Research article

Antibacterial effect of ethanolic fraction of *Medicago sativa* extract on *Escherichia coli* in urinary tract infection

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ABSTRACT

**Introduction and Aim:** Uropathogenic *E. coli* infections of the urinary system have grown to be a serious global public health issue. *Medicago Sativa* has been reported to have antibacterial effects against a variety of harmful microorganisms. Hence, the aim of this study was to investigate *M. sativa*’s potential antibacterial effectiveness against *Escherichia coli* isolated from urine samples in urinary tract infection patients visiting Medical city hospitals in Iraq/Baghdad.

**Material and Methods:** During this study, urine samples were collected from 85 patients visiting various hospitals in Iraq, from December 2021 to May 2022. A semi-quantitative culture technique and a conventional microbiological approach was used to identify *Escherichia coli* in the urine samples. *E.coli* isolates were assessed for their antibiotic susceptibility using the Kirby-Bauer Disk diffusion method. The crude *Medicago sativa* plant extract was screened for its phytochemical constituents. Various ethanol fraction concentrations (25 mg/ml, 50 mg/ml, and 75 mg/ml) were tested for their inhibitory activity of uropathogenic *E. coli*.

**Results:** Only 30 out of the 85 urine samples tested positive for uropathogenic *E. coli*. Among patients, UTI infection with *E.coli* was seen to be more prevalent in females than in males. The *Medicago sativa* extracts demonstrated considerable antibiotic activity against *E. coli* with a MIC of 25 mg/ml for ethanolic fraction.

**Conclusion:** Based on this present study we conclude that *Medicago sativa* extracts have great potential as an antimicrobial agent against uropathogenic *E. coli*.

**Keywords:** *Medicago sativa* extract; *E. coli*; Ethanolic fraction; UTIs.

INTRODUCTION

Urinary tract infections (UTIs) are among the most common infections and a significant cause of death and morbidity (1). Urinary tract infections affect an estimated 150 million people worldwide and are one of the most commonly diagnosed infections in the United States (2). Although UTIs can affect all age groups, females are more likely than males to get an infection at some point in their lifetime (3). The most frequent cause of urinary tract infections is uropathogenic *E. coli* which belongs to the family Enterobacteriaceae (4). These strains have a variety of virulence characteristics, such as adhesion, toxins, and host and avoidance mechanisms, that allow for the establishment of infection (5). For the avoidance of recurrence and complications, such as chronic renal failure and chronic pyelonephritis, it is crucial to identify and treat bacteria in the urine as soon as possible (6). The most frequent reason for prescribing antibiotics is UTIs, and misusing antibiotics results in the growth of multidrug-resistant (MDR) bacteria (7).

As more and more bacterial pathogens quickly develop rapid drug resistance, MDR pathogenic microorganisms are a developing global concern that put a significant burden on clinical care and public health. The WHO recently recognized uropathogenic *E. coli* as a critical priority pathogen (8). Right now, finding new antimicrobial medicines to inhibit the growth of bacteria is of highest importance.

Herbs have been used in traditional medicine for a long time and documented phytochemical and pharmacological investigations confirm this use. In addition, they are effective for clinical research and the creation of commercial medications (6, 7).

Medically, the perennial legume plant alfalfa belongs to the family (Leguminosae). It can reach heights of two to three feet tall with an upright, smooth stem, pinnately trifoliate leaves, racemes of purple-violet flowers, and bear seeds. Due to the presence of phytochemical components like flavonoid, alkaloid, and terpenoid, alfalfa (*Medicago sativa*) has been found to have an antibacterial impact against a variety of pathogens in antimicrobial screening (9). In this study we investigated *M. sativa*’s potential antibacterial effectiveness against *Escherichia coli* isolated from urine samples of urinary tract infection patients, visiting Medical city hospitals in Baghdad/Iraq.

MATERIALS AND METHODS

**Plant material**

Entire *Medicago sativa* (Family: *Fabaceae*) plants were obtained from Kirkuk, Iraq during November 2021. The plant was verified by the Pharmacognosy Department, College of Pharmacy, Baghdad
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University. The plants harvested were cleaned, dried at room temperature in the shade, mechanically ground, and weighed.

**Extraction and fractionation**

The coarsely powdered plant material (350 gm) was defatted using hexane for 2 hours, and allowed to dry further. To obtain the ethanolic extract, the plant dry powder was subjected to extraction using 1.5 liters of 85% ethanol in a Soxhlet apparatus for 24 hours at a temperature of 40°C. The resulting dark greenish-yellow extract was further dried using a rotary flash evaporator. The crude extracts obtained were divided into four separate fractions and suspended in solvents varying in their polarity as petroleum ether fraction (F1), chloroform fraction (F2), ethyl acetate fraction (F3), and ethanolic fraction (F4). All the four fractions were dried using anhydrous sodium sulfate (10) and preserved until further use.

**Preliminary qualitative phytochemical analysis**

Chemical analyses were carried out on crude and fractionated plant extracts to identify the active components such as alkaloids, flavonoids, terpenoids, and steroids using the methods described previously (10, 11).

**Test for alkaloids**

Alkaloids in crude extract were detected by Dragendorff's test and Mayer’s test. One or two drops of Dragendorff's reagent was added to 2 mL of alcoholic extract solution. The presence of an orange precipitate indicated the presence of alkaloids. Further, 1 to 2 drops of Mayer’s reagent was applied to 2 mL of alcoholic extract. The development of a creamy white precipitate confirmed the presence of alkaloids.

**Test for flavonoids**

Flavonoids in the extract were tested by the lead acetate test. 1 ml of a 10% lead acetate solution was added to 2 mL of the alcoholic extract. The appearance of a yellowish-white precipitate was interpreted as positive for the presence of flavonoids.

**Test for steroids**

To detect the presence of steroids, the Liebermann-Burchard test was performed. To 1 ml of the extract solution 1-2 ml of acetic anhydride, 1 ml of chloroform, and 2 drops of concentrated H2SO4 were added. A positive result was indicated by the appearance of a dark green color.

**Test for terpenoids**

To detect terpenoids Salkowski test was performed. To 2 ml of the crude extract, 2ml of chloroform and 2 ml of concentrated H2SO4 was added and blended. The presence of terpenoids was confirmed by the layer of reddish-brown coloration that formed at the interface.

**High-performance liquid chromatography (HPLC) analysis**

HPLC analysis of the ethanolic extract of *M. sativa* was performed to isolate the primary compounds. The instrument employed a nuclear C18-DB, 3 nm particle size (50 x 4.6 mm I.D) column and a linear gradient of the mobile phase consisting of solvent A (trifluoroacetic acid, 0.05%) in deionized water and solvent B (trifluoroacetic acid 0.05%, pH 2.5). The program employed a gradient from 0-100% for 15 minutes, a flow rate of 1.1 ml/min and a UV detection at 355nm. Trifluoroacetic acid and phenolic acid standards were purchased from Sigma-Aldrich (Steinheim, Germany). Each phytochemical constituent was identified by comparing retention times of analyzed sample with reference standards.

**Urine sample collection and culture**

Patients with a clinical feature of a UTI who visited an Iraqi medical city hospital were given clean, dry, sterile containers and asked for a midstream urine sample, which was then checked right away. The container was appropriately labeled with the patient's name and the collection date.

To identify the presence of bacteriuria, a semiquantitative culture method was used to culture urine samples. A loopful of the urine sample was spread plated onto MacConkey agar plates and incubated at 37°C for 24 hours. Typical *E.coli* colonies that developed were further subjected to Vitek-2 examinations to identify and confirm *E.coli*. Samples that were negative for *E. coli* were excluded from the investigation.

**Susceptibility testing of antibiotics**

The Kirby-Bauer disk diffusion method described in CLSI 2012 was used to evaluate the antibiotic susceptibility of the *E. coli* isolates toward eight different antibiotic discs: nitrofurantoin (F), levofloxacin (LEV), amikacin (AK), trimethoprim (TMP), cephalothin (KF), Meropenem (MEM), ceftriaxone (CRO) and ciprofloxacin (CIP). *E. coli* isolates that displayed resistance to equal or greater than 3 antibiotics were determined as multi-drug resistance strains.

**Evaluation of antibacterial activity**

Ethanolic fraction of *M. sativa* was investigated for its antibacterial properties against Gram-negative bacteria *E. coli* isolated from UTI patients in this study. Evaluation of antibacterial properties was done by agar well diffusion method as described previously (12). Pure *E. coli* colonies cultured separately on Muller-Hinton Agar (MHA) at 37°C for 24 hours agar was picked using a sterile loop and transferred to a test tube containing 5 ml of sterile saline (0.9% w/v) solution to obtain a bacterial suspension adjusted to McFarland 0.5 turbidity. The bacterial suspension was spread, plated onto Muller Hinton agar plates. Using a micropipette,
three different concentrations of the crude plant extract (25 mg/ml, 50 mg/ml, and 75 mg/ml) were added to wells (6 mm diameter) made in the agar plates. Meropenem was used as a standard antibiotic and a disc containing 10 mcg meropenem was aseptically placed on the same agar plate. Similarly, dimethyl sulfoxide was used as a negative control. The plates were incubated at 37°C for 24 hours following which the inhibitory zones (in mm) were measured.

Determination of the minimal inhibitory concentration (MIC)

MIC of the plant extract was determined using a 96-well microplate based on the procedure described earlier (12). Briefly, 100 µl of MHB was added to each well of each plate, followed by addition of 100 µl of plant extract (50 mg/ml). The content of each well was serially diluted. A bacterial suspension (50 µl), which was made up by mixing one milliliter of MHB with 100 µl of fresh inoculum, was aseptically poured into each well up to the seventh column. The plates were then incubated for 24 hours at 37°C, following which turbidity measured and MIC calculated.

Data analysis

The Statistical Package for Social Sciences (SPSS) version 26 was used to analyze the data. The values are expressed as mean ± standard deviation (SD) and ranges. The presentation of categorical data uses the difference (LSD) (post hoc test) and analysis of variance (ANOVA) (two-tailed) were employed to compare the continuous variables appropriately. P values less than 0.05 were regarded as significant.

RESULTS

Of the total 85 urine samples tested in this study, only 30 samples tested positive for the presence of E. coli. Among the patients tested positive for E.coli, 56.7% were in the age group of 20-39 years, with the incidence being higher in females than males (ratio of 1.5:1).

E. coli antibiotic susceptibility test

The antibiotic susceptibility pattern of E. coli isolated in this study is shown in Table 1. While all strains were sensitive to the antibiotic Meropenem, 83.3% and 80% of the strains were sensitive to the drugs amikacin and Nitrofurantoin respectively (Table 1). Similarly, highest resistance was seen for Cephalothin (100%), followed by Ceftriaxone (63.4%), Ciprofloxacin (56.7%) and Levofloxacin (56.7%).

Phytochemical screening of M. sativa extract

Phytochemical analysis of M. sativa showed the presence of alkaloid, flavonoid, terpenoid, and steroid in the crude extract (Table 2). As also seen from Table 2, Flavonoids were seen to be present only in ethyl acetate (F3) and ethanolic (F4) fractions and to be absent in the F1 (petroleum ether) and F2 (chloroform) fractions. Similarly, alkaloids, steroids and terpenoids were seen to be absent in the fractions F3 and F4 (Table 2). HPLC analysis of the ethanolic fractions revealed the presence of gallic acid, caffeic acid, salicylic acid pyrogallol, quercetin, apigenin, naringin, and myricetin (Table 3).

Table 1: Susceptibility to antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Symbol</th>
<th>No. of bacterial isolates and their percentages</th>
<th>Sensitive (%)</th>
<th>Intermediate (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meropenem</td>
<td>MEM</td>
<td>30 (100.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>AK</td>
<td>25 (83.3)</td>
<td>3 (10.0)</td>
<td>2 (6.7)</td>
<td></td>
</tr>
<tr>
<td>Ceftriazone</td>
<td>CRO</td>
<td>10 (33.3)</td>
<td>1 (3.3)</td>
<td>19 (63.4)</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>CIP</td>
<td>14 (43.3)</td>
<td>0 (0)</td>
<td>16 (56.7)</td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>LEV</td>
<td>14 (43.3)</td>
<td>0 (0)</td>
<td>16 (56.7)</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>TMP</td>
<td>16 (56.7)</td>
<td>0 (0)</td>
<td>14 (43.3)</td>
<td></td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>F</td>
<td>24 (80.0)</td>
<td>3 (10.0)</td>
<td>3 (10.0)</td>
<td></td>
</tr>
<tr>
<td>Cephalothin</td>
<td>KF</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>30 (100.0)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Phytochemical analysis of crude extract and fractions

<table>
<thead>
<tr>
<th>Crude and fractions</th>
<th>Alkaloids</th>
<th>Flavonoids</th>
<th>Steroids</th>
<th>Terpenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Petroleum ether fraction (F1)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chloroform fraction (F2)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ethyl acetate fraction (F3)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol fraction (F4)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+, - indicates the presence and absence of phytoconstituents.

Table 3: HPLC analysis of ethanolic extract of Medicago sativa

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Phytochemicals</th>
<th>Retention Time</th>
<th>Area Uv</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gallic acid</td>
<td>2.363</td>
<td>24865</td>
</tr>
<tr>
<td>2</td>
<td>Pyrogallol</td>
<td>3.04</td>
<td>25753</td>
</tr>
<tr>
<td>3</td>
<td>Caffeic acid</td>
<td>4.107</td>
<td>18919</td>
</tr>
<tr>
<td>4</td>
<td>Salicylic acid</td>
<td>5.183</td>
<td>20115</td>
</tr>
<tr>
<td>5</td>
<td>Naringin</td>
<td>6.268</td>
<td>18261</td>
</tr>
<tr>
<td>6</td>
<td>Myricetin</td>
<td>7.017</td>
<td>20899</td>
</tr>
</tbody>
</table>
**E. coli inhibitory activity of Medicago sativa ethanolic fraction**

The ethanol fraction of *Medicago sativa*’s antibacterial effectiveness was tested against *E. coli*. The mean of inhibition zones obtained for the various concentrations of the ethanol fraction as well as the antibiotic meropenem is given in Table 4. Meropenem showed a higher mean zone of inhibition (32.85 ± 2.7) as compared to the different concentrations of the ethanolic fraction studied (Fig. 1). Results of post hoc tests (LSD) which was used to find the differences for mean of inhibition zone between the different fractions revealed that the inhibition was higher and significantly (p-value 0.001) proportional to increasing concentration of the ethanolic fractions used. Representative agar plates showing the zone of inhibition is shown in Fig. 1. Our results indicated the MIC of ethanol fraction to be 25 mg/ml and ethanol fraction of concentration 12.5mg/ml and lower did not inhibit the growth of *E. coli* (data not shown).

**Table 4**: Comparison in the mean of inhibition zone between different concentrations of ethanol fraction and meropenem

<table>
<thead>
<tr>
<th>Ethanol fraction Concentration (mg/ml)</th>
<th>Mean of inhibition zone (mm)</th>
<th>F - test</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>21.96 ± 1.9</td>
<td>277.83</td>
<td>0.001</td>
</tr>
<tr>
<td>50</td>
<td>20.07 ± 1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>17.21 ± 2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td>32.85 ± 2.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1**: Sensitivity of *E. coli* to different concentrations of *Medicago sativa* ethanolic fraction. 1: Meropenem (+ve control), 2: Dimethylsulfoxide (-ve control), 3, 4, 5: 25 mg/ml, 50 mg/ml, and 75 mg/ml of the ethanolic fraction respectively.

**DISCUSSION**

Despite the fact that urinary tract infections are treatable, the multidrug resistance (MDR) organisms are increasing at an alarming rate which results in complications, treatment failure, and greater rates of death and morbidity (13). Due to the increased use of antibiotics and the development of multidrug resistant bacteria, there is an urgent need to discover alternate innovative antimicrobials (14). *Medicago sativa*, a medicinal herb has been traditionally used as a diuretic, antibacterial, anti-inflammatory, antifungal, anti-asthmatic, and in improving memory (15). In the present study, we undertook phytochemical screening of the herb *M. sativa* as well as evaluated its crude extract fractions for their potential to inhibit the growth of uropathogenic *E. coli*.

According to our findings, females are more likely than males to have a urinary tract infection caused by the bacteria *E. coli*, which is in accordance with research conducted by Okojie and Omorokpe (16). The higher prevalence of UTIs in females has been attributed to physiological and anatomical differences between the sexes (17). Additionally, females with UTI were most prevalent in the age group of 20-39 years as previously reported (16), wherein the high frequency of UTI among females was linked to their reproductive age.

Preliminary screening of the *M. sativa* plant crude extract revealed the presence of alkaloid, flavonoid, terpenoid, and steroid compounds. Similarly, HPLC analysis revealed the presence of several other phyto components (Table 3). The pathogenic *E.coli* isolated from UTI patients in this study were found to be multidrug resistant to commonly used antibiotics. However, evaluation of *M. sativa*’s crude extract and ethanolic fractions for antibacterial activity revealed that the herb has antimicrobial efficacy against uropathogenic *E. coli* (18). In recent years, several ways including chemical compounds such as plant extraction, nanoparticles or physical ways such as audible sounds and magnetic fields have been shown to be a potential source of bioactive components which are linked to antimicrobial and anti-inflammatory activity (19, 20). The antibacterial activity of the *M. sativa* extract in this study could probably be attributed to its bioactive components such as alkaloid, flavonoid, terpenoid, steroid and other phytocompounds identified for the plant in this study.

Further, an analysis of antimicrobial activity based on different concentrations (25 mg/ml, 50 mg/ml, and 75 mg/ml) of the plant ethanolic extract in inhibiting the pathogen *E. coli* demonstrated that the antimicrobial activity of the extracts increased as the concentration of each extract increased. This finding is supported by previous research that found a direct relationship between antimicrobial activity and extract concentration (21, 22). Phytochemical analysis of the *M. Sativa* ethanolic fraction showed it contains only flavonoids (Table 2). Flavonoids are well known for their strong antibacterial properties, which help to defend plants against plant diseases. They might
therefore be effective in the fight against human infections as well (23).

The presence of myristicin, a naturally occurring compound found in common herbs and spices, was identified by HPLC analysis in the ethanol fraction of *M. sativa*. Myristicin, is considered a strong antimicrobial as it exerts its known to attack the cell wall of bacteria (24).

The MIC of the *M. sativa* ethanolic fraction that inhibited *E. coli* growth was 25 mg/ml. In contrast to our findings, it was discovered that the MIC of *M. sativa* root extract against *Haemophilus influenza, Moraxella catarrhalis* and *Streptococcus pneumonia* was 125 mg/ml. The diverse bacterial strain sources or the usage of a different part of the plant could be the cause of the variations in MIC values (25).

**CONCLUSION**

The current study revealed the relevance of *Medicago sativa* extract as a substitute for the antibacterial agent to combat antibiotic-resistant bacteria that are starting to harm human health. Antibiotic drug resistance has become an emerging challenge for clinicians to overcome UTI-associated pathogens and a global economic burden. The results showed *Medicago sativa* extract has pharmacological utility in the management of uropathogenic *E. coli*–related diseases.

**CONFLICT OF INTEREST**

The authors declare no conflicts of interest.

**REFERENCES**


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