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 12.50 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.
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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 (Alasaba’a hospital, Alasaba’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 37°C. Samples were also sub-culture on other media including Eosin-Methylene Blue Agar (EMB), MacConkey agar and Mannitol Salt Agar (MSA) to confirm the types of bacteria, which had different colony characteristics. In addition, some chemical tests were used to identify these bacteria such as catalase and coagulate 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 37°C. Then, 10 μL of the bacterial suspension was transferred into 10 mL of nutrient broth. The turbidity of inoculum was diluted to approximately 10⁶ 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 37°C.

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 Marrubium vulgare L against S. aureus, K. pneumoniae and E. coli were accomplished in a 96-well microtiter plate with two-fold consecutive standard stock microdilution method and bacterial concentration inoculum of approximately 10⁵ 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 comprised the lowest concentration (19.50 μg/mL). Column 2 has served as a positive growth control among all samples (only NB and inoculum) whilst column contains NB media but no inoculum and no antibacterial agent (as a negative control). Moreover, the microtiter plate was incubated aerobiocally 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 colony morphology as follows; E. coli appeared with large elevated, yellow colony with a metallic green sheen with a smooth surface (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 smooth surface. 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 coagulate tests.
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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 compared to DMSO (10%), which gave negative result.

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

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Mean diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (+)</td>
</tr>
<tr>
<td>E. coli</td>
<td>18.00±0.00</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>17.00±0.00</td>
</tr>
<tr>
<td>S. aureus</td>
<td>11.00±0.00</td>
</tr>
</tbody>
</table>

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 error.

Figure 1 shows the bacterial colonies observed on the CLED agar plate, and Table 2 shows the MIC and MBC of Methanol and Hexane extracts of M. vulgare against tested bacteria.

Table 2: The MIC and MBC (mg/ml) of M. vulgare extracts against tested bacteria.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC MeOH</th>
<th>MBC MeOH</th>
<th>MIC Hex</th>
<th>MBC Hex</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>0.625±0</td>
<td>1.25±0</td>
<td>2.5±0</td>
<td>5.0±0</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>1.25±0</td>
<td>2.5±0</td>
<td>2.5±0</td>
<td>5.0±0</td>
</tr>
<tr>
<td>S. aureus</td>
<td>1.25±0</td>
<td>2.5±0</td>
<td>2.5±0</td>
<td>5.0±0</td>
</tr>
</tbody>
</table>

MeOH: methanol, Hex: hexane.

Figure 4 shows the inhibition zone of Methanol and Hexane extracts of M. vulgare 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 melatonin 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 zones 16 and 20mm respectively. Khled-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.
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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]. Benzidine 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 active ingredient.

6. Acknowledgment
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7. References