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Melissa officinalis Extract as an Antimicrobial Agent Against Clinical Bacterial Strains Isolated from Urinary Tract Infections in Najaf, Iraq

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Abstract

Background: Antibiotics are used to inhibit the growth of bacterial infections. However, antibiotic resistance is increasing due to their use. Plant extracts are natural compounds with unique medical properties. The current study investigated the effect of *Melissa officinalis* collected from Marivan, Iran, against urinary tract infection (UTI).

Methods: The plant extract was prepared using the Soxhlet method. The bacterial strains were isolated from UTI patients at Al-Zahra Hospital in Najaf, Iraq. The antibacterial activity of *M. officinalis* extract was evaluated using the Kirby-Bauer disk diffusion method. The minimal inhibitory concentration (MIC) was also determined by the microdilution method.

Results: According to the inhibition zone results, *M. officinalis* greatly affected *Staphylococcus aureus*, *Proteus vulgaris*, and *Klebsiella pneumoniae*. The plant extract showed the highest antimicrobial activity on the standard and clinical strains of *S. aureus* with the highest inhibition zone record of 8.4±0.163 and 9±0.0 mm and MIC of 0.09 and 0.06 mg/mL, respectively. Also, *M. officinalis* extract indicated an antimicrobial effect against the standard and clinical strains of *K. pneumoniae* with the highest inhibition zone record of 8±0.0 and 9±0.0 mm and MIC of 0.06 and 0.06 mg/mL, respectively.

Furthermore, the plant extract showed the most antimicrobial activity on the standard and clinical strains of *P. vulgaris*, with the highest records of 7.9±0.23 and 9±0.0 mm and MICs of 0.14 and 0.06 mg/mL, respectively.

Conclusion: *Melissa officinalis* extract can be a suitable candidate as an alternative to antibiotics for treating UTIs.

Keywords: *Melissa officinalis*, Antibacterial activity, urinary tract infection

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Introduction

Urinary tract infection (UTI) is a prevalent disease threatening human health. Various factors can cause the disease, including changes in the microbial flora of the urinary tract, bacterial penetration into the urinary tract, a variety of kidney and bladder disorders, and hormonal changes in female and male adults and children.[1]

Many bacteria, including *Staphylococcus aureus*, *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella ozaenae*, and many other pathogenic strains, can cause UTIs.[2,3]

The progression of the infection can lead to permanent kidney damage or pyelonephritis. The treatment strategy against this infection is the consumption of antibiotics such as sulfamethoxazole, trimethoprim, and nitrofurantoin. However, antibiotics can be associated with side effects or add to the global problem of bacterial resistance.[4] According to studies, many plants have a high potential for antimicrobial activity but without the side effects. Extracts of different parts of various plants can fight various bacterial and fungal infections. The results of many experiments have revealed that the antimicrobial activity of these substances is effective on multiple resistant strains.[5,6] The combination of antibiotics and herbal plant extracts has also been shown in successful studies against multi-drug resistant strains.[7,8] Research to discover new efficient extracts on antibiotic-resistant species is in progress.

Melissa officinalis belongs to the Lamiaceae family and is a source of many active compounds. This plant grows in the Mediterranean and Western Asia and is intensively cultivated in Europe. In traditional medicine, this plant is applied to remedy headaches, cancers, mental diseases, and inflammation. However, some biological activities of the extract of this plant against cancer and inflammation have been proven in previous studies.[9-11] More research is needed to study the active compounds in *M. officinalis* extract and their side effects from other perspectives. This

new strategy will require more clinical trials on human cases to investigate the side effects of treating patients. This study could be helpful in subsequent clinical trials to develop a standard herbal medicine to treat UTIs. In this study, we collected 30 strains from UTI patients from Al-Zahra Hospital in Najaf, Iraq, including *S. aureus, P. vulgaris*, and *K. pneumoniae*. Then, the antibacterial activity of *M. officinalis* collected from Marivan city, Kurdistan province, Iran, was evaluated on the strains.

Methods

Sampling

For this study, the aerial parts of *M. officinalis* were gathered during its flowering period in July 2017 from the vicinity of Rikhalan village, a mountainous region of Marivan city, Kurdistan province, Iran. The leaves were separated and dried at 25 °C. They were then powdered and stored at room temperature until they were used (Natural Essential Oil Research Institute, University of Kashan).

Plant extract preparation

After collection, the plant sample of *M. officinalis* was washed and dried at room temperature. To use the plant sample for the Soxhlet method, the completely dried plant material was powdered. Then, 50 g of the powder was mixed with 500 mL of the methanolic solvent. After performing the extraction process, the extract yield was found using the following formula:

$$Yield\,(\%) = weight\ of\ the\ residue\ obtained\ /\ weight\ of\ the\ plant\ material\ taken \times 100$$

The extract was dried under anhydrous sodium sulphate and kept at 4 °C until subsequent analysis.

Microbial sample

The bacterial strains causing the UTIs were collected. The specimens were taken from patients of different ages suspected of UTIs. The sampling area was cleaned with soap and washed with water. The first stream urine was ignored, and then the midstream urine was collected into a sterile cup that the hospital lab supplied. According to the standard method, coding and processing were done, and all samples were transferred to the laboratory for further investigation.

Three hundred sixty-five samples from patients suspected of UTI were isolated at Al-Zahra Hospital in Najaf, Iraq, for over 90 days. Thirty samples containing *P. vulgaris*, *K. pneumoniae*, and *S. aureus* were selected. The bacterial strains were isolated using the culture of urine samples on blood agar (Merck, Germany), MacConkey agar (Merck, Germany), and Mannitol salt agar (Merck, Germany) and incubated at 37 °C for 24 hours. Characteristics such as shape, size, color, odor, colony texture, lactose fermentation, mannitol fermentation, and blood hemolysis were evaluated to recognize the strains.

The next step was using a microscope for Gram staining and bacterial morphology analysis. Biochemical tests included catalase, oxidase, hydrolysis of urea, methyl red, indole, and Kilger's iron agar. API Staph and API 20 E kit (Biomerieux, France) were used to identify and determine the isolated strains. The samples with confirmed bacteria were selected: *K. pneumoniae* ($n = 10$), *P. vulgaris* ($n = 10$), and *S. aureus* ($n = 10$).

The standard microbial strains used in this research included *K. pneumoniae* (ATCC 10031), *P. vulgaris* (PTCC 1182), and *S. aureus* (ATCC 29737). These strains were provided by the Iranian Research Organization for Science and Technology (IROST).

The antimicrobial assays

The antibacterial effect of *M. officinalis* extract against isolated clinical and standard strains was assessed using the Kirby-Bauer disk diffusion method.[12] The extract solution with a 30 mg/mL concentration was prepared by adding dimethyl sulfoxide (DMSO) (Merck, Germany) and using a 0.45 µm sterilized Millipore filter. The discs were saturated with 10 µl of the extract solution and DMSO. The sterile discs with a diameter of 6 mm were used and contained 300 µg/disc of extract solution and DSMO as a negative control. The antibiotics used for the positive control of the test were nitrofurantoin at 300 µg/disc (Acino, Switzerland) and ciprofloxacin at a concentration of 5 µg/disc (Sanavita, Germany). The 0.5 McFarland microbial suspension was prepared by transferring a single colony from each microbial sample to a test tube containing 3 ml of normal saline.

After inoculating Muller Hinton agar medium with a swab impregnated with 100 µL of the suspension, the discs were placed on the plate following incubation for 24 hours at 37 °C. Susceptibility was evaluated by measuring bacterial growth inhibition zones on the agar plate surface. The average zones of inhibition were measured. The zone inhibition results of the samples were compared to the zone inhibition of the antibiotics.

Minimal inhibitory concentration (MIC) and minimum inhibitory concentration (MIC)

The dilution series was performed from 0.062 to 4 mg/mL of the extract solution containing 10% DMSO to evaluate the minimal inhibitory concentration (MIC) and MBC values. For this purpose, 100 µL of each serial was added into each well with 95 µl of brain heart infusion (BHI) (Merck, Germany) and 5 µL of bacterial suspension adjusted to 0.5 McFarland. The final volume in each well was 200 µL. ciprofloxacin (Sanavita, Germany) and nitrofurantoin (Asino, Switzerland) were used for positive control. The MIC value is the lowest concentration required to inhibit the growth of microorganisms. The results were interpreted based on CLSI criteria. Then, 5 µl of the sample from the clear wells were inoculated on nutrient agar and incubated at 37 °C for 24 hours.[13]

MBC was evaluated based on the results from incubated nutrient agar plates inoculated with samples from the wells showing growth inhibition. The MBC was estimated as the least concentrated extract and antibiotics where no visible bacterial growth was determined. All tests were repeated two times.

Results

In the present study, 365 samples were collected from patients suspected of UTIs at Al-Zahra Hospital in Najaf, Iraq. The microscopic urine precipitates analyzed showed bacteria in 187 of the total collected samples (51.2%) through the presence of polymorph nuclear cells (pus cells).[14]

These results should be considered a symptom of infection (pyuria) caused by the attachment of pus cells to urinary infections.[15] The infected samples included 143 (76.5%) females and 44 (23.5%) males with positive urine culture (significant bacteria), which is compatible with Fünfstück et al study.[16] The results can be related to various factors, such as the proximity of the urethra to the anus in females.[16] Table 1 shows the higher rate of UTIs in females than males.

Ten samples of each isolated bacterial strains including *K. pneumoniae*, *P. vulgaris*, and *S. aureus*, were selected from the UTIs for the current study.

The antimicrobial effect of *M. officinalis* extract, ciprofloxacin, and nitrofurantoin was screened against the pathogenic and standard bacterial strains. The range of inhibition zones of the methanolic extract was expressed as 7.9 ± 0.23 to 9 ± 0.0 mm. The maximum inhibitory zones of the methanolic extract were displayed against *K. pneumoniae*, *S. aureus*, and *P. vulgaris* (9 ± 0.0 mm) (Figure 1a). The lowest MIC of the methanolic extract against pathogenic bacterial strains was 0.06 ± 0.00 mg/mL (Figure 2a). According to the results, *M. officinalis* extract affected *S. aureus* and *P. vulgaris* more than it did *K. pneumoniae*. For assessing the effectiveness of ciprofloxacin on *S. aureus*, antibacterial susceptibility was tested based on the Clinical and Laboratory Standards Institute guidelines:

The sensitivity test of *S. aureus* to ciprofloxacin was interpreted according to the Clinical and Laboratory Standards Institute (CLSI). The results showed inhibition zones ≥ 21 mm, 16–20 mm, and ≤ 15 mm were susceptible, intermediate, and resistant, respectively (Figure 1b). Furthermore, the MIC values ≤ 1 μg/mL, 21 μg/mL, and ≥ 41 μg/mL were susceptible, intermediate, and resistant, respectively (Figure 2b).

Table 1. Results of patients suspected of UTIs

Type of culture	Female	Male
Significant bacteria	143 (76.5%)	44 (23.5%)
Non-significant bacteria	18 (66.7%)	9 (33.3%)
Sterile	113 (74.2%)	38 (25.2%)
Total	274 (75%)	91 (25%)

Additionally, the ≤ 26 mm, 22–25 mm, and ≤ 21 mm zone inhibition of ciprofloxacin on *P. vulgaris* and *K. pneumoniae* were interpreted as susceptible, intermediate, and resistant, respectively (Figure 1b). The MIC values of 0.5 μg/mL and 1 μg/mL were intermediate and resistant, respectively (Figure 2b).

The results of nitrofurantoin against *S. aureus*, *K. pneumoniae*, and *P. vulgaris* were as follows: zone inhibition ≥ 17 mm, 15–16 mm, ≤ 14 mm (Figure 1c) were susceptible, intermediate, and resistant, and MIC value ≤ 32 μg/mL, 64 μg/mL, ≥ 128 μg/mL (Figure 2c) were susceptible, intermediate, and resistant, respectively.[13]

During the two repetitions of the tests, the lowest MIC of nitrofurantoin against *K. pneumoniae* and *S. aureus* was 16 ± 0.0 μg/mL, and this value was 32 ± 0.0 μg/mL for *P. vulgaris*. In the case of ciprofloxacin, the lowest MIC against *K. pneumoniae* and *P. vulgaris* was 0.20 ± 0.07 μg/mL, and this value was 0.16 ± 0.07 μg/mL against *S. aureus*. In both tests, the MBC results were equal to the MIC results.

The susceptibility of the strains (%) to *M. officinalis* extract and the mentioned antibiotics were calculated for each strain using the following formula:

$$X\% = (number\ of\ susceptible\ cases \div total\ number\ of\ bacteria) \times 100$$

Discussion

Due to the increased antibiotic resistance of pathogenic bacteria, antibiotic treatment strategies are not a good solution for treating UTIs. However, some antimicrobial plant compounds can inhibit the growth of these pathogens. According to studies, compounds derived from plants, such as oils or plant extracts, have potential therapeutic effects against infectious bacteria resistant to classic antibiotics. In addition, some studies show that plant compounds could work synergistically with antibiotics and enhance their effects against resistant bacteria.

In a similar effort, Al Zuhairi et al[2] examined the antimicrobial properties of *Rosmarinus officinalis* on three bacterial strains causing UTI disease, including *S. aureus*, *K. pneumoniae*, and *P. vulgaris*. The susceptibility of infectious strains isolated from patients with UTI symptoms to *R. officinalis* oil was determined using microwell dilution. The results showed that *R. officinale* oil had the highest antibacterial effect against *S. aureus*. In another study, Lagha et al[17] studied *Origanum majorana*, *Thymus zygis*, and *R. officinalis* plants. The results showed that these plants have antibacterial activity and can prevent the formation of microbial biofilms. Biofilm formation in UTI disease increases pathogens' pathogenicity and resistance to treatment.[18] This study was performed on *E. coli*, one of the most common bacterial causes

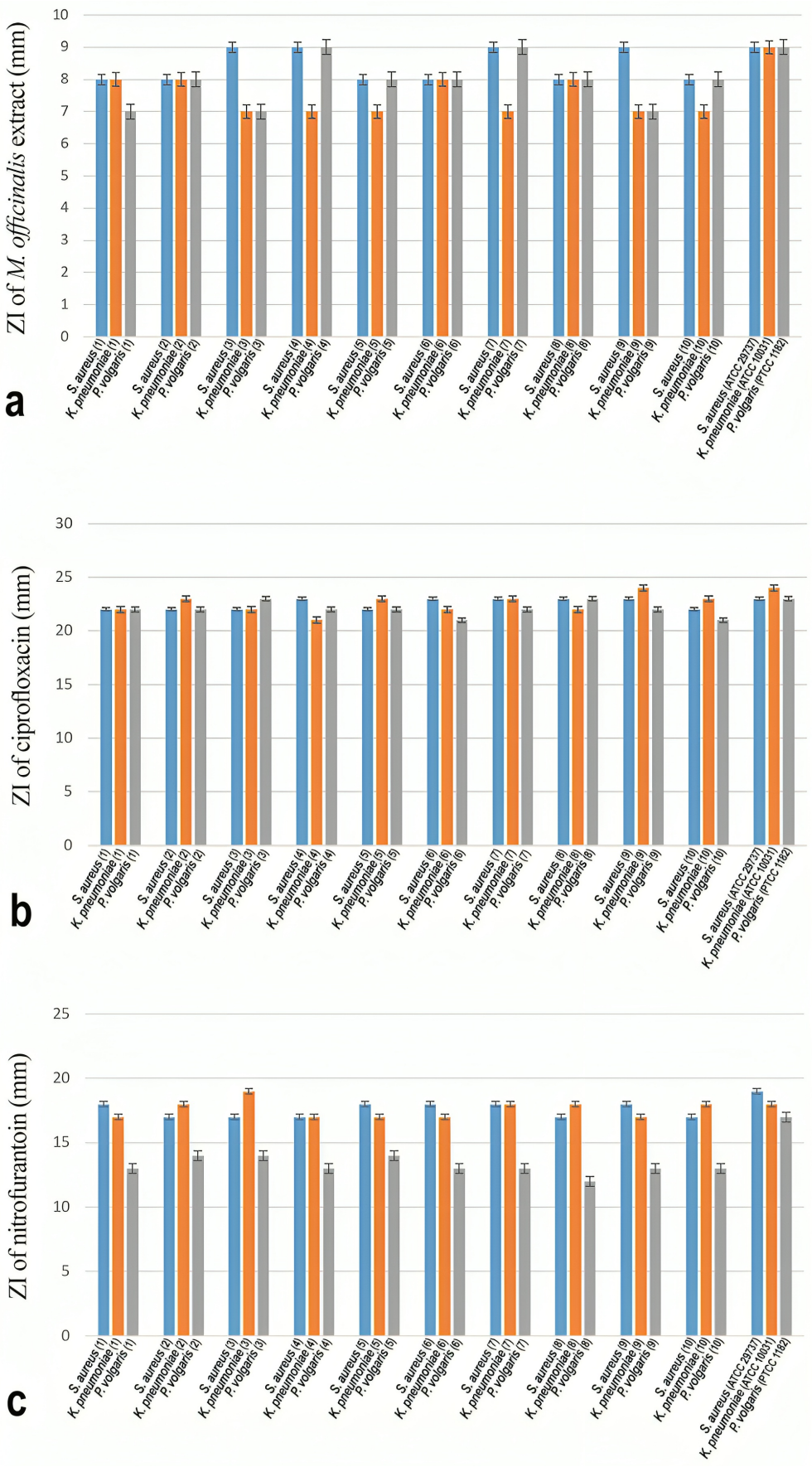

Figure 1. The effect of *M. officinalis* extract, ciprofloxacin, and nitrofurantoin on clinical strains of *S. aureus*, *K. pneumoniae*, *P. vulgaris*, and standard strains are shown in charts a, b, and c, respectively

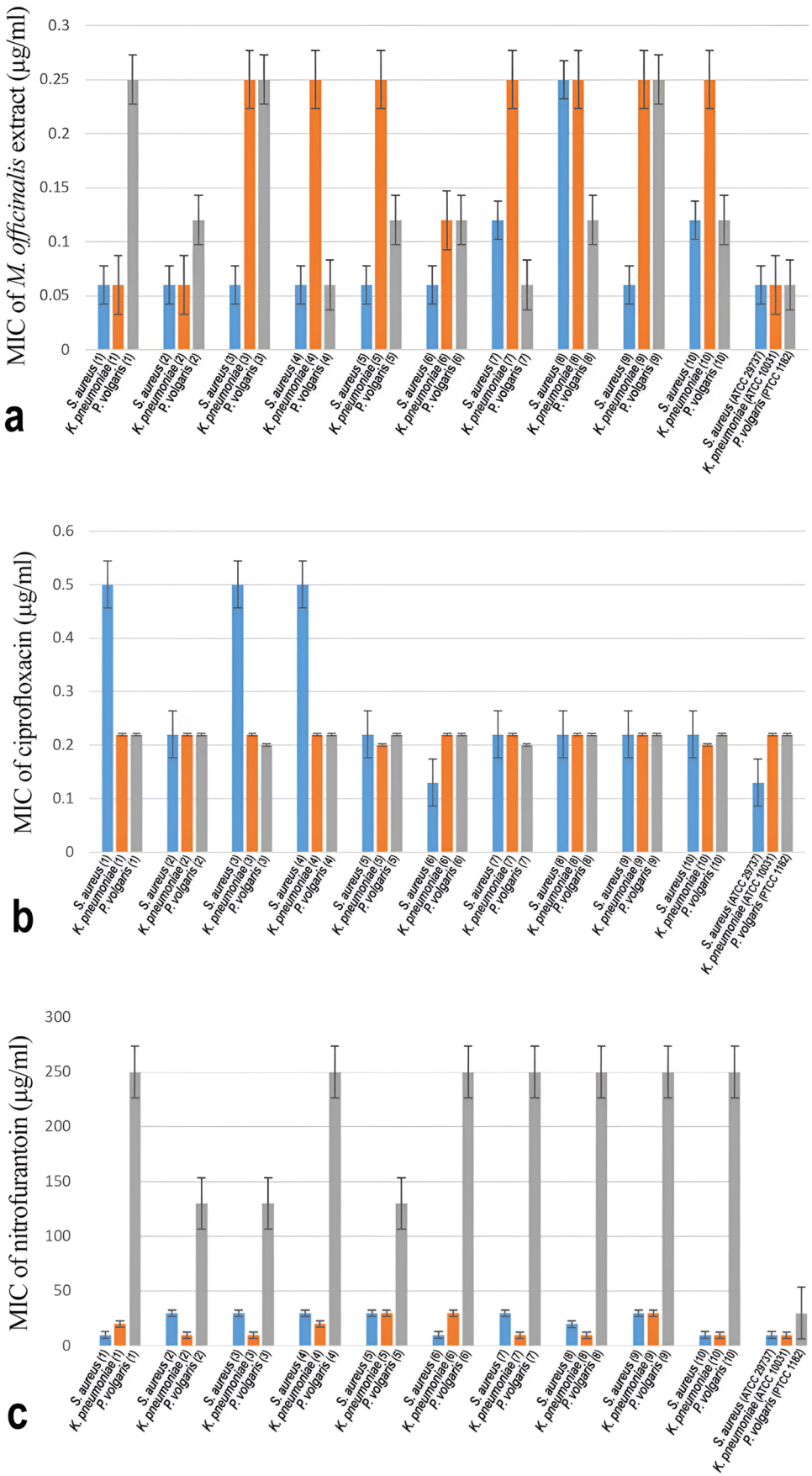

Figure 2. Minimum inhibitory concentration (MIC) of *M. officinalis* extract (part a), ciprofloxacin (part b), and nitrofurantoin (part c) are shown against clinical strains and standard reference strains of *S. aureus*, *K. pneumoniae*, and *P. vulgaris*

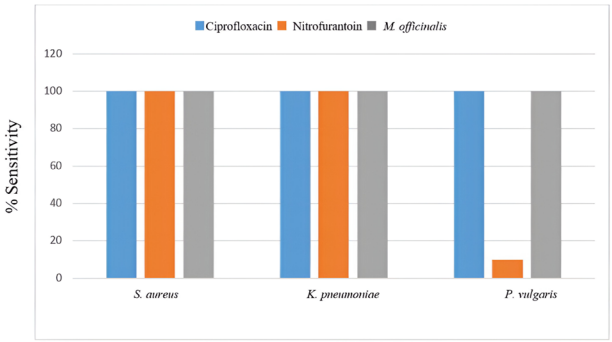

Figure 3. Percentage of susceptibility of the strains (%) to *M. officinalis* extract and the mentioned antibiotics

of UTIs. The highest antibacterial effect was reported for *T. zygis*, *O. majorana*, and *R. officinalis*. According to experiments, the effective composition of *T. zygis* contains a high percentage of linalool alcohol (39.7%), which has antibacterial properties. The most significant effect on preventing biofilm formation was reported for *R. officinalis* oil.

Previous studies on the extract of *M.* officinalis revealed the various compounds effective on bacteria and fungi.[19–21] The number of effective compounds of *M. officinalis* extract varies according to the culture area, the technique used for extraction, and the solvent, which leads to effectiveness in a wide range of bacteria.[22,23]

In another study, the essential oil of *M. officinalis* was evaluated on gram-negative and gram-positive bacteria. Four main substances were determined by GC-MS analysis from 37 components of *M. officinalis* oil. These four major compounds were citronellal (37.33%), thymol (11.96%), citral (10.10%), and caryophyllene (7.27%). The results of the disk diffusion method showed that *S. aureus* and *Listeria* monocytogenes were sensitive to the compounds. Furthermore, the lowest MIC and MBC were related to *S. aureus*. Other strains, such as *E. coli* and *Salmonella typhimurium* showed greater resistance to these antimicrobial compounds. This resistance is related to an outer membrane in gram-negative bacteria. *M. officinalis* extract is used to combat microbial growth and its subsequent effects, such as pathogenicity and food spoilage, in treatment, medicine, and the food industry.[22-24] In the current study, the antimicrobial effect of the *M. officinalis* plant extract was evaluated against bacteria isolated from patients with UTI. The *M. officinalis* sample included the plant's aerial parts, such as foliage and flowers, and was collected from the mountainous areas of Kurdistan city (Iran). The bacterial strains obtained from urine samples of patients with UTI were collected in Al-Zahra Hospital in Najaf (Iraq). *M. officinalis* extract was extracted using the Soxhlet method. Its antibacterial activity was evaluated using the Kirby-Bauer method on clinical bacterial strains. The observations showed that the methanolic extract inhibited the growth of all strains. *K. pneumoniae* and *P. vulgaris* are gram-negative bacteria with an outer membrane barrier around their cells. *S. aureus* is a gram-positive species with one bilayer membrane and thick peptidoglycan.[24,25] This differentiation in the cell envelope determines the bacteria's susceptibility to antibacterial compounds. This structure is a bilayer barrier with a relative thickness that can prevent many compounds, including antibacterial compounds, from entering the cell.[26] The interaction of polar compounds such as polyphenols and triterpenoids makes *M. officinalis* extract more effective against gram-negative strains.[19,22] Fabry et al[27] has reported that the extracts of plants with MIC values < 8 mg/mL has antibacterial activity. If the MIC values of the natural substrates are < 1 mg/mL, they should be considered antimicrobial agents. *S. aureus* and *P. vulgaris* strains were more sensitive to *M. officinalis* extract. In some cases, *K. pneumoniae* species displayed better growth in the presence of the plant extract compounds. The lowest MIC value was equal to the lowest MBC value and was 0.06 µg/mL. In the current study, nitrofurantoin and ciprofloxacin were the antibiotics used to evaluate the susceptibility of the clinical and standard strains. Unlike other strains, *P. vulgaris* displayed resistance to ciprofloxacin. However, another study by M. Rabbani et al. determined that *P. vulgaris* was sensitive to *M. officinalis* extract.[28]

The potential of *M. officinalis* extract to inhibit the growth of bacterial strains was investigated using the micro-dilution procedure. The studies showed that the *S. aureus* strain isolated from burn wound infection had the highest sensitivity to *M. officinalis* hydro-alcoholic extract. In addition, it was determined that the combination of *M. officinalis* extract with *Lawsonia inermis* powder led to an interaction that increased its antibacterial activity.

Conclusion

In this study, 51.2% of the samples from patients suspected of being infected with UTIs showed the presence of polymorph nuclear cells (pus cells) in urine in Al-Zahra Hospital in Najaf, Iraq. The antibacterial activity of the methanolic extract of *M. officinalis* collected from mountainous areas of western Iran was determined against *K. pneumoniae*, *P. vulgaris*, and *S. aureus* isolated from patients suspected of UTIs. This research determined that the methanolic extract of *M. officinalis* had growth inhibitory potential on gram-negative and gram-positive bacteria. The extract of *M. officinalis* could be a good candidate to replace the antibiotic treatment used in UTI treatment.

Authors' Contribution
Conceptualization: Fereshteh Jookar Kashi.
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Formal analysis: Jawad J Mohammed J Al Zuhairi.
Funding acquisition: Fereshteh Jookar Kashi.
Investigation: Fereshteh Jookar Kashi.
Methodology: Fereshteh Jookar Kashi.
Project administration: Fereshteh Jookar Kashi.

Resources: Fereshteh Jookar Kashi.
Software: Fatemeh Koosanjian.
Supervision: Fereshteh Jookar Kashi.
Validation: Fereshteh Jookar Kashi.
Visualization: Fereshteh Jookar Kashi.
Writing–original draft: Fatemeh Koosanjian.
Writing–review & editing: Fereshteh Jookar Kashi.

Competing Interests
None declared.

Ethical Approval
All procedures are fully compliant with applicable institutional, national, and international regulations and standards.

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