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Anti-Pseudomonal Activity of Leaf Extracts of Myrtaceae Plants against β-Lactamase-Producing Strains

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Abstract

***Background*:** Due to occurrence of extended-spectrum β-lactamases (ESBLs) in *Pseudomonas aeruginosa,* There is a need for a reliable method to treat clinical isolates of *P. aeruginosa* that increasingly reported from worldwide. Also the prevalence of multidrug-resistant *P. aeruginosa* producing betalactamase reported from different parts of the world during the last decades. The Eucalyptus contains a number of compounds with antifungal, antiviral and antibacterial properties and used to control several diseases derived from microbial infections.

***Materials and Methods*:** In this experimental study, *Eucalyptus camaldulensis* (*E. camaldulensis*) was used to evaluate the antimicrobial effects against lactamase-producing strains of *P. aeruginosa* methanol extract of the leaves of this plant, with concentrations of 50, 100, 200 and 400 mg/mL were prepared, and antibacterial activities were evaluated by well diffusion method on strains *P. aeruginosa* isolated from patients. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was determine by the microplate method.The frequency of the gene encoding bla OXA-10 was studied by polymerase chain reaction (PCR).

***Results*:** In this study, methanol extract of eucalyptus leaves at concentrations of 400 mg/mL was high activity against *P. aeruginosa*. Minimum inhibitory concentration of the extract on the growth of these bacteria was change to 25 mg/mL. Also MBC of extract showed range from 50 mg/mL respectively.

***Conclusion*:** Result from these finding suggest that, eucalyptus extract, has an inhibitory effect on strains that carrier betalactamase. Also, the effect of extract, risesed by increasing the concentration. The results of this study suggest the traditional use of *E. camaldulensis* leaves as an antibacterial agent.

Introduction

Iran with rich natural has been a source of medicinal agents for thousands of years. In Iran, almost majority of plants are medicinal and the application of medicinal plants especially in traditional medicine is constantly established. Plant extracts and essential oils in many researches have shown high effect in the treatment of infectious diseases including viral, bacterial and fungal infections [1]. Many studies have been done to extract herbal ingredients for survey antimicrobial activity [2-4]. The *Eucalyptus camaldulensis* (belongs to the family Myrtaceae) that contains a number of compounds with antispasmodic, anti-inflammatory, antifungal, antiviral, and antibacterial properties are used to control several diseases derived from microbial infections. This benefit is due to an ingredient called cineole that is typically cited as the active material in eucalyptus. Recently some article in medicine examined the antimicrobial effects of cineole [5, 6].

Today, there is a need to search for new antibacterial agents due to development of bacterial resistance. *Pseudomonas aeruginosa* is an important pathogen that frequently involved in infections of weakened immune system [7]. *P. aeruginosa* is the most common pathogen isolated from hospitalized patients and frequent cause of nosocomial infections such as pneumonia, urinary tract infections (UTIs), and bacteremia. Attempts to treat of *P. aeruginosa* from patients through intense antimicrobial therapy may lead to significant selection of resistance strains in care unit of the hospitals [7, 8]. β-lactamases are enzymes produced by bacteria that inactivate β-lactam antibiotics. Extended-spectrum β-lactamases (ESBLs) have the ability to hydrolyse and cause resistance to various types of the modern β-lactam antibiotics. Many *P. aeruginosa* strains can produce different class of ESBLs that enable organism to resistant against many of cephalosporins, such as cefotaxime, ceftriaxone and ceftazidime.

These enzymes are encoded by chromosomal or plasmid-born genes that are inactivated by the β-lactamases inhibitor such as clavulanate. The OXA-type ESBLs have been found mainly in *P. aeruginosa* isolates from Turkey and France. Recently, however, several class A, B and D extended spectrum β-lactamases have been reported in *P. aeruginosa* [8-11]. In the study of Jouki and Khazaei, the ethanolic extract of this plant inhibited the growth of *Bacillus subtilis* and *Staphylococcus aureus*

respectively. But had no inhibitory effects on *Escherichia coli* [12]. In the study of Ayepola and Adeniyi the methanol extracts showed greater activity against *Salmonella typhi, S. aureus* and *B. subtilis* than Klebsiella spp., *Yersinia enterocolitica* and *P. eruginosa*. The methanol residue had a lower activity against all the test organisms except Klebsiella spp. and *S. typhi* [13]. Mumtaz et al. reported that extracts showed varying degrees of inhibition on the tested Gram-positive and Gram-negative bacteria. The methanol extracts showed greater activity against *E. coli* and *B. subtilis* [14]. This study was done to detection of *P. aeruginosa* strains harbouring β-lactamase and the effect of eucalyptus extract on β-lactamase-producing strains, respectively. To date, few studies have been conducted in this area.

Materials and Methods

This experimental study was conducted at microbiology research laboratory, department of microbiology; Islamic Azad university of Falavarjan in 2013. Herbal samples were collected and identified in cooperation with herbarium and herbal research center of Isfahan. In each experiment, three iterations were employed to do statistical calculations.

The bacterial strains was collected from Al-Zahra hospital in Isfahan, and then revived by standard methods.

One microliter of *P. aeruginosa* fresh cultures in Brain-Heart Infusion Broth (BHI) medium (Scharlau, Spain) were transferred into 1.5 mL sterile microfuge tubes and centrifuged at 13 g for 10 min. The pellets were dissolved in 500 µL of lysis buffer (NaCl 1 M, Tris-HCl 100 mM, EDTA 0.5 M), SDS (10%), 3 µL of proteinase-K (20 mg/mL) and incubated at 55ºC for 2 h. After the lysis, 500 µL of phenol/chloroform/isoamyl alcohol (25:24:1 Volume/Volume) were added to the above solutions, vortexed, and centrifuged at 13 g for 10 min. The supernatants were transferred to other sterile microfuge tubes. One microliter 95% cold ethanol was added and stands for 1 h in refrigeration condition (4ºC). DNA was then precipitated in each tube by centrifugation at 12 g for 10 min. The precipitated DNA was dissolved in 50 µL of 10 mM Tris EDTA-buffer (TE) as described by Shahcheraghi and Nikbin used for further investigation [15].

PCR was used to detect OXA10 gene, in the multidrug resistant bacterial strains using the following primer (designed in this study): PaOxa10 F 5ATTATCGGCCTAGAAACTGG-3 And PaOxa10 R 5-CTTACTTCGCCAACTTCTCTG-3 PCR was carried out with 1 µL of the template DNA, 0.4 pM of each primer, PCR buffer 1X, 200 µM dNTP, 1.5 mM $MgCl_2$ and 1 U of Taq DNA polymerase (Sinaclon) in a total volume of 25 µL. Amplification was carried out in a thermocycler (Eppendorf Mastercycler®, Massachusetts, USA). Agarose gel electrophoresis (1%) of PCR products was carried out using 1 mM Tris-borate-EDTA (TBE) buffer at 85 V for 1 h and the DNA bands then stained with ethidum bromide (Sinaclon). One hundred bp DNA ladder was used to confirm the size of specific bla gene. Simultaneously, a positive control was used for blaOXA10 gene. The reaction conditions were as follows: pre-denaturation at 94ºC for 4 min, followed by 35 amplification cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1.5 min, with a final extension step of 72ºC for 5 min [15, 16].

The fresh leaves of *E. camaldulensis* were collected and dried in a place not exposed to sun light. Then the dried leaves were crushed to powder. Fifty gram of leaves were weighted and poured into a sterilized erlenmeyer flask. In the next step, 250 mL methanol (98%) was added so that the herb's compounds were dissolved. Erlenmeyer flask containing alcohol and herb powder was placed into shaker for 48 h until the solvent may exert their effect at the temperature of 40ºC. Next, rotary was employed to eliminate the solvent. Finally, the *E. camaldulensis* extracts were preserved in sterilized dishes at refrigerator and to prevent from the light effects, they were wrapped with aluminum covers. Extracts with concentrations of 50, 100, 200 and 400 mg/mL were provided and solved in 5% dimethylsulfoxide (DMSO). Then they were used in well diffusion and MIC tests. To prepare a microbial suspension, several colonies from 24 h newly cultured were transferred into Mueller Hinton Broth medium. The turbidity was 0.5 McFarland standard (1.5×10^8 bacteria/mL).

This suspension was then 0.01 diluted to achieve the turbidity of 1.5×106 CFU (Colony-forming unit) because the turbidity of suspension has considerable influence on accuracy of results. In this research, the antibacterial effect of methanol extracts from *E. camaldulensis* was examined through well plate and micro plate methods. In well plate method, suspension with turbidity of 1.5×106 CFU was steadily cultured in the surface of Mueller Hinton Agar plate. Then some 6 mm diameter wells were made in appropriate intervals and 100 µL extract was poured into each. DMSO was used as negative control. Next, the cultured media were heated in 37ºC incubator for 24 h. Ultimately, plates were evaluated in terms of inhibition zone, and inhibition zone diameter was measured by millimeter [17, 18].

The dilution in microplate method was used to determine Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of different leaves concentration extracts of *Rosmarinus officinallis*. The strains of mentioned pathogenic bacteria were put under a 24 hours culture process in Mueller Hinton Broth medium at the temperature of 37ºC. Dilution serials of 6.25, 12.5, 25, 50, 100, 200 and 400 mg/mL were provided from the extract and 70 µL of them were added into 96 cells micro plates containing 70 µL bacteria suspension with turbidity of half McFarland. Then the similar tests were done for positive control (medium with bacteria and without extract) and negative control (medium without bacteria). Afterwards, micro plates were heated in a 37ºC incubator. The minimum dilution with no seen turbidity was reported as MIC. Tests were repeated and the average was presented.

Considering MIC results, the MBC was also specified. From all cells in which the growth of bacteria was

stopped, plates containing Mueller Hinton Agar were cultured and heated at 37ºC for 24 hours. Concentrations without bacteria growth were reported as MBC [19-22]. All the tests were repeated three times and the results were averaged. Data were analyzed by statistical SPSS-14 software. To study the significant difference, variance analysis and χ^2 tests were used and their difference was calculated in a significant level ($p \leq 0.001$).

Results

PCR reaction confirmed to detection of betalactamase producing strains of *P. aeruginosa*. Then antimicrobial susceptibility pattern of strains was evaluated (Fig. 1). The results of antibacterial activity of *E. camaldulensis* leaves extracts were shown in table 1. All extracts showed dose dependent activity which increases with increase in concentration. As the table shows, methanol extract has prevented from the growth of *P. aeruginosa* producing beta lactamase. Also, by increasing the concentration of methanol extract, the inhibition zone increased ($p \leq 0.001$). Additionally, the results revealed that, *E. camaldulensis* extract inhibited growth of *P. aeruginosa* with multiple drug resistance (Fig. 2). Moreover, the concentration of 400 mg/mL has more effect on tested bacteria. The values of MIC and MBC of methanol extract of eucalyptus leaves have been presented in table 2 against the referred bacteria. The results determined that in tested bacteria, there was a significant difference ($p \leq 0.001$) in terms of sensitivity to methanol extract. In other words, the most sensitivity was observed in H9 and S19 strains. So, as it shown in this table, MIC is 25 mg/mL and MBC is 50 mg/mL respectively ($p \leq 0.001$).

Discussion

The results indicated that methanol extract of *E. camaldulensis* with concentration about 100 mg/mL has prevented from the growth of tested bacteria. Thus, the research represents the antibacterial effects of this medical herb on betalactamase producing bacteria. Growth inhibitory effect of the extract starts from the concentration about 20 mg/mL and by gradual increase of concentration, inhibition zone increases as well. Inhibition zone *E. camaldulensis* methanolic extract.

It was observed in *P. aeruginosa* between 9 to 27 mm. Because of the method of extraction and preventing from using high temperature to decrease the rate of destruction of effective herbal compound, there is a partial difference between these results and the similar studies. In the study of Jouki and Khazaei [12] the phytochemical analysis of the crude extract revealed the presence of saponin, steroid, cardiac glycoside, tannins, volatile oils, phenols and balsam (gum). The ethanolic extract of this plant inhibited the growth of *B. subtilis* and *S. aureus* respectively. But had no inhibitory effects on *E. coli*.

The minimum inhibitory concentration (MIC) of the extract ranged from 1.25 g/mL to 5 g/mL. The results obtained suggest that *E. camaldulensis* can be used in treating diseases caused by the test organisms. In the study of Ayepola and Adeniyi [13] antibacterial screening of the plant leaves revealed that the methanol extract, dichloromethane fraction and the methanol residue represent a broad spectrum of activity. All extracts showed varying degrees of inhibition on the tested microorganisms at a concentration of 10 mg/mL.

Table 1. Antibacterial activity of Eucalyptus camaldulensis leaves extracts against tested bacteria

P. aeruginosa producing β-lactamase	50 (mg/mL)	100 (mg/mL)	200 (mg/mL)	400 (mg/mL)
A3 (mm)	-	12	18	22
H9 (mm)	18	20	27	27
S29 (mm)	16	20	22	23
S32 (mm)	-	-	-	-
H2 (mm)	10	20	24	25
S18 (mm)	9	18	20	23
S19 (mm)	-	16	22	27

Table 2. MIC and MBC of leaves extracts against beta lactamase producing bacteria

Bacteria	MIC (mg/mL)	MBC (mg/mL)
S19	25	50
H2	25	50
H9	25	50
S29	25	50

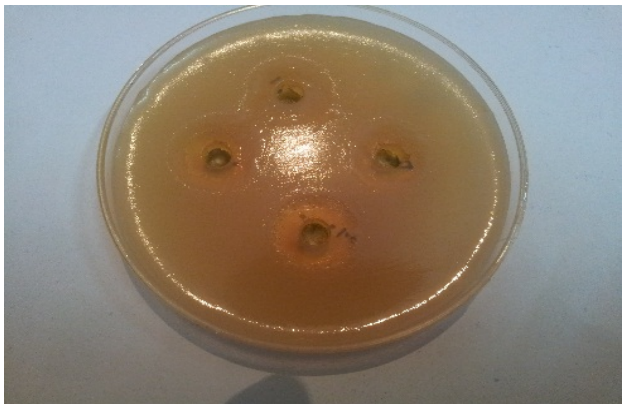

Figure 1. Zone of growth inhibition of Pseudomonas aeruginosa at different concentration

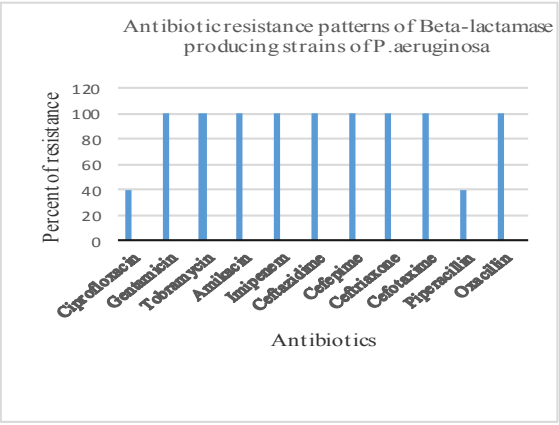

Figure 2. Antimicrobial susceptibility pattern of strains producing β-lactamase

The methanol extracts showed greater activity against *S. typhi, S. aureus* and *B. subtilis* (15-16 mm) than Klebsiella spp., *Yersinia enterocolitica* and *P. aeruginosa*

(14 mm). The dichloromethane fraction exhibited higher activity against Klebsiella spp., *S. typhi, Y. enterocolitica* and *B. subtilis* (15-16 mm) than *S. aureus* and *P. aeruginosa* (13-14 mm). The methanol residue had a lower activity against all the test organisms except Klebsiella spp. and *S. typhi*. The result also showed that Klebsiella spp. and *Y. enterocolitica* used as a positive control in this study were inhibited by the extracts. However the petroleum ether fraction showed no activity on all test organisms. The methanol extract, the MIC of the methanol extract and dichloromethane fraction determined by the agar dilution method ranged between 0.04 and 10 mg/mL with that of *B. subtilis* being the least. Phytochemical screening of the plant revealed the presence of tannins, saponins and cardiac glycosides. Mumtaz et al. [14] reported that extracts solvents (aqueous, methanol, acetone) were found to have antimicrobial activity against *E. coli* and *B. subtilis*. All extracts showed varying degrees of inhibition on the tested Gram positive and Gram negative bacteria.

The methanol extracts showed greater activity against *E. coli* and *B. subtilis*. In the study of Babayi et al. [23] methanolic extracts of leaves of *E. camaldulensis* and *Terminalia catappa* were studied. The phytochemical analysis of the crude extracts of the medicinal plants, similar to another studies revealed the presence of saponin, saponin glycosides, steroid, cardiac glycoside, tannins, volatile oils, phenols and balsam (gum). The methanolic extracts of the two plants inhibited the growth of *B. subtilis* and *S. aureus*. But had no inhibitory effects on *P. aeruginosa, S. typhi* and *E. coli. Candida albicans* was inhibited by the crude extracts of *E. camaldulensis* only. Accelerated gradient chromatography (AGC) gave fractions of the extract of *T. catappa* that were more active on *C. albicans* and *E. coli* than the crude extract. The MIC of the extracts ranged from 1.25 g/mL to 5 g/mL. The results obtained suggest that *T. catappa* and *E. camaldulensis* can be used in treating diseases caused by the test organisms. But in present study, as indicated, methanol extract had inhibitory effect on growth of *P. aeruginosa* [23]. It may be due to use of high concentration of this plant.

Abubakar [24] proved the leaves of *E. camaldulensis* can be used as a source for the development and formulation of antibacterial drugs, thus justifying the use of the leaves in herbal medicines to treat a variety of infectious conditions. The effectiveness of the extracts was more pronounced under alkaline conditions and lower temperatures. MIC values ranged from 6.25-50 mg/mL and MBC values ranged from 6.25-100 mg/mL [24] that was similar to our study. Uzama et al. [25] indicated that the extract of the leaves of eucalyptus was very active against *C. albicans* at 25 mg/mL, but has no activity with *S. aureus* at the same concentration. The phytochemical screening of eucalyptus revealed presence of flavonoids. The MIC of the crude extracts were determined for the various organisms which ranged between 0.01 and 2.5 mg/mL, while the MBC and minimum fungicidal concentration ranged between 0.02 and 2.5 mg/mL, and 0.01 and 2.0 mg/mL respectively. In their research, the eucalyptus crude water extracts offer a potential antifungal property against *C. albicans* while Basil offers a potential antibacterial property against *S. aureus* [25].

In another study Elaissi et al. [26] investigated the essential oils of fifteen eucalyptus species (North East Tunisia) were screened for their antibacterial activities by the agar disc diffusion method. The main one was 1, 8-cineole, followed by spathulenol, trans-pinocarveol, α-pinene, *p*-cymene, globulol, cryptone, *β*-phellandrene, viridiflorol, borneol, limonene and isospathulenol. The chemical principal component analysis identified five species groups and subgroups, where each group constituted a chemotype, however that of the values of zone diameter of the inhibition (ZDI) identified six groups of eucalyptus oils, characterized by their antibacterial inhibition ability. The strongest activity was shown by *E. platypus* oil against *E. faecalis* and by *E. lamannii* oil against *S. aureus*, *P. aeruginosa* and *E. coli*. A correlation between the levels of some major components and the antibacterial activities was observed [26]. The results of this study support the traditional use of *E. camaldulensis* leaves as an antibacterial agent.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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