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Frequency of Broad-Spectrum Beta-Lactamase Gene and Evaluation of Antimicrobial Effect of *Teucrium Polium* Extract and Essential Oil in Clinical Isolates of *Klebsiella Pneumonia*

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Abstract

Background: β-lactamase enzymes are one of the most important factors in generating antibiotic resistance among gram-negative bacteria. *Klebsiella pneumoniae* is an opportunistic pathogen from the *Enterobacteriaceae* family, which plays a major role in the development of hospital infections and people with underlying diseases. This study was aimed to investigate the antimicrobial effect of vitreous essential oil on strains containing antibiotic resistant genes in clinical isolates of *Klebsiella pneumoniae* in Zahedan.Methods and Materials: In order to identify chemical compounds and to investigate the antibacterial effects of *Teucrium Polium* extract, the leaves of this plant were collected from their natural habitat in May 2018 at full flowering stage. Extraction was carried out by water distillation method. Antibacterial activity of the extract of this plant, the least inhibitory concentration of growth on the resistant strains of *Klebsiella pneumoniae* was performed.Results: The essential oil yield of the *Teucrium Polium* was 75%. Of the 29 compounds identified in the essential oil of the *Teucrium Polium*, the combination of alphapinene with 12.52%, linalool was the highest in the essential oil of 10.63%. Of the 120 isolates of *Klebsiella pneumoniae*, 52 isolates in the initial screening were positive for ESBL production, of which 39 isolates (32.5%) were positive in the phenotypic confirmatory assay. 11 isolates (10.8%) had AmpC gene. Based on the results of PCR, 76.9%, 12.8%, 30.8%, 28.2%, 25.7%, 56.4% of the isolates were carriers of MOX, CIT, DHA, ACC, EBC genes and FOX, respectively.Conclusion: The results of antimicrobial resistance study of isolates showed that the highest resistance to Erythromycin Antibiotics (92.5%), Cefotaxime (38.4%) and Ceftriaxone (35.9%), respectively, while the highest susceptibility was observed in Colistin antibiotics (98.3%), Imipenem (90%) and Amikacin (88.3%), respectively. The essential oil of the *Teucrium Polium* had a significant antibacterial effect. Due to the high percentage of high concentrations of alphapinene and linalool in the essential oil of the *Teucrium Polium* with antibacterial properties and can be used to cope with certain pathogenic bacteria.

Keywords: Antibacterial, Drug resistance, *Klebsiella pneumoniae, Teucrium Polium*

Introduction

Klebsiella pneumoniae is a gram-negative pathogen of opportunistic Enterobacteriaceae family that plays a major role in the development of various hospital infections such as pneumonia, sepsis, urinary tract infections, and intra-abdominal infections in hospitalized patients as well as patients with underlying diseases (Hassan et al., 2015). β-lactam antimicrobial agents are currently the most commonly used treatment for bacterial infections. Their use leads to the emergence of resistance to bacterial beta-lactam antibiotics worldwide.

AmpC plasmids enzymes are divided into six families of MOX, FOX, CIT, DHA, EBC and ACC. A small change in the sequence of their amino acids leads to different varieties among the family (Shanthi &. Balagurunathan, 2014). Over the decades after the discovery of antibiotics, their uncontrolled administration to treat bacterial infections has led to the selection and spread of resistant strains of bacteria, so that bacterial resistance to antibiotics has now become a global dilemma. The major consequence of this misguided strategy is to replace and create clones of resistant strains of bacteria instead of susceptible strains.

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Teucrium Polium is herbaceous, persistent, ramose with 10 to 35 cm height and has a white cotton appearance, usually in the arid, rocky, and sandy beaches of the world. Its leaves are narrow, long and covered with cotton flakes on both sides of the surface. The use of it is effective in the headache, weakness of the digestive system, urinary tract disorders, and delayed or absent menstruation due to general weakness. It also has anticonvulsant effects. This bitter plant in Baluchistan, Iran and India is used to relieve the pain of the heart, as well as antipyretic and is consumed by the boiling water in the water and soaked or caught in the water in cases of colds. In Zahedan, people also use it to relieve the discomfort of the digestive system and the heart. This plant contains alphapinene and linalool, which enhances the probability of bacterial sensitivity. So far, studies have been done on its antibiotic properties on other species (Mohammadi et al., 2013).

The purpose of this study was to determine the antibiotic resistance pattern of *Klebsiella pneumoniae* strains isolated from clinical specimens of patients in educational hospitals of Zahedan, studying the frequency of AmpC β-lactamase genes in clinical isolates of *Klebsiella pneumoniae*, and comparing the therapeutic properties of *Teucrium Polium* with modern antibiotic therapy under laboratory conditions. Considering the creation of new gaps between conventional medicine and molecular medicine in the present age, antibiotic therapy can be selected by identifying the resistance gene in the bacteria in the shortest possible time based on the molecular analysis of the polymerase chain reaction. Introducing the results of this study will help the medical community to select the type and dose of medicine appropriately (Jalalpoor, 2011). If the results of the infection with the *Teucrium Polium* be positive, it can be helpful to identify and isolate the effective substance for introduction into the pharmaceutical industry.

Method and Materials

This was a descriptive-analytical and laboratory study. Klebsiella pneumoniae samples were included urine, blood and respiratory tract secretions samples during April to September, 230 samples of clinical isolates of *Klebsiella pneumonia* from microbiology laboratories of Zahedan hospitals including Bu Ali Infectious hospital, Imam Ali hospital, Khatam hospital and Nabi Akram hospital. After sample collection, suspected sample were transferred to the microbiology laboratory of the Infectious Disease Research Center of Bu Ali hospital of Zahedan. All patient characteristics including the patient's first name and surname, hospitalization ward, sampling site, and other required specifications were recorded. Then, in the laboratory, the collected samples were confirmed by standard and differential microbiological methods. Finally, 120 isolates were identified as Klebsiella pneumoniae.

The samples were inoculated on blood and MacConkey agar culture and incubated at 37 ° C. After 24 h incubation, the first clone was extracted, the lam was prepared and the germ staining was performed. The color of the colony was examined either on the face or on color on the MacConkey agar. Then catalase and oxidase tests were performed on single colonies (Manoharan et al., 2012). The following biochemical tests were performed for the final diagnosis of bacteria using the standard table; 1) Antibacterial culture in a TSI agar, 2) Bacterial culture of the bacteria in Simon Citrate agar, 3) Culture of the bacteria in the SIM, 4) Endodontic test using Kovacs, 5) Culture of bacteria in MR-VP liquid, 6) Culture of bacteria in urea agar, 7) Culture of bacteria in lysine-decarboxylase, 8) Culture of bacteria in ornithine decarboxylase, 9) Culture of bacteria in arginine dehydrolases and 9) Investigation of fermentation of sugars.

The leaves of the Teucrium Polium from their natural habitat were collected from the villages of Sistan and Baluchistan province in April, and after identification, they were cleaned and washed and in shadow conditions due to hydrolysis of the compounds, the plants were dried at ambient temperature and then 150 gr of crushed samples were extracted by water distillation using Clevenger for three hours. Then, the extract was injected into a mass spectrometer (GC / MS) coupled with the mass spectrometry and corresponding chromatograms (Mohammadi et al., 2013). Antibiotic resistance and susceptibility patterns for 120 clinical isolates of *Klebsiella pneumonia* by disc diffusion method with antibiotic discs were as following: ceftriaxone (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), amikacin (30 µg), imipenem (10 µg), chloramphenicol (30 µg), colistin (10 µg) and erythromycin (15 µg) (prepared by MAST UK). In accordance with the CLSI guidelines, the disk propagation method was used (Patel, 2017).

Disk Diffusion

The microbial suspension prepared with dilution of interest was applied to the surface of the agar (next to the flame). Rotational rotations were done in the right and left directions to be uniformly distributed at the agar surface. After 5 minutes, the plates were placed in a slope and the microbial suspension was removed using the remaining sampler. Then, using sterile pins, the antibiotics were placed on the surface with proper spacing from each other and from the plate wall. Plates were placed at 37 ° C for 18 hours (Ahmedet al., 2013).

Result of disc diffusion

After 18 hours' incubation at 37 ° C, using a ruler under the light bulb, the diameter of the inhibition zone around the antibiotic discs was measured and evaluated using the CLSI instruction. The specimens were resistant, intermediate and sensitive. In each series, the standard strain of E. coli ATCC 25922 was used to control the quality of the discs (Fig. 1) (Sękowska et al., 2012).

Fig 1. The result of disc diffusion

Results

Clinical samples were collected from different parts of the hospital including 75 isolates (62.5%) from outpatients, 23 isolates (19.2%) from ICU, 8 isolates (6.7%) from emergency department, 6 isolates (5%) from infectious ward, 3 isolates (2.5%) from the internal section, 4 isolates (3.3%) from the women's department and 1 isolate (0.8%) from the pediatric ward. Of the 120 samples of *Klebsiella pneumoniae*, 73 samples (60.8%) were related to the female population and 47 samples (39.2%) were related to the male population.

All strains of *Klebsiella pneumoniae* were analyzed after definitive isolation and diagnosis for disk diffusion test. The diameter of the inhibition zone around the discs was measured using a millimeter ruler and was evaluated in accordance with the CLSI 2017 standard table. The samples were resistant (R), intermediate (I) and sensitive (S) (Table 1) (Patel, 2017).

Table 1. Frequency distribution of resistance pattern and antibiotic senility in *Klebsiella pneumoniae* strains isolated from educational hospitals in Zahedan according to the type of used antibiotics

Antibiotic type	Abbreviated code for antibiotics	Disc concentration	Resistant		Intermediate		Sensitive	
			%	N	%	N	%	N
Chloramphenicol	C	30µg	15	18	0	0	85	102
Ceftazidime	CAZ	30 µg	25.8	31	5.8	7	68.4	82
Ciprofloxacin	CIP	5 µg	23.4	28	5	6	71.6	86
Ceftriaxone	CRO	30 µg	35.9	43	1.6	2	62.5	75
Cefotaxime	CTX	30 µg	38.4	46	1.6	2	60	72
Gentamicin	GM	10 µg	32.5	39	2.5	3	65	78
Amikacin	AK	30 µg	8.3	10	3.4	4	88.3	106
Imipenem	IMI	10 µg	7.5	9	2.5	3	90	108
Colistin	CO	10 µg	1.7	2	0	0	98.3	118

The results showed that the yield of *Teucrium Polium* collected from Sistan and Baluchistan province was 0.75%. Of the 28 compounds identified in the *Teucrium Polium* extract with 99.75%, alphapinene compounds with 12.52%, linalool with 10.63%, and caryophyllene oxide with 9.69% have the highest percentage of extract. The extract of this plant on the gram-negative bacteria of *Klebsiella pneumoniae* has a diameter of 15 mm growth inhibitory effect. As shown in Figure 2, the frequency distribution of GOXs, FOX, EBC, ACC DHA, and CIT in *Klebsiella pneumoniae* isolates were 23.1%, 2.2%, 7.87%, 71.69%, 74.3% and 43.6%, respectively.

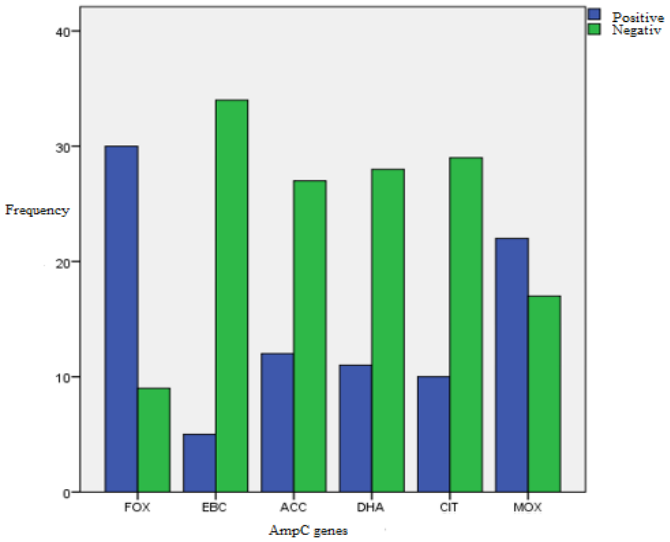

Fig 2. Frequency of genes producing broad-spectrum beta-lactamase enzymes in *Klebsiella pneumoniae* isolates

Discussion

Medicinal plants such as *Teucrium polium L*. are one of the great important plants in the field of traditional medicine and modern medicine, with important pharmaceutical and nutritional compounds and antibacterial effects in botanical matters. (Baher & M. Mirza, 2003). The results of *Teucrium polium* analysis collected from Sistan and Baluchistan province showed that the extract yield of this species was 75%, which was less efficient than the study of Mohammad et al. that was carried out by steam distillation method (Mohammadi et al., 2013). The investigations showed that the total of 29 compounds identified in the *Teucrium polium* extract with 99.75%, alphapinene compounds with 12.52%, linalool with 10.63%, and caryophyllene oxide with 9.69% had the highest percentage which had similarities and differences in comparison with other researchers.

In Iran and other parts of the world, a lot of research has been carried out on the different species of *Teucrium*, including the identification of *T.Stocksianum* compounds by Jaimand et al. that reported Camphen (20.6%), α-Cadinol (19.7%), Myrcene (10.2%) and Carvacrol (9.9%) as the main components (Jaimand et al., 2006). In another study, T.flavum was shown to be the most active ingredient in α-caryophyllene (30.7%), Germacrene (21.3%) α -humulenea (8.8%) (Baher, &. Mirza, 2003). *T.orientale.L. subsp Orientale* has been reported with caryophyllene (33.5%), linalool (17.0%) and ß-caryophyllene (9.3%) (Javidnia & Miri, 2003). In the study of T.persicum, the compounds of Caryophyllene oxide (10.6%), α-pinene (9.4%), Linalool -α (7.8%), Cadinene (7.4%), Elemol (9 / 6%), α-cadinol (5.5%) had the highest levels (Javidnia et al., 2007). In the study of T.orientale.L. subsp Taylori of Lorestan province of Iran, Linalool (28.66%), Caryophyllene oxide (15.62%), 3-octanol (5.55%), ß-pinene (8.75%) and ß-caryophyllene (7.33%) were identified as major combinations (Amiri, 2008). In the study of the Teucrium species in Italy, the T.fruticans species had major components including ß-pinene (21.0%), Germacrene D (18.1%), ß-Myrcene (13.0%) and ß-caryophyllene (12.0%) (Flamini et al., 2001). In another study, the main component of T.polium subsp.Capitatum were α-pinene (28.8%), ß-pinene (7.2%) and p-cymene (0.07%) (Cozzani et al., 2005). In another study in Jordan, Teucrium polium showed the highest levels of 8-cedern-13-ol (24.8%), ß-caryophyllene (7.8%), Germacrene D (6.8%) and Sabinene (5.2%) (Aburjai et al., 2006; Cakir et al., 1998).

In this study, the combination of α-pinene with 12.52%, linalool with 10.63%, and Caryophyllene oxide with 9.69% constitute the highest percentage of *Teucrium polium*, which are similar to other researchers in Iran and elsewhere in the world, which can be compared to The cause of the climatic and geographical conditions. The results of the study of antibacterial effects of *Teucrium polium* plant in Sistan and Baluchistan province showed that the plant's antiseptic effect on gram-negative bacteria of *Klebsiella pneumoniae* with a growth inhibitory diameter of 15 mm had significant antibacterial effects compared to selective antigens. In another study, the antihypertensive effect of the *Teucrium polium* was experimentally approved (Suleiman et al., 1988). In another study, *Teucrium polium* significantly reduced cholesterol and triglyceride levels in experimental mice (Rasekh et al., 2001).

Klebsiella pneumoniae is a gram-negative bacterium due to a range of diseases such as pneumonia, urinary tract infections, septicemia, soft tissue infections, bacterial meningitis and hepatitis. It is also the cause of hospital infections and acquired diseases. *Klebsiella pneumoniae* in the hospital, where colonization is directly related to the length of hospitalization, dramatically increases. Long stay in a hospital increase the percentage of the presence of *Klebsiella pneumoniae* on the skin of patients and even hospital staff (Pourali Sheshblouki & Mardaneh, 2016)

In the present study, 120 isolates of *Klebsiella pneumoniae* in hospitalized patients studied and the highest number was related to ICUs with 23 cases (19.2%). In a study conducted in Iran in 2010, 200 clinical isolates of *Klebsiella pneumoniae*, which were collected from the ICU, urology, respiratory and surgical departments during a year, the highest frequency was isolated from the ICUs (Nasehi et al.,2010), which is consistent with our study.

Our results showed that the level of resistance to third-generation cephalosporin is relatively high among clinical isolates of *Klebsiella pneumoniae* in Zahedan, and due to the lack of effective antibiotics for the treatment of these pathogens, this level of resistance can have many problems in the future in the treatment of infection caused by these bacteria and other bacteria of the genres and species due to the ease of transferring the genes producing these enzymes. Finally, it should be noted that endemic control of ESBLs is difficult and may only be possible through the use and organization of health and medical programs considering high cost. Therefore, the primary frequency control of the organisms producing ESBLs in a hospital or special department of the hospital is very important.

Conclusion

The results indicated the high inhibitory and microbial strength of *Teucrium Polium*. The antibacterial effects of *Teucrium Polium* can be attributed to the compounds of alphapinene and linalool, which have been approved the antibacterial effects of these compounds. Therefore, due to the antibacterial effects of *Teucrium Polium* extract in comparison with the third-generation cephalosporin antigens, this antibacterial agent can be used as a combination with antibacterial effects of natural origin. Our results showed that the level of resistance to third-generation cephalosporin among the clinical isolates of *Klebsiella pneumoniae* in Zahedan was relatively high and due to the lack of effective antibiotics for the treatment of these pathogens, this resistance could be a major problem in the future in treatment infections caused by these bacteria as well as other bacteria, genera and species due to the ease of transferring the genes producing these enzymes.

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Conflicts of interest

The authors declare no conflict of interest.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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