Developing New Antimicrobial Therapies: as a Candidate for the Treatment of Bacterial Infection with PER-1 gene Resistance to Antibiotics

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Abstract

The emergence of multidrug resistance in bacteria due to the overuse of antibiotics has become an important health concern in recent years, which requires the development of novel alternatives to fight against. Essential oils (EOs) are secondary metabolites that have different components and chemical compositions, which may provide promising solutions to the problem of the rising number of drug-resistant bacteria. This study aims to determine the efficacy of Eucalyptus and Geranium oil against *Klebsiella pneumoniae*, antibiotic-sensitive profile, and detect the molecular of the *PER-1* gene by PCR method. In total 60 isolates of *K. pneumoniae* were collected from different hospitals in Ahvaz, (Golestan and Imam, Iran). Clevenger-type apparatus was used for oils extraction of hydro-distilled plants aerial, to determine the efficacy of essential oils. The agar well diffusion method and disc diffusion method were applied for five antibiotic discs to detect the profile of antibiotic sensitivity. Molecular detection of the *PER-1 gene* was done by PCR method. The results indicated that the oil extract of each plants inhibit the growth of *K. pneumoniae* with zones of inhibition ranging from 25 mm. *Klebsiella pneumonia* isolates were highly resistant to ceftazidime (83.3%), and more sensitive to *Eucalyptus camaldulensis* Dehnh Molecular detection of extended-spectrum β-lactamase bla PER-1 gene was recorded 12 (20%). The Geranium and Eucalyptus essential oil can be used as an alternative treatment for diseases caused by resistance to clinical isolation of *K. pneumoniae.*

Keywords: *Klebsiella pneumonia*, PER-1 gene, Essential oils

Introduction

Pneumonia causes a wide range of infections, both in the community and in a healthcare setting, leading to increased morbidity and mortality. The pathogenicity of *K. pneumoniae* is due to the presence of different virulence factors such as capsules, endotoxins, siderophores, iron-scavenging systems, and adhesions. These factors help this bacterium evade the immune system and cause various infections(Remya, et al., 2019). *K. pneumonia* has become a

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significant clinical and public health problem due to the increasing frequency of infections caused by newly discovered multidrug-resistant strains (Odari et al., 2022). *K. pneumoniae* has resistance mechanisms that include β-lactamase production, the absence of membrane porins, and active drug efflux. The majority of *K. pneumoniae* bacteria can produce the extended-spectrum β-lactamase (ESBL), which inactivates most β-lactam antibiotics and broad-spectrum cephalosporins. They also exhibit cross-resistance to a wide range of other antibiotic classes, such as aminoglycosides and fluoroquinolones(Tran et al., 2023). The most common β-lactamases detected in *K. pneumoniae* are ESBL enzymes. These enzymes can hydrolyze beta-lactam antibiotics of a broad spectrum, like penicillins, cephalosporins, and aztreonam. Infections caused by ESBL-producing isolates can increase medical costs, prolong hospital stays, and increase morbidity and mortality (Abaas et al., 2018). The initial documentation of PER-1 β-lactamase occurred in clinical isolates of *Pseudomonas aeruginosa* in France in 1993 (Jalal et al., 2023). PER-1 exhibits strong hydrolytic capabilities against cephalosporins, however, it is unable to break down antibiotics such as carbapenems and cephamycins, unlike the TEM and SHV types of ESBLs (Abaas et al., 2018). It is crucial to discover antimicrobial compounds with minimum side effects due to the rise of bacterial resistance to common antibiotics, the covert use of antimicrobial drugs by the general population, and the high incidence of sensitivity and side effects associated with chemical treatments. Hence, there is an urgent requirement to introduce novel and efficient antibiotics that possess reduce side effects, antibiotics derived naturally from plants(Shaik et al., 2014).

Eucalyptus camaldulensis Dehnh. is an indigenous tree species in Australia that is globally cultivated. It belongs to the Myrtaceae family, which encompasses approximately 800 species. Eucalyptus oil, which is secreted and stored in sub-dermal cavities, contains the plant's medicinal properties(Khalaf et al., 2020). Eucalyptus oil contains a wide range of compounds, including monoterpenes, sesquiterpenes, aromatic phenols, oxides, ethers, alcohols, esters, aldehydes, and ketones. The oil contains certain compounds including 1,8-cineole (Eucalyptol), citronellal, citronellol, citronellyl acetate, p-cymene, eucamalol, limonene, linalool, α-pinene, γ-terpinene, α-terpineol, and aromadendrene (Almas et al., 2021). This plant is rich in terpenoids and polyphenols. The primary component of the plant leaves is eucalyptol or cineole (which makes up 70–80) % of the composition (Jafari et al., 2020). The oil exhibits several biological effects, such as antibacterial, antiviral, antifungal, and antioxidant properties(Khalaf et al., 2020). Geranium, or *Pelargonium graveolens* L., is regarded as a highly significant food and medicinal plant. It belongs to the Gerianceae family, and they are hardy perennial herbs with purplish flowers. The leaves are green to gray-green and can be hairy or smooth (Abaas et al., 2018). The Essential oil (EO) extracted from *Pelargonium graveolens* is known to have numerous anti-infective ., This oil possesses a robust hydrophilic and

is rich in acyclic monoterpene alcohols, geraniol, citronellol, and linalool, resulting in high bioactivity (Dumlupinar et al., 2023). The hydrophilic nature
of essential oils makes them permeable to bacteria allowing them to effortlessly enter lipid membranes and disrupt cell walls. This leads to a decreased membrane potential, and causing coagulation of cellular components. As a result, all of this leads to the demise of bacteria (Srivastava et al., 2022).

The study aimed to evaluate the antibacterial activity of essential oil from the leaves of *E. camaldulensis* and *P. graveolens* (Geranium) in Tehran, Iran, on inhibiting the proliferation of *K. pneumoniae* clinical isolates carrying the ESBL PER1 resistant gene.

Material and methods:

Samples collection and Isolation

In total, 60 isolates of *K. pneumonia* were collected from various clinical samples in two hospitals located in Ahvaz, Iran, the isolated bacteria were identified using conventional methods such as colony morphology, gram stain, oxidase test, citrate utilization test, motility test, urease test, indole test, and sugar fermentation tests between February and May 2023.

Antibiotic Sensitivity Test

The Kirby-Bauer disk diffusion method was used to study the sensitivity pattern of these isolates to 5 disc antibiotics, namely cefotaxime (CTX 30 µg), ceftazidime (CAZ 30µg), ciprofloxacin (CIP 5 µg), gentamicin (GM 10 µg), and amikacin (AK 30µg). Mueller-Hinton agar (Merck, Germany) was utilized as the medium, following the guidelines set by the Clinical Laboratory Standards Institute (CLSI) guidelines. The agar was inoculated with a *K. pneumoniae* isolate at a density equivalent to 0.5% MacFarland standard turbidity and the Plates were incubated for 24 hours at 37 °C. The measurement of the diameters of zones of inhibition was conducted accordingly.

DNA extraction and PCR

DNA was extracted from *K. pneumoniae* using the Sina Pure DNA Kit (Sinaclon, Iran). The PER-1 gene, with a size of 933 bp, was amplified using a special primer as follows: Forward Primer (F): 5'- ATGAATGTCATTATAAAAGC-3' and Reverse Primer (R): 5'-AATTTGGGCTTAGGGCAGAA -3'. The PCR was conducted in 25 µl reaction volumes, consisting of 0.5 µl each for the Forward and Reverse Primers, 3 µl of DNA template, 9 µl of nuclease-free water, and 12 µl of Taq Red Master Mix (Ampliqon, Denmark). DNA amplification was performed using a Thermal Cycler (Tobi Nagin, Germany) under the following conditions: pre-denaturation at 95 °C for 3 min, denaturation at 93 °C for 45 s, annealing at 58 °C for 1 min, 35 cycles, and extension at 72° C for 5 min. After that, 5 µl of the amplification product was transferred to the wells of a 1.5% Agarose gel electrophoresis (Invitrogen, USA) to analyze the results for 45 minutes using 1X TBE running buffer. The determination of size and detection of gene presence was facilitated by utilizing a 100-bp DNA ladder (Sinaclon Iran). In order to ensure accuracy, each amplification experiment incorporated a negative control blank that consisted of all PCR materials

without DNA.

Essential oil extraction

The aerial parts of each plant were subjected to hydro-distillation using a Clevenger-type apparatus for 3 hours, with 100 g of plant material used for each extraction according to the European Pharmacopoeia procedure (2006). The essential oil was stored in sealed vials, kept in a dark place, and stored in a refrigerator at 4 °C until needed.

Antibacterial effect of Essential Oil extraction on clinically isolated

Agar well diffusion method

Essential oil antibacterial activities of *E. camaldulensis* and *P. graveolens* were determined by the standard disk diffusion susceptibility method on Mueller Hinton agar (Assaggaf et al., 2022). The antimicrobial activity of PGEO and ECEO was tested in the microbiology lab at the Department of Microbiology and Microbial Biotechnology at the Faculty of Life Science and Biotechnology, Shahid Beheshti University in 2023. The tests were conducted against 10 strains of *K. pneumoniae* clinical isolates. An equal suspension of every individual sample, matching the 0.5 McFarland turbidity standards, was introduced onto Mueller-Hinton agar plates. Using sterilized cotton swabs, the suspensions were distributed over the agar plates. Then, a well was created in the middle of the culture medium, and 50 microliters of the oil extract were applied to the well in the center of the plates. The plates were then incubated at 37 °C for 24 h for bacterial isolates.

Statistical Analysis

Data results from this study were analyzed using the GraphPad Prism 9 program and Microsoft Excel 2016 for each biological replicate. A probability level of $P \leq 0.05$ was used to identify a significant difference.

Results

In this study, 60 samples of *K. pneumoniae* were collected from the laboratories of the investigated hospitals. Among 60 clinical samples, 21 samples were isolated from women (35%), and 39 samples were isolated from men (65%), as shown in Figure 1.

The frequency of Klebsiella pneumonia infections by sample type

These samples include 27 urines (45%), 20 tracheal secretions (33.3%), 7 wounds

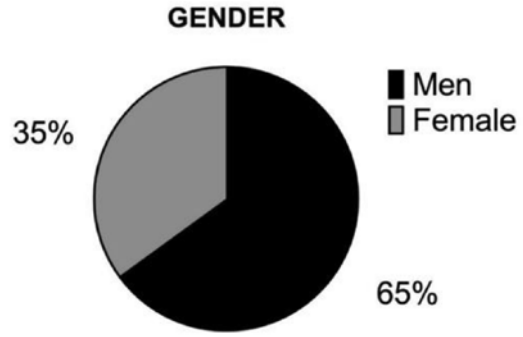

Fig. 1. Frequency of samples according to the gender of patients

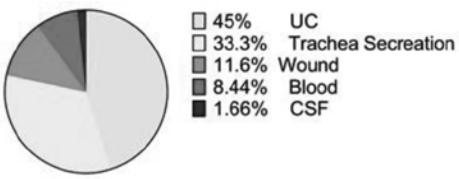

Fig. 2. Distribution of frequency of clinical sample type

(11.6%), 5 blood samples (8.44%), and 1 cerebrospinal fluid (CSF) sample (1.66%) divided by type and number. Additionally, the highest frequency of the samples was related to the urine sample, and the lowest was related to the CSF sample, as shown in Figure 2.

Antibiotic sensitivity test results

K. pneumoniae exhibited the greatest resistance to ceftazidime (83.3%), while displaying the lowest resistance to amikacin (51.6%). Ceftazidime resistance was observed in 83.3% of the isolates, while cefotaxime resistance was found in 80% of them. Additionally, 78.3% of the isolates exhibited resistance to ciprofloxacin, 73.3% to gentamicin, and 51.6% to amikacin, as depicted in Figure 3.

Effect The antimicrobial effect of essential oils on clinical isolates of Klebsiella pneumoniae

The results show that the oils extracted from (*E. camaldulensis* and *P. graveolens*) inhibited the growth of (*K. pneumoniae*) used in this study. Table 1 and Figure 5 demonstrate that *K. pneumoniae* exhibited the highest sensitivity to the essential oil extract. The zone of inhibition for *E. camaldulensis* ranged from 8–25 mm, while for *P. graveolens* the growth of *K. pneumoniae* was inhibited with zones ranging from 8-14 mm.

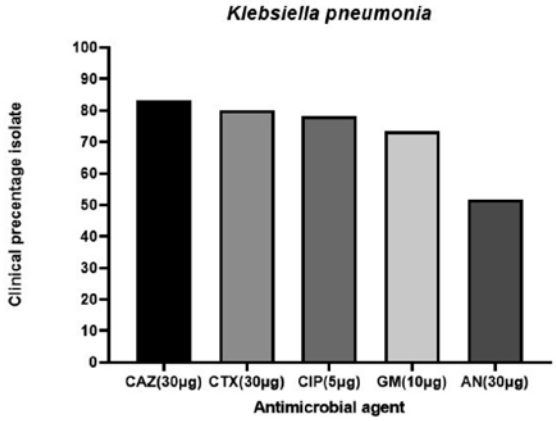

Fig. 3. The percentage of antibiotic-resistant clinical isolates of *Klebsiella pneumoniae* CAZ (ceftazidime), CTX (cefotaxime), CIP (ciprofloxacin), GM (gentamicin), and AN (Amikacin)

Table 1. Antibiotics-resistant profile of multidrug-resistant *Klebsiella pneumonia* isolated

Antibiotic	N19 (mm)	N33 (mm)	N35 (mm)	N36 (mm)	N41 (mm)	N45 (mm)	N50 (mm)	N54 (mm)	N56 (mm)	N57 (mm)
Eco	24	25	23	25	18	20	24	15	11	15
Pgo	11	12	14	12	11	8	10	13	13	10
Ceftazidium	S	R	R	R	R	R	R	R	R	R
Cefotaxime	S	R	R	R	R	R	R	R	R	R
Ciprofloxacim	S	R	R	R	R	R	R	R	S	S
Gentamicin	S	R	R	R	R	S	R	R	R	R
Amikacin	S	S	S	R	R	R	R	R	S	S

Abbreviations: ECO (*E. camaldulensis* oil); PGO (*P. graveolens* oil); R (resistance); S (sensitive); N (isolate number)

Discussion

Pneumonia is one of the pathogens on the WHO's list of antibiotic-resistant diseases of critical priority, meaning that developing new antibiotics is necessary to fight them (Oliveira *et al.*, 2022). Therefore, studies regarding the antimicrobial properties of novel antimicrobial products are of great interest. Using natural products such as essential oils is one possible solution (Łysakowska et al., 2015)it is essential to identify novel ways of eradicating them from infected root canals. One such approach may be the use of antimicrobials such as plant essential oils. Enterococcal strains were isolated from endodontically treated teeth by standard microbiological methods. Susceptibility to antibiotics was evaluated by the disc-diffusion method. The minimal inhibitory concentration (MIC. Essential oils and their constituents have a wide range of antipathogenic effects(Ahmad et al., 2023). The antibacterial activity of essential oil extracts of *E. camaldulensis* and *G. graveolens*) was determined against *K. pneumoniae* clinically isolated from urine, trench secretion, wounds, blood, and from csf in this study. The results showed the antimicrobial activities of essential oils in *E. camaldulensis* and *P. graveolens* were robust and efficient against *K. pneumoniae* clinical isolates as determined through the agar well diffusion method due to their intricate composition (Galovičová et al., 2023)antibiofilm, antimicrobial (in situ and in vitro. The essential oil of *E. camaldulensis* oil exhibited the highest sensitivity among strains, growth inhibition zone on the disk measuring between (11–25 mm), on the other hand, strains showed sensitivity to the essential oil of *G. graveolens* oil growth inhibition zone ranging (from 8 to–14 mm). These results are detailed in Table 2 and Figure 5.

Mehani et al. (2012)they are used for their antiseptic properties against infectious diseases of fungal origin, against dermatophytes, those of bacterial origin [3, 4]. The aim of our study is to determine the antimicrobial effect of essential oils of the

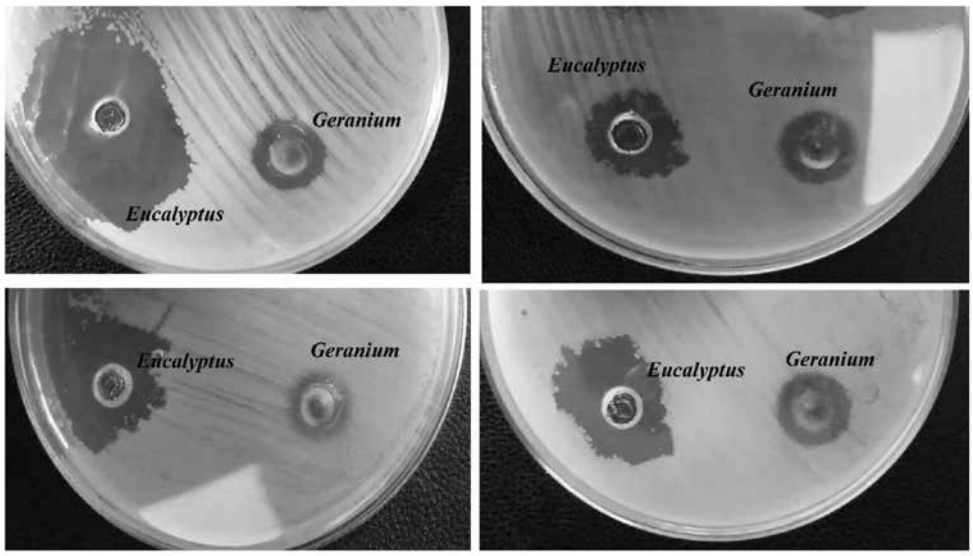

Fig. 5. antibacterial activity of *P. graveolens* and *E. camaldulensis* oil extract against *Klebsiella Pneumonia* clinical isolates

plant Eucalyptus camaldulensis on some pathogenic bacteria which is a medicinal plant used in traditional therapy. The test adopted is based on the diffusion method on solid medium (sensitivity also reported similar results, showing that, the essential oil of *E. camaldulensis* exhibited a zone of inhibition of 11.66 mm, against the Antirobactère strain, indicating a higher sensitivity to essential oils. Additionally, the essential oils of *E. camaldulensis* exhibited a 10 mm inhibition zone for Proteus and a 9.69 mm for Escherichia coli strains (Mehani et al., 2012). Fenghour et al. (2021) reported similar results and found that the essential oils of *E. camaldulensis* have a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria. Opi et al. (2023) showed that 1,8-cineole, a particular oil component, accounts for a considerable portion of the therapeutic benefit of eucalyptus oil. Elaagib et al. reported that cymene in Eucalyptus essential oil may be responsible for its antibacterial activities against *S. aureus*, *Bacillus cereus*, and *E. coli* (Khalaf et al., 2020). In a study reported by Yakowska et al. (2015) against reference strains of *Escherichia coli* ATCC 25922, *K. pneumoniae* ATCC 15380, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923, Geranium oil has been demonstrated to have an antibacterial impact. The primary components that exhibit biological activity are citronellol, geraniol, linalool, isomenthone, nerol, and citronellyl formate. These constituents give *P. graveolens* essential oil its potent antibacterial properties, as evidenced by its low minimal inhibitory concentration (MIC) values of 0.72 mg/mL against *S. aureus*, 0.36 mg/mL against Bacillus cereus, and 0.72 mg/mL against *B. subtilis* (Bigos et al., 2012). *K. pneumoniae* has emerged as one of the most problematic and highly antibiotic-resistant pathogens worldwide (Zhang et al., 2023). This study showed that the highest level of resistance of *K. pneumoniae* was against ceftazidime (83.3%), as opposed to the lowest level of resistance being against amikacin (51.6%). This result is in agreement with the study conducted by (Kadivarian et al., 2023) west of Iran. Materials and Methods: Identification and antibiotic susceptibility pattern of 165 isolates were performed by biochemical and disk diffusion methods, respectively. Screening and confirming the presence of ESBL genes were performed according to the double disk combination test (DDCT in Iran, where they show the highest resistance in ESBL-producing isolates was observed for ceftazidime (n = 88, 53.33%) (Kadivarian et al., 2023)west of Iran. Materials and Methods: Identification and antibiotic susceptibility pattern of 165 isolates were performed by biochemical and disk diffusion methods, respectively. Screening and confirming the presence of ESBL genes were performed according to the double disk combination test (DDCT This result was similar to the study conducted by Al-Saady (2023) in Iraq, where the lowest level of resistance was against Amikacin (56.25%) (Al-Saady, 2023).

The investigation involved the extraction of essential oils from the two plant species (*E. camaldulensis* and *P. graveolens*) followed by their analysis of *K. pneumoniae* clinical

isolates. The essential oils obtained from both plants showed noteworthy biological. Activity against the bacteria. Suggesting their potential use as a natural antibacterial agent.

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