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The effect of Marrubium vulgare L extracts against urinary tract bacteria pathogens infection in Alassaba'a hospital, of west Tripoli

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ABSTRACT

Urinary tract infections (UTIs) are common infections that happen by many types of bacteria. Some of bacteria have resistance against several types of antibiotics. Many medicinal plants used as an alternative treatment around the world. *Marrubium vulgare* L has been reported to exhibit several biological properties. Thus, the aim of current study is estimating antimicrobial activities of *M. vulgare* against *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, which caused urinary tract infections, isolated from infected patients. In the results, the growth of all tested bacteria was inhibited strongly by methanol extract at 1.0% (w/v) with inhibition zones 11.00, 11.00 and 15.50 mm against *E. coli*, *k. pneumoniae* and *S. aureus* respectively. Besides, Minimum Inhibitory Concentration (MIC) values were ranged between 0.625 to 1.25 mg/mL. While, Minimum Bactericidal Concentration (MBC) values were ranging between 1.25 to 2.5 mg/mL. On the other hand, hexane extract also was inhibited the growth of *E. coli*, *k. pneumoniae* and *S. aureus* with inhibition zones 10.00, 11.00 and 12.50 mm respectively. In addition, MIC values ranging 2.5 mg/mL, while MBC values of 5 mg/mL against all bacteria. In conclusion, the methanol extract of *M. vulgare* had a strongly effects on all bacteria compared to hexane extract, thus it can be developed as anti-bacterial agent.

دراسة تأثير مستخلصات نبات الروبيا (Marrubium vulgare L) ضد البكتيريا المسببة للالتهابات المسالك البولية في مستشفى الأصابعة غرب طرابلس

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الكلمات المفتاحية:
النشاط المضاد الميكروبات
التهاب المسالك البولية
نبات الروبيا

الملخص

التهابات المسالك البولية هي عدوى شائعة تسببها أنواع عديدة من البكتيريا. تتمتع بعض البكتيريا بمقاومة أنواع عديدة من المضادات الحيوية. لذلك تستخدم العديد من النباتات الطبية كعلاج بديل في انحاء كثيرة من العالم. من بين هذه النباتات نبات الروبيا *Marrubium vulgare* L لما يمتلكه من خصائص بيولوجية متنوعة. و بالتالي، فإن الهدف من هذه الدراسة هو تقدير النشاط المضاد للبكتيريا لمستخلصات مختلفة لنبات الروبيا *M. vulgare* ضد بكتيريا *Escherichia coli* , *Klebsiella pneumoniae* و *Staphylococcus aureus* ، التي تسبب التهابات المسالك البولية ، معزولة عن المرضى المصابين بهذه الالتهابات. حيث أظهرت النتائج أنه تم تثبيط نمو جميع البكتريا المختبرة بمستخلص الميثانول لنبات الروبيا بنسبة 1.0٪ مع مناطق تثبيط 11.00 و 11.00 و 15.50 ملم ضد *E. coli* , *K. pneumoniae* و *S. aureus* على التوالي. إلى جانب ذلك، تراوحت قيم الحد الأدنى للتركيز المثبط للنمو MIC بين 0.625 إلى

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1.25 مجم / مل. بينما كانت قيم الحد الأدنى من التركيز المبيد للبكتريا MBC بين 1.25 إلى 2.5 مجم / مل. من ناحية أخرى، ثبط مستخلص الهكسان لهذا النبات نمو *E. coli* ، *K. pneumoniae* و *S. aureus* بمناطق تثبيط بلغت 10.00 و 11.00 و 12.50 ملم على التوالي. بالإضافة إلى ذلك، كانت قيم MIC 2.5 مجم / مل، بينما قيمة MBC 5مجم / مل ضد جميع البكتيريا. في الختام، لوحظ أن مستخلص الميثانول من نبات الروبيا *M. vulgare* كان اكثر تأثيرا على جميع البكتيريا مقارنة بمستخلص الهكسان، وبالتالي يمكن تطويره كعامل مضاد للبكتيريا.

Introduction

Urinary tract infections (UTIs) are a public health problem, which caused by many pathogens, but most commonly by *E. coli*, *S. aureus*, *K. pneumoniae*, and *P. mirabilis*. Increasing antimicrobial resistance among these pathogens and high recurrence rates intimidate to increase the economic burden of these infections. Many types of medicinal plants are used for their antibacterial, antifungal and antiviral properties in many parts of the world. Plants are rich in a wide variety of activity compounds, which have been found in many studies to have antimicrobial properties [1].

Marrubium vulgare is perennial herbaceous belongs to the Lamiaceae family and is known as Rubia in Libya [2]. Its Long-lived herbaceous plant. It has been used as traditional medicine to cure a variety of diseases such as dyspeptic complaints and for loss of appetite. Also, *M. vulgare* has been known as a general tonic, stomachic tonic, antipyretic and as external use, *M. vulgare* has been used as an anti-septic and healing agent [3]. Furthermore, *M. vulgare* is helpful for bronchial asthma and dry cough [4]. *M. vulgare* has also displayed antibacterial activity against many types of gram-positive combined with gram-negative bacteria [5,6].

The aim of this study was to evaluate the antimicrobial activities of the methanol and hexane extracts of *M. vulgare* against selected bacterial pathogens isolated from patients with UTIs.

2. MATERIALS AND METHODS

2.1 Plant Collection and Preparation

M. vulgare leaves were collected from forest of Shabbab in Atmaan Yefren-Libya, in March 2022. Plants were authenticated plant identity and a voucher specimen (RM961) by a botanist (Prof. Sh-hoob Elahmir) faculty of sciences, Gharyan University. Samples were cleaned with distilled water, then dried for 5 days in oven drying at 40°C. For extraction, the dried *M. vulgare* leaves were grinded into fine powder using a mixer grinder, and then the samples were stored in plastic bags, at 4°C.

2.2 Bacterial isolation, identification and preparation of Bacterial Inoculum

Clinical isolates of the following bacteria: *E. coli*, *K. pneumonia* and *S. aureus* were isolated from urine culture of patients suffered from urinary tract infections in (Alassaba'a hospital, Alassaba'a, west of Tripoli) during the years 2022. Samples were cultured on fresh media Cystine Lactose Electrolyte Deficient agar (CLED agar), and incubated for 24 h at 37C°. Samples were also sub-culture on other media including Eosin-Methylene Blue Agar (EMB), MacConkey agar and Mannitol Salt Agar (MSA) to confirmed the types of bacteria, which had different colony characteristics. In addition, some chemical tests were used to identify these bacteria such as catalase and coagulase tests**.**

Then, two to three bacterial colonies were transferred into 1 mL of nutrient broth by using a sterile wire loop and the bacterial suspension vortexed for 10 min and subsequently allowed for the growth for one day at 37C°. Then, 10 µL of the bacterial suspension was transferred into 10 mL of nutrient broth. The turbidity of inoculum was diluted to approximately 10^6 colony-forming unit/mL (CFU/mL), utilizing a standard broth microdilution [7] and inoculum quantification methods [8]. Inoculum quantification was performed by plating 20 µL of bacterial suspension on Mueller Hinton Agar (MHA) and counting the colonies formed after incubation for one day at 37C°.

2.3 Preparation of Extracts

For extraction, 10 g portions of the powdered leaf material was mixed with 150 mL of each solvent (pure hexane and absolute methanol). Each solvent mixture was put in a dark place at room temperature for 24 h. The mixtures were subsequently filtered through Whatman No 1 filter paper, the collected filtrates concentrated using vacuum rotary evaporator, to yield the crude extracts and were stored at 4°C until used. [9].

2.4 Sample Preparation for Antibacterial Assay

The stock solutions (100 mg/mL) of each solvent extracts were prepared by dissolving 10 mg of the crude extract in 100 µL dimethyl sulfoxide (DMSO). Then, 1% test stock solutions were prepared by diluting 100 µL of the stock solution in 900 µL distilled water. The solutions were placed at 4°C prior to the assay.

Plant extracts were screened for antimicrobial activity by using the disc diffusion method which described by the Clinical and Laboratory Standards Institute [10]. All bacteria were streaked on MHA plates using sterile cotton swab. Sterile filter paper discs (6 mm) (Whatman, Germany) were pre-wetted with 10 µL aliquots of the test extracts, prepared at a concentration of 10 mg/mL (1%). The discs were subsequently put on the inoculated plates at a good distance from each other. Positive control 10µg Vancomycin (VA), 300µg Streptomycin (S) and negative c (10% DMSO) control discs were put on the inoculated plate. The plates were incubated at 37 °C for 24 h. The clear zone indicates the inhibition of bacterial growth and the diameter of the zone was measured in millimetres.

The Minimal inhibitory concentration (MICs) and minimum bactericidal concentrations (MBCs) were defined as described by CLSI [10]. The MICs and MBCs of methanol and hexane extracts of *M. vulgare L* against *S. aureus*, *K. pneumoniae and E. coli* were accomplished in a 96-well microliter plate with two-fold consecutive standard stock microdilution method and bacterial concentration inoculum of approximately 10^6 CFU/mL. One hundred microliters of each methanol and hexane extracts of 1% stock solution (10 mg/mL) were mixed and diluted in two-folds with testing bacteria in nutrient broth (NB) (100 µL). Column 12 of the microtiter plate included the highest extracts concentration (500 µg/mL) whilst 3rd column comprises the lowest concentration (19.50 µg/mL). Column 2 has served as a positive growth control among all samples (only NB and inoculum) while1st column contains NB media but no inoculum and no antibacterial agent (as a negative control). Moreover, the microtiter plate was incubated aerobically at 37 °C for 24 h [11].

2.5 Statistical Analyses

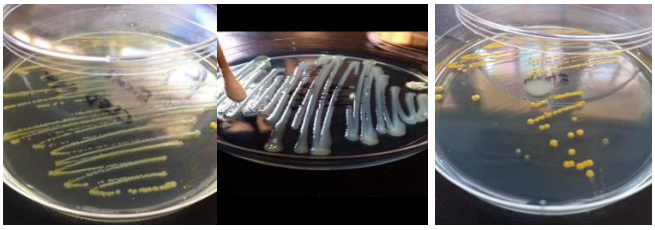
Windows Excel 2010 was intended for the analysis of antimicrobial outcome data. The results were expressed as mean ± SD of 3 replicates.

3. RESULTS

3.1 Isolated and identified the bacteria

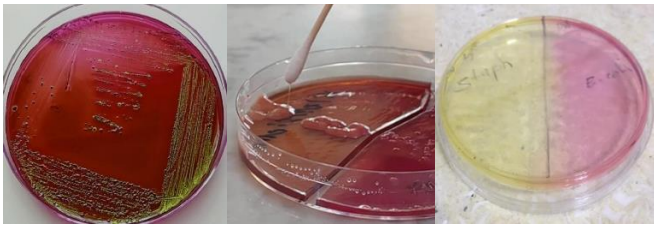
On CLED agar, the bacteria were identified based on colonial morphology as follows; *E. coli* appeared with large elevated, yellow with center more intense yellow, while *K. pneumonia* extremely mucoid colonies varying in color from yellow to whitish-blue and yellowish medium. On the other hand, *S. aureus* appeared with deep yellow colonies about 0.75 mm (Figure 1). In addition, sub-culture on other media such as Eosin-Methylene Blue Agar (EMB) used to confirmed. *E. coli*, which grow with a metallic green sheen with a dark center. Whilst, *K. pneumoniae* had large mucoid dark pink colony on MacConkey agar (Figure 2). Furthermore, *S. aureus* on Mannitol Salt Agar (MSA) displayed a yellow. In addition, some chemical tests were used to identify these bacteria such as catalase

and coagulase tests for *S. aureus*, which gave positive results of both tests. As well as, *E. coli* gave positive results of catalase test a long with *k. pneumoniae*.



E. coli ***K. pneumoniae*** ***S. aureus***

Fig 1: Isolated the bacterial tests on CLED agar



E. coli ***K. pneumoniae*** ***S. aureus***

Fig 2: Identified the bacterial tests on different types of media

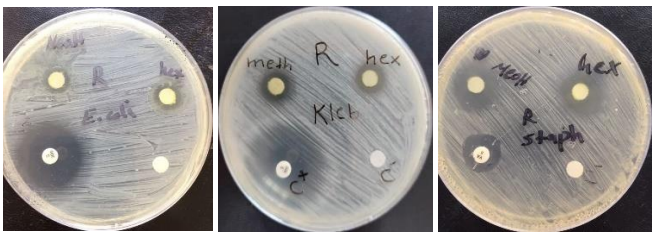
3.2 Antibacterial Activities of methanol and hexane extracts of *M. vulgare*

The antibacterial activities of the crude methanol and hexane extracts of *M. vulgare* were evaluated based on their diameter of clear zones against *E. coli*, *K. pneumonia* and *S. aureus*. The diameters of inhibition zones against all bacterial tests are given in Table 1 and Figure 3. The results show that, the methanol extract had greater effect against *E. coli* and *S. aureus* compared to hexane extract, while the both extracts were equal effect against *K. pneumonia.* However, the inhibition zone produced by Vancomycin and Streptomycin (10, 300 mg/mL) as the positive control was 18.00 mm, 17.00 and 11.00 compered to DMSO (10%), which gave negative result.

Table 1: Disc diffusion of *M. vulgare* extracts against tested bacteria.

Bacteria	Mean diameter of inhibition zone (mm)		
	Control (+)	MeOH	Hex
E. coli	18.00±0.00	11.00±0.00	10.00±1.00
K. pneumoniae	17.00±0.00	11.00±1.00	11.00±1.00
S. aureus	11.00±0.00	15.50±0.70	12.50±0.70

Positive control (Vancomycin against S. aureus and Streptomycin against E. coli and K. pneumonia), MeOH; methanol, Hex; hexane. The diameter of inhibition zones in mm (including disc). Results were expressed as means ± standard deviation.



E. coli ***K. pneumoniae*** ***S. aureus***

Fig 3: Disk diffusion test of M. vulgare MeOH, Hex extract, control positive (Vancomycin and Streptomycin) and DMSO 10% as control negative against E. coli, K. pneumoniae and S. aureus

Generally, disc diffusion test showed that, the methanol extract of *M. vulgare* observed inhibition zones of 11.00 to 15.50 mm, while hexane extract showed inhibition zones of 10.00 to 12.50 mm. However, the positive standard gave inhibition zones of 18.00 to 17.16 mm against of *E. coli* and *K. pneumonia* respectively, and 11.00 mm against *S. aureus*.

The susceptibility of *E. coli*, *K. pneumoniae* and *S. aureus* to *M. vulgare* crude extracts were also determined. The MICs values of the *M. vulgare* methanol and hexane extracts are presented in Table 2 and Figure 4 and 5. The obtained results showed that, the tested extracts had a potential for antibacterial against all evaluated bacteria. Current research demonstrated that, the results of methanol extract with MICs ranging between 0.625 and 1.25 mg/mL, while the MICs of hexane equal to 2.5 mg/mL. However, all bacterial strains showed higher MBCs of hexane extract 5.0 mg/mL compared with those of the MIC, while the methanol extract had a strong growth static (MBC) activity against all bacteria along with MBC varying between 1.25 to 2.5 mg/mL.

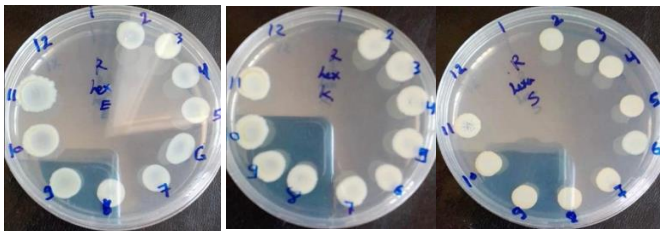
Table 02: The MIC and MBC (mg/ml) of *M. vulgare* extracts against tested bacteria.

Bacteria	MeOH		Hex	
	MIC	MBC	MIC	MBC
E. coli	0.625±0	1.25±0	2.5±0	5.0±0
K. pneumonia	1.25±0	2.5±0	2.5±0	5.0±0
S. aureus	1.25±0	2.5±0	2.5±0	5.0±0

MeOH; methanol, Hex; hexane.

E. coli ***K. pneumoniae*** ***S. aureus***

Fig 4: MBC of M. vulgare methanol extract against tested bacteria.



E. coli ***K. pneumoniae*** ***S. aureus***

Fig 5: MBC of M. vulgare Hexane extract against tested bacteria.

4. DISCUSSION

Urinary tract infection (UTIs) is one of the common infections. Recently, there was a significant increase in the incidence of antibiotic resistant bacteria causing UTIs. *E. coli* is the predominant uropathogen responsible for approximately 80% of UTIs, followed by *Staphylococcus* and *Klebsiella* [12].

The extracts of many parts of medicinal plants are used for their antimicrobial properties in several countries around the world [13]. Besides, the antibacterial mode of action of the plants is poorly understood and remains in debate [14]. In current study, *M. vulgare* was chosen, which used to treat many diseases in Libya and other countries. The methanolic extract of leaves showed higher activity compared to hexane extract against all tested bacteria. These effects might be linked to different quantity of compounds, which have a different polarity depends on the solvent. In addition, the differences in the ability of *M. vulgare* to react against pathogenic strains can be explained by the locality from the environmental effects on its chemical composition. As well as, the method of extraction the solvent used can have an effect on the antimicrobial activity of the plant extract [15].

Compared to other studies which reported the antimicrobial activities of different extracts of *M. vulgare*, Al-Snafi *et al.* (2021) [16] informed that the methanolic extract of *M. vulgare* against *E. coli* and *S. aureus* had high activity with inhibition zones16 and 20mm respectively. Khaled-Khodja *et al*. (2014) [15] also reported the 2 mg/disk methanolic extract of *M. vulgare* from Algeria inhibited the growth of *S. aureus* and *E. coli* with inhibition zones of 15.5 ± 0.7 mm, and 18.5 ± 1.04 mm, respectively. Similarly, the antimicrobial of methanol extract of *M. vulgare* with some Libyan medicinal plants have been reported against *S. aureus* with clear zone of 10.00 mm,

while no effect against *Salmonella* species and *E. coli* [17], which were less than of current study results. The phytochemical analysis of plant extracts indicates that the presence of one or more groups of phytoconstituents like flavonoids, tannins, glycoside, phenols, etc. is responsible for antibacterial activity [9]. Benzidane *et al*. (2020) [9] showed that Leaves methanol extract contains tannic acid, caffeine and ferulic acid, and gallic acid, which had the antimicrobial activities. In current results, might be linked to different quantity of compounds, which have a different polarity depends on the solvent that showed different effects on these bacteria.

On the other hand, the current study results are agreement with results of Khaled-Khodja *et al*. (2014) which showed that, the 2% of methanol extract of *M. vulgare* inhibits the growth of *E. coli* and *S. aureus* with MIC values of 0.9 and 20 mg/mL respectively [15]. Additionally, Radojević *et al*.(2013) [18] described that the 1.0 % methanolic extract of *M. vulgare* constrains the growth of *E. coli*, *S. aureus*, with MIC values of 5 and 1.25 mg/mL respectively, while the MBC values of 5 mg/mL for the both. Mostafa *et al*. (2018) [19], have reported that the difference in MIC of plant extracts is because due to the unstable nature of chemical components.

5. Conclusion

The results of current study showed that, the methanol extracts of *M. vulgare* were a very good source of antimicrobial drug against the three urinary pathogens that were tested tests.

This is particularly important in the fight against the recent resistant organisms with multiple drugs. These results suggest that future researches should be done to investigate the *in vivo* activity of this plant, toxicity and and active ingredient.

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