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Research article

Antibacterial Activity of *Gaultheria procumbens*, *Thymus vulgaris*, *Peganum harmala*, and Bergamot (*Citrus bergamia*) Essential Oils against *Enterococcus raffinosus* Isolated from Urinary Tract Infections

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ABSTRACT

The discovery of alternative antimicrobial agents against resistant bacteria has been a major target for microbiologists and physicians, especially in recent years, because the resistance to antibiotics has increased. No previous study has investigated the antibacterial effects of essential oils prepared from *Gaultheria procumbens, Thymus vulgaris, Peganum harmala,* and Bergamot (*Citrus bergamia*) against *Enterococcus raffinosus*. The present study aims to investigate the antibacterial activity of the essential oils of the above medicinal plants against *E. raffinosus in vitro*. In the present study, essential oils were extracted from the above four medicinal plants. For evaluating the antibacterial effect of the essential oils, three isolates of *E. raffinosus* were isolated from 44 urine samples collected from patients with urinary tract infections (UTIs). Only one isolate was multidrug-resistant (MDR) to commonly used antibiotics. The microdilution method on microtiter plates and the agar diffusion method were used to check the susceptibility of *E. raffinosus* to the essential oils. The study showed that the essential oil of *T. vulgaris* showed the highest antibacterial activity against *E. raffinosus*, followed by *P. harmala*. The present study showed a moderate antibacterial effect of *G. procumbens* essential oil against *E. raffinosus*. The present study showed no antibacterial effect of the essential oil of Bergamot against *E. raffinosus*. It can be concluded from the study that the antibacterial effect of the essential oils prepared from *G. procumbens, T. vulgaris,* and *P. harmala* against MDR-*E. raffinosus*, that is why these oils can serve as a novel antibacterial agent against *E. raffinosus* infections.

Keywords: Antibacterial effect, *Enterococcus raffinosus,* Essential oil, Urinary tract infections.

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1. INTRODUCTION

Urinary tract infections (UTIs) are the most common bacterial infections globally and a major public health concern worldwide. The bacteria responsible for UTIs and antibiotic resistance have increased over time due to the misuse of antibiotics by patients [1]. Antibiotic misuse is widespread in developing countries. This represents one of the biggest issues in the Middle East especially in Iraq [2]. Several types of bacteria are responsible for UTIs, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Enterococcus faecalis* and *E. raffinosus* [3,4]. These pathogens were isolated from clinical samples collected from various bacterial infectious diseases. They were reported to be isolated from UTIs, bacteremia, and other opp-

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ortunistic infections, particularly in hospitalized and immunocompromised patients. Their pathogenicity and susceptibility to antibiotics vary from isolate to isolate [5].

The resistance of bacteria that cause UTIs represents a challenge for physicians. Resistance of *E. raffinosus* to antibiotics, including β-lactams, has been reported by different investigators [6]. This problem encourages the search for alternative medicinal agents against highly resistant bacteria. Essential oils of medicinal plants are complex mixtures of terpenoids, phenolics, and alkaloids, many of which have antimicrobial properties [7]. *Gaultheria procumbens* essential oil is rich in methyl salicylate, which has antibacterial and anti-inflammatory activities. T*hymus vulgaris* essential oil contains high concentrations of thymol and carvacrol [8]. Both are reported previously as antibacterial agents against different bacterial species. *Peganum harmala* essential oil contains alkaloids, such as harmine and harmaline, which exhibit antimicrobial activity, particularly against Gram-positive pathogens [9]. Bergamot (*Citrus bergamia*) essential oil contains linalool and limonene, which have antimicrobial properties [10].

Several previous studies have investigated the antibacterial effects of essential oils; however, data on the antibacterial activity of Gaultheria procumbens, Thymus vulgaris, Peganum harmala, and Bergamot (Citrus bergamia) essential oils against uncommon enterococcal species, including *E. raffinosus*, remain unaddressed.

Therefore, the present study aims to evaluate the in vitro antibacterial activity of the selected essential oils against *E. raffinosus* isolated from patients with UTIs. This investigation will help address antibiotic-resistant bacteria. The high safety of medicinal plants provides strong support for these agents as the best choice for treating infectious diseases in the future.

2. MATERIALS and METHODS

2.1. Clinical samples

Morning midstream urine samples were collected under sterile conditions from 44 hospitalized patients with UTIs at two hospitals in Baghdad (Baghdad Teaching Hospital and Al-Yarmouk Teaching Hospital, Baghdad, Iraq). All patients were withheld from antibiotics for 72 h before providing urine samples. Written informed consent was obtained from each participant prior to study inclusion. The ethical standards of the Helsinki Declaration and those of the Ministry of Health, Baghdad, Iraq, were strictly adhered to during clinical sample collection. The average age of the patients was 39.4 ± 12.7 years; 29 patients were female (36.4 ± 11.8 years), and 15 were male (49.3 ± 19.2 years). All samples were collected in sterile special urine containers. The samples were immediately transferred to the laboratory for analysis.

2.2. Bacterial Isolation and Identification

Urine samples were inoculated onto different culture media, including MacConkey agar (HiMedia, Mumbai, India) and cysteine-lactose-electrolyte-deficient (CLED) agar (HiMedia, Mumbai, India). After that, the plates were incubated for 24 h at 37 °C. Bacterial cell morphology was determined by assessing the bacterial shape after Gram staining. Various biochemical tests were performed to identify the bacterial isolates, including growth in 6.5% NaCl, response to catalase reaction (negative), and growth on bile esculin medium (Hardy Diagnostics). The VITEK 2 DensiCheck instrument (bio-Mérieux, Marcy-l'Étoile, France) was used to confirm isolate identification. The bacterial isolates were subcultured onto the nutrient agar plates for short-term storage [11].

2.3. Preparing essential oil

Standard methods were used to prepare essential oils from *P. harmala, G. procumbens, T. vulgaris*, and bergamot (*C. bergamia*). The plant samples included the aerial parts of *G. procumbens* (fruit), *T. vulgaris* (flower), P. harmala (flower), and bergamot (*C. bergamia*) (fruit), purchased from a local market. The plants were cleaned, washed with tap water, dried at room temperature, and stored in a clean environment until use. The essential oils of these plants were prepared by drying the plant parts and then extracting them by steam distillation (Cleavenger) using DMSO (Dimethyl Sulfoxide). The prepared essential oils were dried at 37 ºC and kept at 4º C until use [12,13].

2.4. Antibiotics susceptibility

The Kirby–Bauer disk diffusion method was applied to identify the multidrug-resistant *E. raffinosus*. The bacterial suspension (turbidity equivalent to a 0.5 McFarland standard) was spread onto Muller-Hinton Agar (MHA, Himedia). Antibiotic disks [imipenem (IMP 10 µg), tobramycin (TOB 10 µg), tetracycline (TE 10 µg), vancomycin (VA 30 µg), levofloxacin (LEV, 5 µg), amikacin (AK 10 µg), gentamycin (GM, 10 µg), ceftriaxone (CRO 10 µg), cefepime (FEP 30 µg)] were placed on the MHA and incubated for overnight at 37 ºC. The diameters were compared with the diameters on the Clinical and Laboratory Standards Institute (CLSI) breakpoint charts to determine the sensitivity (S), intermediate (I), and resistance (R) of bacteria to imipenem [14,15].

2.5. Minimum Inhibitory Concentrations (MICs)

The microdilution method in a polystyrene microtiter plate was used to determine the MICs of *P. harmala*, *G. procumbens*, *T. vulgaris*, and bergamot (*C. bergamia*) against an MDR-*E. raffinosus* isolate. A 1/10 stock of essential oil from medicinal plants was prepared in DMSO. Two-fold serial dilutions (100 µL) were prepared in a microtiter plate using sterile Mueller–Hinton broth (MHB; HiMedia). Five microliters of the MDR-*E. raffinosus* isolate were added to each well. The bacterial inoculum was prepared by washing an overnight culture with sterile phosphate-buffered saline (PBS; 0.1 M, pH 7.2) by centrifugation at 7,000 g for 10 min (Mini Centrifuge CFG-15M; Model: CFG-15M, USA), and the optical density of the bacterial suspension was adjusted to 0.1 at 600 nm (spectrophotometer; Bioevopeak, Jinan, China). Three controls were included. Control 1: wells containing MHB and the bacterial inoculum (5 µL of Pa13) without EOs. Control 2: wells containing MHB only. Control 3: wells containing different dilutions of EOs without bacteria. The plates were incubated at 37 °C overnight. The MIC was defined as the lowest concentration of the agent that completely inhibited visible bacterial growth.

2.6. Well diffusion method

In this method, the standard inoculum of MDR-*E. raffinosus* isolate was prepared. The bacterial inoculum was prepared by washing an overnight culture with sterile PBS (0.1 M, pH 7.2) by centrifugation at 7,000 g for 10 min (Mini Centrifuge CFG-15M; Model: CFG-15M, USA), and the optical density of the bacterial suspension was adjusted to 0.1 at 600 nm (spectrophotometer; Bioevopeak, Jinan, China). The 100 microliters of standard inoculum were spread on the MHA. Six wells (6 mm) were done in the Muller-Hinton agar plates. Fifty microliters of 1/10 of the essential oil were added to the wells. The plates were incubated at 37 ºC for 2 h, then inverted at the same temperature for 18 h. The scale was used to measure the inhibition diameter. The experiment was repeated using different dilutions of essential oils.

2.7. Statistical analysis

The data from the present study are presented as mean ± standard deviation (SD). A one-way ANOVA was used to assess statistical significance. P values less than 0.05 are considered significant differences.

3. RESULTS

3.1. Bacterial Isolates

From 44 urine samples collected from hospitalised patients, three *E. raffinosus* isolates were identified and tested for antibiotic susceptibility. One isolate exhibiting resistance to the highest number of antibiotics (MDR) was selected for testing susceptibility to essential oils from four medicinal plants (*P. harmala, G. procumbens, T. vulgaris*, and *C. bergamia*).

3.2. Susceptibility to Essential Oils

The MICs were measured for four essential oils against MDR *E. raffinosus*. Fig. 1 shows that the highest MIC value ($^1/_{1280}$) was observed with *T. vulgaris* essential oil, followed by *P. harmala* essential oil. However, the current study did not show any antimicrobial effect of *C. bergamia* essential oil against *E. raffinosus*. Fig. 2 shows the diameter of the inhibition zone used to evaluate the antibacterial effects of *G. procumbens* essential oil, *T. vulgaris* essential oil, *P. harmala* essential oil, and Bergamot (*C. bergamia*) essential oil against *E. raffinosus*. Consistent with the MIC results, the largest inhibition zone diameter was observed with *T. vulgaris* essential oil ($P<0.05$) compared with the inhibition zone diameters of the other agents, followed by *P. harmala* essential oil. No inhibition zone was seen around the well that was filled with the essential oil of *C. bergamia*.

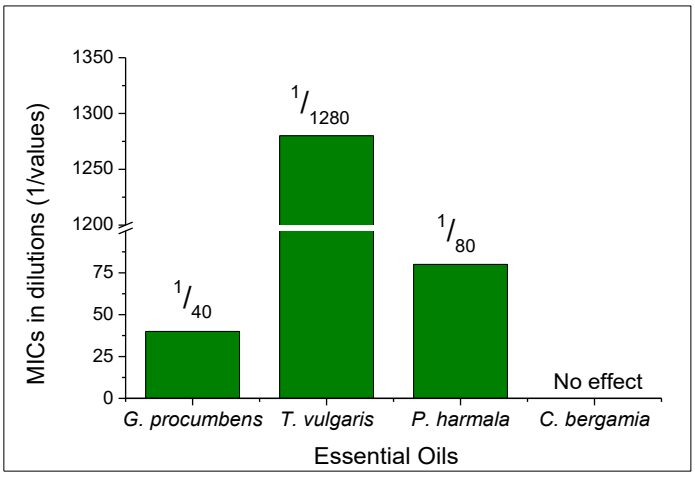

Fig. 1 Minimum inhibition concentrations (MICs) of *G. procumbens* essential oil, *T. vulgaris* essential oil, *P. harmala* essential oil, and Bergamot (*C. bergamia*) essential oil against MDR-*E. raffinosus*. The values of MICs were presented in the dilution of the stock solution of the essential oils of medicinal plants.

4. DISCUSSION

Antibiotic resistance has become a serious public health problem, particularly in developing countries. Emerging opportunistic pathogens, such as MDR-*E. raffinosus*, are associated with UTIs, especially in immunocompromised patients who are hospitalised for long periods [16]. The present study highlighted the isolation of *E. raffinosus* from urine samples and the evaluation of the antibacterial effects of essential oils from medicinal plants, including *G. procumbens*, T. vulgaris, P. harmala, and Bergamot (C. bergamia), against MDR-*E. raffinosus* isolated from urine samples. The current study demonstrated the potential of medicinal plant-derived essential oils as antibacterial agents against MDR-*E. raffinosus*. The safety of plant extracts enhances the possibility of using these agents as novel antimicrobial drugs.

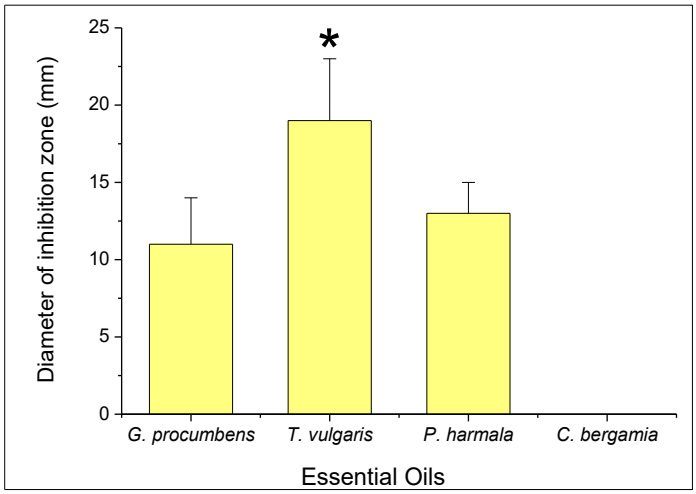

Fig. 2 Diameter of inhibition zones of the essential oils of the extractions of four medicinal plants (*G. procumbens*, *T. vulgaris*, *P. harmala*, and Bergamot (*C. bergamia*)) against MDR-*E. raffinosus*. An asterisk indicates a significant difference from the diameters of the inhibitory zone made by the *G. procumbens* essential oil and *P. harmala* essential oil.

In the present study, three isolates of *E. raffinosus* were isolated from 44 urine samples. Previous studies have shown that *E. raffinosus* is responsible for different infectious diseases [11, 16]. The isolation of MDR-*E. raffinosus* isolate indicates that this species is responsible for serious clinical problems.

The essential oil of *T. vulgaris* was an effective antibacterial agent against MDR *E. raffinosus*, supported by the lowest MIC value ($^1/_{1280}$) and the highest inhibition zone diameter (19 mm). These findings support previous studies reported the antimicrobial effects of *T. vulgaris* essential oil against pathogenic bacteria [17]. That because of containing this extract high levels of phenolic compounds, such as thymol and carvacrol. There is no previous study showed the antibacterial effect of *T. vulgaris* against *E. raffinosus*. The active compounds in the *T. vulgaris* essential oil disrupt bacterial cell membranes, increase membrane permeability, and interfere with the main bacterial cell pathways. That is leading to bacterial death [18]. The essential oil of *P. harmala* showed a moderate to low antibacterial activity compared the effectivity of *T. vulgaris* essential oil. The antibacterial effect of *P. harmala* has been reported because it has high alkaloid content, including harmine and harmaline, which exhibit antibacterial and antimicrobial activity. The findings of the present study suggest that *P. harmala* exhibits promising properties, particularly in combination therapies or as a single agent, for improving a novel antimicrobial medicine [19].

On the other hand, *G. procumbens* essential oil exhibited a moderate antibacterial effect, that shown because the low diameters of the inhibition zones was yielded compared to inhibitory zone that those formed around the wells filled with *T. vulgaris* and *P. harmala*. Earlier studies have documented the antimicrobial efficacy of *G. procumbens* against pathogenic bacteria, with effects mediated by multiple mechanisms [9]. The moderate antibacterial effect of *G. procumbens* essential oil

indicates an antibacterial secondary impact that may be less effective against MDR-*E. raffinosus*.

It was observed that *C. bergamia (bergamot)* essential oil did not show any antimicrobial activity against *E. raffinosus*. The results of the present study contrast with previous reports that showed antibacterial effects of bergamot essential oil against various pathogenic bacterial species [20]. The absence of an antibacterial effect of the essential oil of this herbal medicine may reflect intrinsic resistance mechanisms of *Enterococcus* species, alterations in essential oil composition, or inadequate concentrations of active compounds effective against *E. raffinosus*. The results of the current study highlight the central role of selected herbal essential oils as alternative antimicrobial agents, particularly for treating infections caused by MDR enterococci.

However, this study has heaps of limitations. A single MDR *E. raffinosus* isolate was involved in this study and tested against the essential oils, which may limit the generalizability of the outcomes. Furthermore, the study focused on an *in vitro* assessment of antibacterial activity; future investigations are encouraged to evaluate the in vivo safety, toxicity, and efficacy of the essential oils included in the current study. Future studies have to explore the synergistic effects of essential oils in combination with antibiotics to improve the antimicrobial efficacy and reduce antibiotic resistance to the antibiotics that are commonly used in treating bacterial infectious diseases.

5. CONCLUSION

The present study shows that the essential oils of *T. vulgaris* and *P. harmala* exhibit significant antibacterial activity against MDR-*E. raffinosus* isolated from UTIs. However, *C. bergamia (bergamot)* essential oil did not show any antimicrobial activity against *E. raffinosus*. The findings of the current study support continued investigation of medicinal plants as promising sources of alternative antimicrobial agents and provide a scientific basis for further research into their therapeutic applications against resistant enterococcal infections.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

This study was approved by the Ethics Committee of the University of Baghdad, Baghdad, Iraq (CSEC/0125/007 on January 20, 2025), since it was a retrospective analysis of routinely collected clinical data; individual patient consent was waived in accordance with national ethical guidelines and the Declaration of Helsinki.

CRediT authorship contribution statement

Seema Kumari: Roles/Writing, Writing–review, Investigation; Project administration; Roles/Writing - original draft; and Writing-review & editing.

Hwazen A. Shnyoor: Administration, Resources; Methodology, Supervision; Validation; Roles/Writing, Writing–review, Investigation; Project administration; Roles/Writing - original draft; and Writing-review & editing.

All authors have read and agreed to the published version.

Availability of data and materials

Data will be made available on request

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