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Assessment of Antimicrobial and Antibiofilm Effect of *Salvia officinalis* Extract Against Multidrug Resistant Pathogenic *Escherichia coli*

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Abstract:

Fifty-five *Escherichia coli* isolates were obtained from urinary tract infections using classical methods and the VITEK system. Antibiotic sensitivity testing revealed that 33 (60%) of the pathogenic *E. coli* isolates were classified as multidrug-resistant (MDR). The study found that the majority of the isolates revealed resistance to ampicillin and ceftazidime, and their resistance ratios were 94.54% and 72.72%, respectively. In contrast, the most effective antibiotics were ertapenem, imipenem and tigecycline, and their resistance ratios were 5.45%, 9.09% and 10.9%, respectively. The aim of the current study is to assess the antimicrobial and antibiofilm formation activity of *Salvia officinalis* extract against MDR pathogenic *Escherichia coli*. All 33 isolates of MDR pathogenic *E. coli* displayed biofilm formation ability extended from moderate to strong. Ethanol extract of *Salvia officinalis* plant leaves revealed antibacterial activity against all MDR *E. coli* isolates at the concentration of 100 mg/ml, 200 mg/ml, and higher than these concentrations. Additionally, *Salvia officinalis* extract also exhibited antibiofilm formation activity against all MDR pathogenic isolates at the concentration of 50 mg/ml.

Keywords: Pathogenic *E. coli*, Multidrug resistant, *Salvia officinalis*, Biofilm, UTI

تقييم تأثير الضد ميكروبي وضد الغشاء الحيوي لمستخلص المريمية ضد الايشريكية القولونية الممرضة المتعددة المقاومة للمضادات الحيوية

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الخلاصة:

تم الحصول على خمسة وخمسين عزلة من الإشريكية القولونية من التهابات المسالك البولية باستخدام الطرق الكلاسيكية ونظام الفاتك. كشف اختبار حساسية المضادات الحيوية ان 33 (60%) من عزلات الإشريكية القولونية المسببة للأمراض تم تصنيفها على انها مقاومة للأدوية المتعددة (MDR). وجدت الدراسة ان غالبية العزلات أظهرت مقاومة للامبيسلين والسيفتازيديم، وكانت نسب مقاومتها 94.54% و72.72% على التوال. في المقابل، كانت المضادات الحيوية الأكثر فعالية هي الارتابينيم والايميبينيم والتيجيسيكلين، وكانت نسب مقاومتها 5.45% و9.09% و10.9% على التوالي. الهدف من الدراسة الحالية هو تقييم الفعالية الضد ميكروبية وضد الغشاء الحيوي لمستخلص المريمية ضد الإشريكية القولونية الممرضة المقاومة للأدوية المتعددة.

أظهرت جميع عزلات الإشريكية القولونية المسببة للأمراض المقاومة للأدوية المتعددة البالغ عددها 33 عزلة قدرة على تكوين الأغشية الحيوية تتراوح من المتوسطة الى القوية. أظهر مستخلص الإيثانول لأوراق نبات المريمية نشاطاً مضاداً للبكتيريا ضد جميع عزلات الإشريكية القولونية المقاومة للأدوية المتعددة بتركيز 100 ملغم/مل و200 ملغم/مل واعلى من هذه التراكيز. بالإضافة الى ذلك، أظهر مستخلص المريمية ايضاً فعالية ضد تكوين الغشاء الحيوي ضد جميع العزلات الممرضة المقاومة للأدوية المتعددة بتركيز 50 ملغم/مل.

1. Introduction

Urinary tract infections (UTIs) are a significant public health concern, and are most commonly caused by uropathogenic *Escherichia coli* (UPEC) in both simple and complex cases. UPEC is the primary bacterial agent responsible for UTIs, posing a substantial threat to human health. Uropathogenic *E. coli* (UPEC) have many virulence factors that enable this bacterium from colonization and causing UTIs, such as adhesion and invasions [1, 2]. Pregnant women experience various physiological changes, particularly to the urinary system, which increases their susceptibility to UTIs [3]. *Escherichia coli* is classified as Multidrug Resistant due to its numerous resistance mechanisms, such as the enzyme β-lactamase, which increases resistance to β-lactam antibiotics [4]. The incidence of infection varies across different environments and individuals based on factors such as geography and health status. A considerable proportion of UTIs may be attributed to closely related UPEC groups, infection prevalence in women is greater than men [5, 6]. *Salvia officinalis* extract proven effective as an antibacterial and antifungal, *Salvia officinalis* extract contain phenolic acids that have antimicrobial activity against many pathogens, especially against *Pseudomonas aeruginosa* and *Staphylococcus aureus* [7, 8]. The aim of the current study is to assess the antimicrobial and antibiofilm formation activity of *Salvia officinalis* extract against MDR pathogenic *Escherichia coli*.

2. Materials and Methods

2.1 Isolation of E. coli

Specimens from UTIs, were collected under sterile conditions from several hospitals of Karbala, totaling 130 samples. In macroscopic examination, the samples were immediately streaked onto on MacConkey and Eosin methylene blue (EMB) agar, then incubated at 37 °C and for 24 hours. Pink colonies were selected and re-cultured on new sterile MacConkey and EMB agar. Single colonies were then chosen for diagnosis tests such as biochemical, morphological, oxidase, catalase, and then microscopic examination by Gram staining. Diagnostic results confirmed by Vitek 2 compact system test [9].

This study was approved by the Ethical Committee, University of Al-Zahraa for Women, Karbala, under the number 1 in November 13, 2024

2. 2 Susceptibility test

The sensitivity test was conducted using VITEK 2 compact system with antibiotic sensitivity card that includes 16 antibiotics and test for the presence of extended-spectrum beta-lactamase (ESBL) enzyme which responsible for resistance to many beta-lactam antibiotics (penicillins, cephalosporins, carbapenems, or monobactams) [10].

2.3. Biofilm formation assay

The *E. coli* isolates were tested for their ability to produce biofilm by using Microtiter plate method.

- All the isolates were cultured for 24 hours at 37°C in Muller Hinton broth (MHB). Following this, two mL of normal saline were added to a tube with 100 microliters of bacteria, the turbidity was visually using a turbidity meter, which should fall between 0.5-0.63.

- A sterile flat bottom 96-well polystyrene microtiter plate method was filled with 180 µl of MHB containing 1% glucose.
- Three wells of micro-titer plate were filled with 20 µl of bacterial suspension (turbidity should be between 0.5-0.63), negative control was MHB only.
- After 24 h from incubation in 37 °C, distilled water was used to wash the plate three times and then dried.
- 200 µl crystal violet dye was applied to the plates and left for 15 minutes in room temperature, then washed and dried for 30 minutes in 37°C.
- Then add 200µl ethanol 96% to wells for 15 min to extraction of the stained adherent.
- Microtiter plate method reader used at 630 nm to read optical density (OD).

Results were divided into four categories according to their optical densities as show in table 1 [11].

Table 1: Biofilms categories according to optical density

Optical Densities	Results
OD ≤ ODc	Non producers
ODc < OD ≤ 2×ODc	Weak producers
2×ODc < OD ≤ 4×ODc	Moderate producers
4×ODc <OD	Strong producers

2.4. Preparation of leaves extract

Leaves from *S. officinalis* plant were collected from local plant stores in Kerbala. Initially, in the first, the leaves were washed multiple times with water to remove dust and any other particles, then dried in an air oven at 60 °C for three days. One, the air-dried, the leaves were grounded in the blender and 10 gm of the plant powder was extracted with 200 ml aqueous ethanol (80%) using Soxhlet for 8 hours. The extract was then dried using a rotary evaporator at 60 °C and the resulting powder was kept in closed glass container in refrigerator until use [12].

2.5. Minimum inhibitory concentration of Salvia officinalis

The antibacterial activity of *S. officinalis* leaf extract was assessed using the macro-dilution method as recommended by CLSI [13]. Two-fold serial dilutions were prepared with Muller Hinton broth with final concentrations 800, 400, 200, 100, 50, 25, 12.5, and 6.25 mg/ml of the leaf extract.

A fresh bacterial suspension of *Escherichia coli* was prepared, ensuring the turbidity measured between 0.5 and 0.63 (about $1.5*10^6$ CFU/ml), and 10 µl this suspension was inoculated into each dilution across all eight test tubes. The tubes were then incubated for 24 hours in 37°C. After incubation, turbidity was examined and the lowest concentration of *S. officinalis* leaves extract that inhibit the bacterial visible growth was considered as minimum inhibitory concentration (MIC). Two tubes were prepared as negative control (only MHB) and positive control (MHB and 10µl ($1.5*10^6$ CFU/ml) bacteria inoculum) [14].

2.6. Antibiofilm activity of Salvia officinalis

The assay was performed in micro-titer plate as described by [15], with some modification. Fifty microliters of MHB containing 1 % glucose were added to each well of the microtiter plate, followed by the addition of 50µl of an alcoholic leaves extract (100 mg/ml concentration) in MHB was added to wells and 10µl from bacterial inoculum was added. Negative control was MHB only. After incubating at 37 °C for 24 h from incubation, distilled water was used to wash the plate three times and then dried. Two hundred microliters of crystal violet dye were

applied to the plates and left for 15 minutes in room temperature, then washed and dried for 30 minutes in 37°C. Subsequently, 200 µl of 96% ethanol was added to each well for 15 minutes to extract the stained adherent material.

A second microtiter plate was prepared and inoculated with the same method but without treated with alcoholic leaves extract, used to comparison the results for each isolate before and after processing with alcoholic leaves extract to identify antibiofilm like activity of alcoholic leaves extract to prevention biofilm formation by *E. coli*. MTP reader used at 630 nm to read optical density (OD). Results were determined according to the absorbance of media control [11]. The percentage reduction in biofilm formation was measured using the following formula [15]:

$$\% \text{ Reduction} = \frac{\text{Control OD} - \text{Test Sample OD}}{\text{Control OD}} \times 100$$

3. Results and Discussion

3.1 Isolation and Identification of Escherichia coli

Fifty-five uropathogenic *E. coli* isolates were obtained, with a higher number of isolates from female (n= 31) compared to male (n=24). This difference is attributed to the anatomical and physiological characteristics of female that make it more susceptible to infection with UTI than male. These findings agreed with study by Joya *et al*., [16], which reported that the number of *E. coli* isolates from UTI in female more than male, and also with many studies [17,18].

3.2 Susceptibility test

The sensitivity test conducted using VITKE system demonstrated variations in the resistance of *E. coli* isolates to 16 antibiotics (Figure 1), and confirmed the presence of the extended-spectrum beta-lactamase enzyme in uropathogenic *E. coli*.

High resistance ratio was observed against ampicillin and ceftazidime, with resistance ratios of 94.54% and 72.72%, respectively. Ertapenem, imipenem, and tigecycline showed lowest resistance ratio were 5.45%, 9.09% and 10.9%, respectively. Specifically, 52 isolates (94.54%) were resistant to ampicillin, 40 (72.72%) isolates to ceftazidime, 37 (67.27%) isolates to ceftriaxone, 33 (60%) isolates to cefepime, 5 (9.09%) isolates to imipenem, 7 (12.72%) isolates to amikacin, 11 (20%) isolates to gentamicin, 14 (25.45%) isolates to ciprofloxacin, and 9 (16.36%) isolates to levofloxacin. The results of resent study for these antibiotics were in agreement with many studies [16,19-21].

Twenty-four (43.63%) and 18 (32.72%) isolates were resistant to piperacillin/tazobactam and nitrofurantoin, respectively. These results contrast with the findings of Kulkarni *et al*., [19], who reported that the resistance ratios to piperacillin/tazobactam and nitrofurantoin were 19.24% and 7.59%, respectively. However, they agreed with the study by Al-Hasnawy *et al*., [20] which found that 44.8% of isolates were resistant to nitrofurantoin, and agreed with another local study by Ahmed and Nsaif [22] that revealed 40% from isolates were resistant to piperacillin/tazobactam.

In this study, *E. coli* isolates exhibited varying resistance rates to cefazolin, ertapenem, and trimethoprim/Sulfamethoxazole were 22 (40%), 3 (5.45%), and 31 (56.36%) isolates, respectively. These results are consistent with findings from a local study by Naqid *et al*., [23]. Thirty-three isolates (60%) resistant to cefoxitin antibiotic and only six isolates (10.9%) resistant to tigecycline, that agreed with the results of Ahmed and Nsaif [22] and Musa *et al*., [24], who reported resistance rate of 63.3% and 10% from isolates were resistant to these antibiotics, respectively.

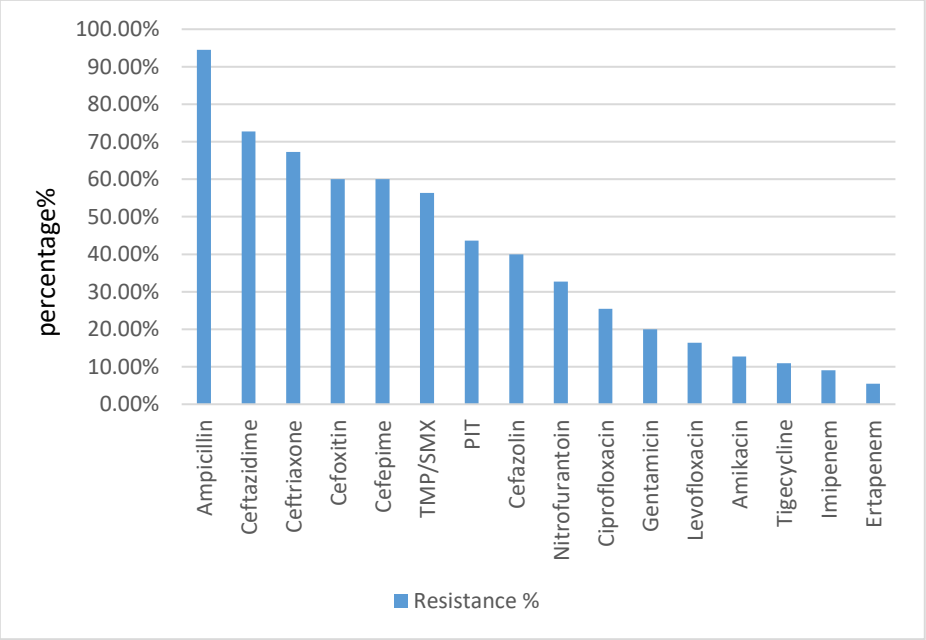

Figure 1: Antibiotics sensitivity test of *E. coli* isolates.

Escherichia coli produce extended spectrum β-lactamase which enables this bacterium to resistant different types of antibiotics such as ceftazidime, ampicillin, cefotaxime, and ceftriaxone. This enzyme contributes to the multidrug resistant isolates, complicating the infection and making treatment more challenging. The ESBL producing *E. coli* responsible for high death rates [25, 26, 27]. In this study, thirty-three isolates (60%) were identified as ESBL producers, while a study by Khan *et al*., [21] revealed 57% of isolates from UTI were ESBL producers. Thirty-three (60%) isolates were identified as Multidrug Resistant (MDR) *Escherichia coli* based on the results antibiotics sensitivity test to 16 antibiotics. Several studied have shown an increase in the MDR ratio of *E. coli* especially that isolated from UTI samples. For instance, a study by Khan *et al*., [21] found that 84% of isolates were MDR, while another study by Al-Hasnawy *et al*., [20] was showed 88.09% of isolates were MDR.

3.3. Biofilm formation capacity of Uropathogenic E. coli

Fifty-two isolates (94.55%) of all UPEC isolates showed biofilm formation capacity by MTP method and 3 (5.45%) were non-producer. This high percentage was distributed across three groups: 12 (21.82%) weak isolates, 29 (52.73%) moderate isolates, and 11 (20%) strong isolates (Figure 2). All these findings were in agreement with many study that showed high percent from biofilm formation ability in *E. coli* isolates especially, that isolates from UTIs, making it more resistant to wide range of antibiotics and complicates infection and their treatment, this explains why the most isolates from UTIs were MDR [28,29,30,31]. Notably, all the MDR isolates in this study were biofilm-former, moderate (n= 22) and strong (n=11).

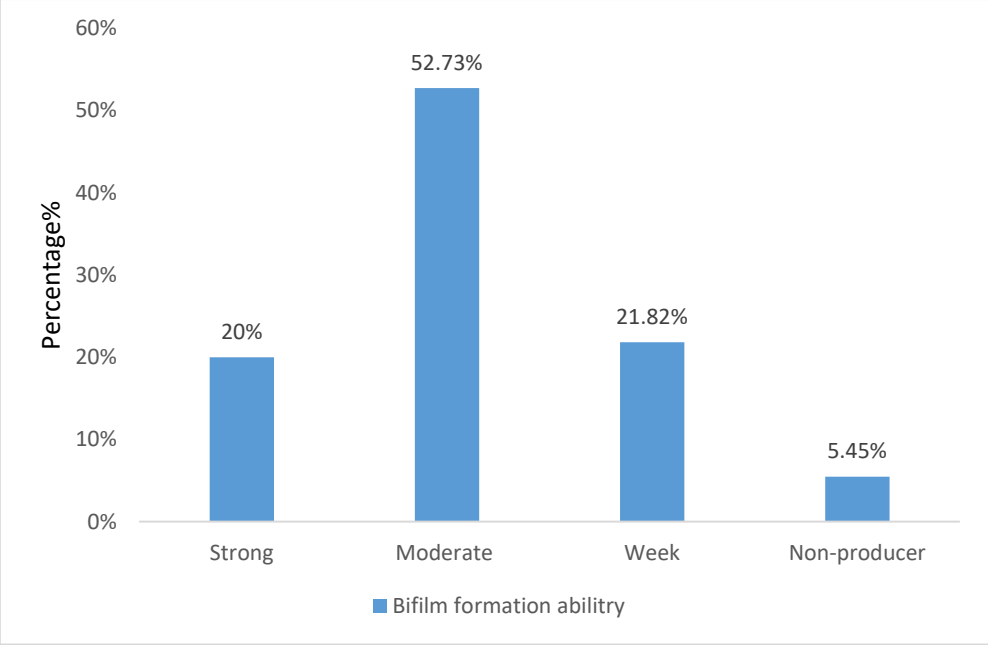

Figure 2: Biofilm formation ability in Uropathogenic *E. coli*

3.4 Minimum inhibitory concentration and antibiofilm activity of Salvia officinalis extract

The extract *of Salvia officinalis* leaves demonstrated antibacterial activity at the concentrations of 100 and 200 mg/ml and higher than these concentrations, while 50 mg/ml and below showed no inhibitory effect against all MDR Uropathogenic *E. coli* isolates. The antibiofilm activity of this plant leaves extract was assessed by MTP method at concentration of 50 mg/ml (equal to subMIC) against all MDR isolates, this extract led to high reduction percentage in biofilm formation of all MDR isolates table 2, when all these isolates become non-producers to biofilm after processed with this extract.

Table 2: Reduction percentage of biofilm

Reduction percentage rate %	(50-59)%	(60-69)%	(70-79)%	(80-89)%
No. of isolates	2	13	10	8

These findings are consistent with several studies that demonstrated that the *S. officinalis* extract have antibacterial and antibiofilm activities against wide range of bacteria such as *E. coli*, *P. aeruginosa, Bacillus subtilis, Enterococcus faecalis, S. mutans* and *Staphylococcus aureus* [32-38]. Selim *et al*., showed antimicrobial properties of *S. officinalis* extract against MDR bacterial isolates [39]. Many of the medicine plant have different compounds which responsible for antibacterial and antibiofilm activity such as chlorogenic acid, rosmarinic acid, flavonoids and rutin in *S. officinalis* [40, 41].

Chlorogenic acid in *S. officinalis* with potent biological activity. This phenolic compound exhibit antibacterial activity against *E. coli*, by disrupting the integrity of the cell wall and membrane because of the high polarity and affinity of chlorogenic acid for lipids, led to binds effectively to the surface of Gram-negative bacteria, and alterations in the membrane structure. This process leads to an increase in membrane permeability, resulting in the leakage of cellular contents [42]. Rutin, very important compound in *S. officinalis* due to their antibacterial and antifungal activity and for use in the drug delivery system [43]. Terpenes are a key class of compounds found in many medicinal plants, including *Salvia officinalis*, and are responsible for their antibacterial properties [44, 45]. The presence of notable amounts of terpenes in *S. officinalis* is thought to contribute to its antibacterial activity.

In the same manner, polar compounds in *S. officinalis* such as alcohols and phenols were excellent active ingredients against many bacterial species [46]. Cineol (1,8-cineol) one of the antibacterial active compounds in *S. officinalis* [46], against different bacterial species especially *E. coli* [47, 48]. Manool is one of the key compounds that enables *S. officinalis* to resist and eliminate microbes [49],

Flavonoids derived possess antibacterial activity against bacterial biofilms, because these flavonoids were found to reduce the metabolic activity of the biofilm by 28% and 29%, that indicate their potential in combating biofilm-related infections [50]. Carvacrol and thymol were very important components that present in *S. officinalis* extract [46], responsible for the efflux pumps inhibition, prevent the biofilm formation and disruption of preformed biofilms, inhibition of bacterial motility and membrane ATPases, and the disruption of bacterial membrane that leads to bacterial lysis and leakage of intracellular contents resulting in death. Thus, when carvacrol interacts with the lipid bilayer and penetrate the fatty acid chains, led to expand and destabilization of the cytoplasmic membrane [51, 52].

Camphor, 1,8-cineole, and α-pinene all these compounds found in high concentration in *S. officinalis* extract. All these compounds were reported with antibacterial and anti-biofilm activity [39, 53].

Conclusion

We can conclude that the *S. officinalis* ethanoic leaves extract had antimicrobial and anti-biofilm activity against MDR uropathogenic *E. coli*. High resistance ratio was achieved by *E. coli* against ampicillin and ceftazidime. While the most effective antibiotics were ertapenem, imipenem and tigecycline. Uropathogenic *E. coli* isolates have high biofilm formation capacity (weak, moderate, and strong).

"Conflict of Interest: The authors declare that they have no conflicts of interest."

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