbidity of no. 0.5 McFarland criteria for the dilution of 1x 10^{8} cell / ml) were spread on the agar surface. Wells with diameter of 0.5 cm were created on agar plates by sterile cork-borer. The extract was prepared at concentrations 200, 100, 50, 25, 12.5, and 6.25 mg/ml. After loaded the wells with concentrations of extract, the plates were incubated overnight and the antibacterial activity was evaluated by the millimeter measurement of the diameter of the inhibition zones around wells. The inhibition zones were assumed to indicate the effects of the MIC of the leaves extract against (MDR) *Pseudomonas aeruginosa* [12] as compared to control.

Determination of the synergetic effects of *Salvia officinalis* and antibiotics:

To determine the synergistic effects of alcoholic leaves extract with three antibiotic discs (Ciprofloxacin (5mg), Cefotaxime (30 mg), Ticarcillin + Clavulanic acid (10/10 mg)), the MIC concentration of leaves extract was added to the antibiotic discs. After that, the plates were incubated overnight. Then, a comparison was made between the regions of inhibition of antibiotic discs alone with those of antibiotic discs with alcoholic leaves extract, to observe the synergistic effects of alcoholic leaves extracts with antibiotics. The test was performed at the same conditions as previously described [15].

Results and discussion:

Isolation and identification of *Pseudomonas aeruginosa*:

Thirty urine samples were collected and cultured on MacConkey agar, blood agar. But only two isolates were belonged to *Pseudomonas aeruginosa*. The isolates are gram negative bacteria appeared as pale colonies. On blood agar, the beta type of haemolysis was observed, while *P. aeruginosa* isolates were positive for the oxidase test. Many strains of *P. aeruginosa* produce various species of pyocyanin on Cetramide agar [8]. The result shown in Table-1 indicate that these tests identified only two isolates belonging to *Pseudomonas aeruginosa*, and the detection was confirmed by VITEK 2 System.

Table 1-The results of Cultural and Morphological tests of *P. aeruginosa:*

The test name	Results
Microscopically	**Gram negative**
MacConkey agar	**Pale colonies**
Blood agar	**Beta haemolysis**
Cetramide agar	**Pyocynin pigment production**
oxidase	**positive**

The sensitivity test of *P. aeruginosa* to antibiotics:

The sensitivity test of two isolates (*P. aeruginosa* 1 and *P. aeruginosa* 2) isolated from UTI patients was conducted against fifteen antibiotic discs. The results of sensitivity to Ciprofloxacin showed that the inhibition diameter is 28 mm for *P. aeruginosa* 1 and 25mm for P. *aeruginosa* 2. Both isolates of *P. aeruginosa* were showed inhibition zone in the diameter of 25 mm in response to Cefotaxime. *P. aeruginosa* 1 was sensitive to Imipenem/Cilactin with a diameter of 27 mm, while *P. aeruginosa* 2 showed a diameter of 11 mm, indicating resistance. It was also found that the two isolates were sensitive to Ticarcillin + Clavulanic Acid with inhibition zone diameter of 27 mm (Table-2). This agrees whit the results of another study which reported that that *P. aeruginosa* isolates, especially those isolated from UTI are resistant to high concentrations of salts and dyes, weak antiseptics, and commonly used antibiotics [16]. *P. aeruginosa* showed the highest resistance rate to Erythromycin, Clindamycin, Cefoxitin, Cephalothin, and Trimethoprim, while the lowest resistance rate was to Ticarcillin, and Imipenem, respectively, according to the antibiotic sensitivity test. Also, the highest sensitivity rate was observed against the antibiotics Ciprofloxacillin, Cefotaxime, Ticarcillin + Clavulanic Acid for both isolates. In a previous study, *P. aeruginosa* isolated from the eyes showed resistance to Cephalothin, Ofloxacin, Piperacillin, and Moxifloxacin, but was sensitive to Chloramphenicol and Ciprofloxacin [17]. In another report of sensitivity test, Imipenem and Gentamicin showed antimicrobial effects on gram positive bacteria, while Imipenem was more effective against gram positive bacteria [15]. The isolates in this study were resistant to different groups of antibiotics more than four groups. Therefore, these isolates were identified as MDR *P. aeruginosa*, as described in CLSI instructions [11]. Other studies have specifically reported on patients with infection caused by MDR *P. aeruginosa*. However, as the resistance to many of these antibiotics increases, it becomes imperative that more antibiotics be discovered as an effective treatment for infections caused by MDR *P. aeruginosa*. [18].

Table 2-The results of sensitivity test to antibiotics against *P. aeruginosa:*

ID	Antibiotic	*P. aeruginosa* 1	*P. aeruginosa* 2
1	Cephalothin	0mm (R)	0mm (R)
2	Clindamycin	0mm (R)	0mm (R)
3	Cefalexin	9mm(R)	10mm(R)
4	Amikacin	18mm(R)	17mm(R)
5	Ciprofloxacin	28mm(S)	28mm(S)
6	Erythromycin	0mm(R)	0mm(R)
7	Norfloxacin	19mm(R)	20mm(R)
8	Amoxicillin + Clavulanic Acid	13mm(R)	12mm(R)
9	Cefotaxime	25mm(S)	25mm(S)
10	Ticarcillin + Clavulanic Acid	27mm(S)	27mm (S)
11	Cefoxitin	0mm (R)	0mm (R)
12	Trimethoprim	0mm (R)	29mm(S)
13	Imipenem/Cilactin	27mm(S)	11mm(R)
14	Ticarcillin	13mm(R)	11mm(R)
15	Imipenem	10mm(R)	5mm(R)

MIC determination of *Pseudomonas aeruginosa* by E-test:

The E-test by diffusion method was used to evaluate the MIC. The results showed elliptical inhibition zones around the strips, as in Figure-1 and Table-3. In this study, Cefoxitin and Cefoperazone , Levofloxacin strips were used for two isolates of P. aeruginosa, and they were giving MIC values (4, 8, 6 µg/ml, respectively) only for the isolate of P. aeruginosa 1;whereas P. aeruginosa 2 did not show inhibition for these antibiotic strips. Gentamicin showed MIC values of 6 and 4 µg/ml for both isolates respectively. MIC Strip is a rapid and reliable method for determining the antimicrobial susceptibility of different microorganisms against antibiotics. The E-test for Cefotaxime/Cefotaxime + Clavulanic acid was also used to determine ESBLs (Extended spectrum beta-lactamases) production by P. aeruginosa. According to CLSI results, when the MIC value of Cefotaxime/Cefotaxime + Clavulanic acid was lower than 8, this indicated that the isolate is resistant to these antibiotics [11].And these isolates were ESBLs producers.

Table 3-The results of E-test antibiotic strips against MDR *P. aeruginosa*:

Names of E-test antibiotic strips	MIC of *P. aeruginosa* 1 in µg/ml	MIC of *P. aeruginosa* 2 in µg/ml
Cefoxitin	**4**	**R**
Cefoperazone	**8**	**R**
Cefotaxime/ Cefotaxime +	**1.5/0.16**	**0.32/2**
Gentamycin	**6**	**4**
Levofloxacin	**6**	**R**

A **B**

Figure 1-E-test strips of antibiotics against MDR *P. aeruginosa*. A: Gentamycin E-test strip; B: Cefotaxime/ Cefotaxime + E-test strip

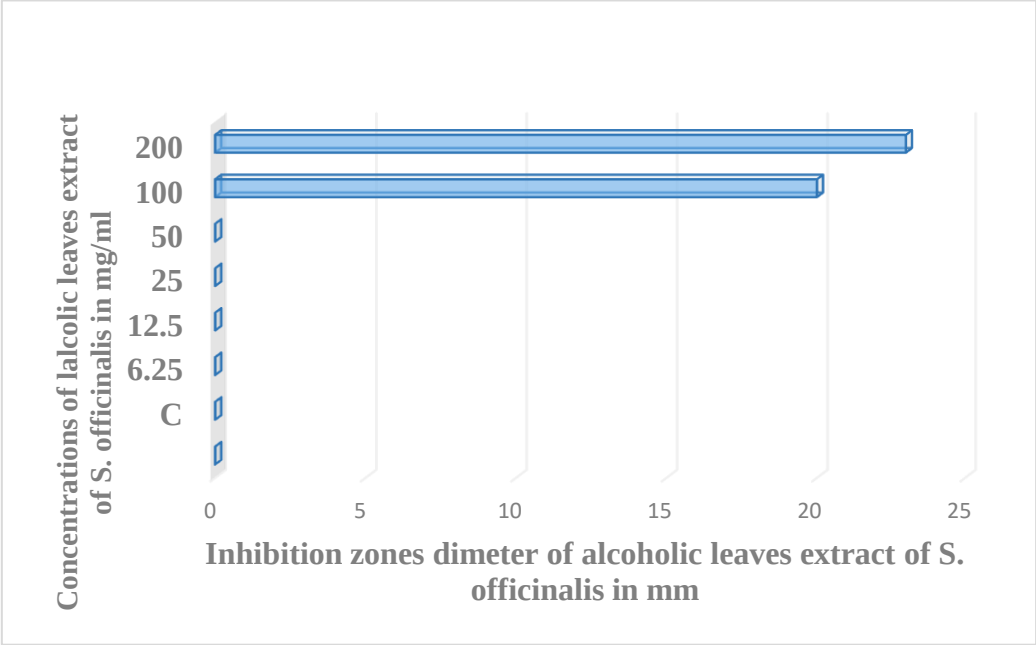
Antibacterial activity of alcoholic leaves extract of *Salvia officinalis*:

The data showed a clear inhibitory effect of *Salvia officinalis* leaves extract at concentrations of 200 m g/ml was(23mm) but, in 100 mg/ml concentration was (20mm), while the other concentrations showed no inhibitory activity against both *P. aeruginosa* isolates. These results agree with those reported by another study which found that the plant has antibacterial activity against *P. aeruginosa* [13, 14]. Other herbal plants, such as methanolic alcohol extract and fractions of *C. longa*

L. rhizomes, *C. myrrha L. gums* showed biological activities against *P. aeruginosa* and *S. aureus* [19]. The results of this study agree with another study which found that the flavonoids of *S. officinalis* had antibacterial activities against *S. aureus* and *P. aeruginosa* [20]. The MIC value of *S. officinalis* was 100 mg/ml against *P. aeruginosa*, but the results of Muttalib and Naqishbandi were found that of 75% *S. officinalis* alcoholic leaves extract in 100 mg/ml concentration did not exert activity against *P. aeruginosa* [21].

Comparing inhibitory activity of antibiotic discs and plant extracts of *S. officinalis* against MDR *P. aeruginosa*:

As observed in Figure-2, alcoholic leaves extract *of S. officinalis* at a concentration of 200 mg/ml, and 100 mg/ml had an inhibition zones diameter 23 mm, 20 mm respectively. These results demonstrate more potent inhibition activity as compared to those of antibiotics as Erythromycin (10µg/ml) and Cephalothin (30µg/ml), against *P. aeruginosa.* However, the inhibition zone value was less than that caused by Ciprofloxacin and close to those caused by Cefotaxime. These results indicate the possibility of the use of S. officinalis leaves extract to treat UTIs caused by MDR P. aeruginosa instead of using antibiotics, especially that the plants extracts have less side effects than those caused by antibiotics. The *S. officinalis* extract was found to have antibacterial activities against *P. aeruginosa* with the ability of preventing biofilm formation also [22].



C: Control

Figure 2-The inhibitory activity of alcoholic leaves extract of *Salvia officinalis* against MDR *P. aeruginosa*.

Determination of the synergetic effects of alcoholic leaves extract of *Salvia officinalis* with antibiotics:

After adding the MIC concentration of alcoholic leaves extract to antibiotic discs, the results were presented in Table-4. Only the antibiotics which showed sensitivity effects against the bacteria were then added to MIC concentrations of the plant extract. The results showed an increased inhibition zones of antibiotic discs with added to plant extract, while the inhibition zones of antibiotics without plant extract were smaller. Other studies showed synergistic interactions between amoxicillin and acetone or ethyl acetate extract of *Salvia officinalis* and between chloramphenicol and ethyl acetate extract of *Salvia officinalis* against *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Enterobacter cloacae,* and *Klebsiella pneumoniae*. But the same study excluded that alcoholic plant extract of *Salvia officinalis* can initiate the activity of antibiotics against the resistant bacteria, such as *Pseudomonas aeruginosa*, when used in combination with antibiotics [23], however

this study's result which showed that alcoholic leaves extract *Salvia officinalis* has synergetic effects when used with antibiotics such as (Ciprofloxacin, Cefotaxime, Ticarcillin + Clavulanic Acid).

Table 4-The synergetic effects of alcoholic leaves extract of *Salvia officinalis* with antibiotic discs against MDR *Pseudomonas aeruginosa*:

Antibiotics discs	The diameter of inhibition zone of antibiotic discs without *S. officinalis*	The diameter of inhibition zone of antibiotic discs with *S. officinalis*
Ciprofloxacin	**28mm**	**30mm**
Cefoxtaxime	**25mm**	**33mm**
Ticarcillin+Clavulanic Acid	**27mm**	**31mm**

In this study, we can conclude that alcoholic leaves extract of *S. officinalis* had antimicrobial effects on MDR *P. aeruginosa* that were similar to those caused by antibiotics. Therefore, it is important to replace antimicrobial therapy (antibiotics) by treatment with plant extracts or pharmaceutical preparations. The latter can cause an effective elimination of MDR *P. aeruginosa*, whereas the repeated use of antibiotics stimulates the emergence of new strains of resistant pathogenic bacteria. The side effects of plants are less than those of chemotherapeutic agents, especially when taking into account the proof that concentrations less than 300 µg/ml of *S. officinalis* leaves extract had not cause injury on the cell membrane solidity and maintenance [24].

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