اثر عصاره متانولی کلپوره به عنوان یک گیاه ضدباکتریایی بومی علیه سویه‌های MRSA

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چکیده

زمینه: عفونت‌های ناشی از سویه‌های مقاوم *استافیلوکوکوس آرئوس* سالیانه در حال افزایش است. بنابراین یافتن منابع جدید آنتی‌بیوتیکی اهمیت ویژه‌ای دارد. گیاهان دارویی به‌عنوان منابع طبیعی آنتی‌بیوتیک‌ها گزینه مناسبی برای این منظور هستند. کلپوره، گیاه بومی استان خوزستان، مدت مدیدی است که در طب سنتی استفاده می‌شود. هدف از مطالعه حاضر بررسی فعالیت ضدباکتریایی این گیاه علیه سویه‌های *استافیلوکوکوس آرئوس* مقاوم به متی‌سیلین (MRSA) می‌باشد.

روش‌ها: برای این منظور عصاره متانولی از گل‌های کلپوره تهیه شد و فعالیت ضدباکتریایی آن علیه 50 جدایه MRSA از طریق روش انتشار دیسک کربای- باثر بررسی شد. این جدایه‌ها از نظر حضور ژن‌های *mec*A و *pvl* نیز غربال‌گری شدند.

یافته‌ها: 20 جدایه مقاوم به متی‌سیلین برای انجام تست حساسیت به عصاره متانولی انتخاب شد که از بین آن‌ها 14 جدایه دارای ژن *mec*A و 2 جدایه دارای ژن *pvl* بودند و 4 جدایه فاقد هر دو ژن بودند. هیچ‌کدام از نمونه‌ها همزمان دو ژن *mec*A و *pvl* را نداشتند. نتایج نشان داد که عصاره متانولی کلپوره حتی در کم‌ترین غلظت به‌کار رفته اثر مهاری قابل‌توجهی علیه جدایه‌های MRSA دارد. سویه‌های *mec*A و *pvl* منفی نسبت به عصاره متانولی حساس‌تر بودند ولی حضور *mec*A اثر قابل‌توجهی روی مقاومت آن‌ها نداشت.

نتیجه‌گیری: بر اساس این نتایج و افزایش روند عفونت‌های *استافیلوکوکوس آرئوس* اکتسابی از بیمارستان می‌توان بیان کرد که کلپوره یک گیاه دارویی مؤثر برای درمان عفونت‌های ناشی از این باکتری است و از آن به‌عنوان یک منبع طبیعی برای تولید آنتی‌بیوتیک جدید می‌توان استفاده کرد.

کلیدواژه‌ها: کلپوره، *استافیلوکوکوس آرئوس* مقاوم به متی‌سیلین، *mec*A، *pvl*، طب سنتی

«دریافت: 1393/6/25 پذیرش: 1393/9/25»

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The antibacterial properties of methanolic extract of *Teucrium polium* against MRSA

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Received: 16 September, 2014 Accepted: 16 December, 2014

Abstract

Background: Infections due to resistant *Staphylococcus aureus* strains are progressively increased annually. So, finding new antibiotic resources is of great importance. Medicinal plants as a natural source of antibiotics are considered a good option. *Teucrium polium*, a native plant in Khouzestan, has been used for a long time in folk medicine. The aim of the present study was to investigate the antibacterial potential of this plant against methicillin resistant *S. aureus* (MRSA) strains.

Methods: Methanolic extract was prepared from the flowers of *T. polium*, and its antibacterial activity was evaluated against 50 MRSA isolates by Kirby-Bauer disc diffusion method. These isolates were also screened for *mec* and *pvl* genes.

Results: From 50 isolates, 20 isolates were selected and subjected to antibacterial analysis. From these, 14 isolates were positive for *mec*A gene, 2 of them were positive for *pvl* gene and 4 of them didn't have *mec* or *pvl* genes. None of MRSA isolates were positive for both genes. The results showed that the methanolic extract of *T. polium* has considerable inhibitory effect against MRSA, even at the lowest concentration. *mec*A and *pvl* negative strains were more sensitive to methanolic extract, but the presence of *mec*A did not have any significant effect on their resistance.

Conclusion: Based on these findings and with regard to the increasing trend in hospital-acquired S. aureus infections, it can be suggested that *T.polium* is an effective medicinal plant for treatment of infections caused by this bacterium and can be used as a natural source to produce new antibiotics.

Keywords: *Teucrium polium*, MRSA, *mec*A, *pvl*, natural medicine.

J Kermanshah Univ Med Sci. 2015; 18(10): 557-562

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Introduction

In recent years, drug resistance of human pathogenic bacteria has been commonly reported. Due to the side effects and resistance of pathogenic microorganisms to antibiotics, many researchers have recently focused on the extracts and biologically active compounds extracted from plant species used in herbal medicine (1). It has been detected that plants produce different compounds to protect themselves against different pathogens; so, we can use these compounds as antibacterial compounds (2).

Teucrium polium L. (family Lamiaceae) is one of the 300 species of the Teucrium genus and commonly found in Mediterranean and western Irano-Turanian sphere (3). *T. polium* is a durable grass plant, with 10-30 cm height and white callous exterior, that is usually dispersed in rocky and sandy areas of Europe, North Africa and Southwest Asia, including Iran. The medical properties of this plant were considered in traditional medicine by Socrates and Jalinous (2). In Iran, this medicinal plant is called “kalpooreh”, which is commonly used by native inhabitants as herbal tea, spice or a hypoglycemic agent that is recommended by herbalists. Among the species, *T. polium* L. is mainly used in traditional medicine for many therapeutic purposes. Some biological and medical effects have been reported for *T. polium* L. such as anti-oxidant, anti-inflammatory, anti-rheumatoid, anti-nociceptive, anti-pyretic, anti-microbial, hypolipidemic, hepatoprotective, anti-gastric ulcer, cytotoxic and apoptotic effects (3, 4, 5). Several compounds have been extracted from different parts of this plant that are structurally characterized as iridoids, flavonoids, eudesmane, clerodane and

abietane derivatives (4). Methicillin resistant *Staphylococcus aureus* (MRSA) strains are one of the most important agents of hospital-acquired infections. Resistance to methicillin is caused by *mec*A gene which encodes an alternative penicillin-binding protein (PBP2a) (6). PVL (Panton- Valentine leukocidin) is a cytotoxin that can destroy white blood cells and cause tissue necrosis and severe infection. Firstly, this toxin was detected by Panton and Valentine in 1932. This toxin, as a virulent factor, is a member of synergohymenotropic toxins family, which creates pores in the leukocytes of the host. Fortunately, PVL is produced by fewer than 5% of S. aureus strains (7).The emergence and progress of MRSA in hospital and health care units can worry the clinicians due to treatment failure. However, those MRSA strains that are PVL positive can cause life-threatening infections with high mortality. So, frequent monitoring of the presence and distribution of these strains must be considered in the control and prevention programs. Furthermore, finding new antibacterial agents for fighting these infections is also of great importance. Natural remedy is one of the suitable options for this purpose; hence, there is low possibility of resistance in these strains against active constituents of medicinal plants. Our previous study on the antibacterial activity of *T. poluim* showed that this plant has a potential to be used for treatment of infectious diseases (2). Thus, the aim of this study was to investigate the antibacterial activity of T. polium methanolic extract against methicillin resistant *S. aureus* strains.

Materials and Methods

Sampling

S. aureus isolates were collected from the patients in three educational hospitals of Ahvaz, including Imam Khomeini, Golestan and Taleghni hospitals. These samples were selected from different wards of the mentioned hospitals including, OPD, dermatology, men, ophthalmology, nephrology, pediatrics, obstetrics, orthopedics, ENT, neonates and internal medicine. Samples were collected from the skin lesions, blood cultures, burns, intravenous catheters, wound drainages, abscesses, tracheal secretions, synovial fluids, ocular secretions and urines. These isolates were identified based on standard morphological and biochemical tests (8). In order to determine the methicillin resistance of isolates, MHA (Muller-Hinton Agar) screening test was used according to CLSI (Clinical and Laboratory Standards Institute) (9). Furthermore, PCR (polymerase chain reaction) assay was performed for *mec*A and *pvl* genes using specific primers, and the amplification was confirmed through electrophoresis in 1% agarose gel(10).The susceptibility of isolates to oxacillin was also surveyed by standard Kirby-Bauer disc diffusion method (11).

Extract preparation

The flowery parts of *T. polium* were shade dried at room temperature for ten days and then ground to a fine powder. One gram of the powder was extracted using 10 ml of methanol-distilled water solution (8:2 v/v), 1 min vortexing, centrifugation (3000 rpm, 15 min) and harvesting the supernatant. This process was repeated for three times. The solvents were then evaporated (2).

Antimicrobial sensitivity assay

The MRSA strains under study were cultured in Muller Hinton-Broth (Merck, Germany) medium to obtain 0.5 McFarland turbidity. Then, a lawn culture was prepared on Muller-Hinton Agar (Merck, Germany) medium using a sterile cotton swab. Different concentrations, including 100, 200, 400 and 600 mg/ml of methanolic extract were prepared. The sterile blank paper discs (6.4 mm diameter) were then saturated with these solutions. So, the effective concentrations of extracts in the discs were 4, 8, 16 and 24 mg, respectively. The discs remained at room temperature (30min) for solvent evaporation. Then, these discs were put on the lawn culture of bacteria. Simultaneously, one sterile paper disc was saturated with methanol and used to control the solvent. These media were incubated at 37˚C for 24 h, and then the inhibition zone diameter of the bacterial growth around each disc was measured and recorded (mm) (2).

Results

Fifty *S. aureus* isolates were obtained from different clinical samples. All of these isolates were resistant to methicillin and were regarded as MRSA. From these isolates, 20 isolates were selected for antibacterial susceptibility test, from which 14 isolates were positive for *mec*A gene, 2 of them were positive for pvl gene and 4 of them did not have *mec* or *pvl* genes (Figure 1). None of MRSA isolates was positive for both genes. (Table1).

Table 1. The results of *mec* A and *pvl* genes screening in MRSA isolates

Gene	Isolate																			
	1	8	9	12	15	18	21	23	24	28	30	33	36	42	45	46	47	48	49	50
*mec*A	-	+	+	-	-	+	+	-	+	+	+	+	-	-	+	+	+	+	+	+
pvl	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-

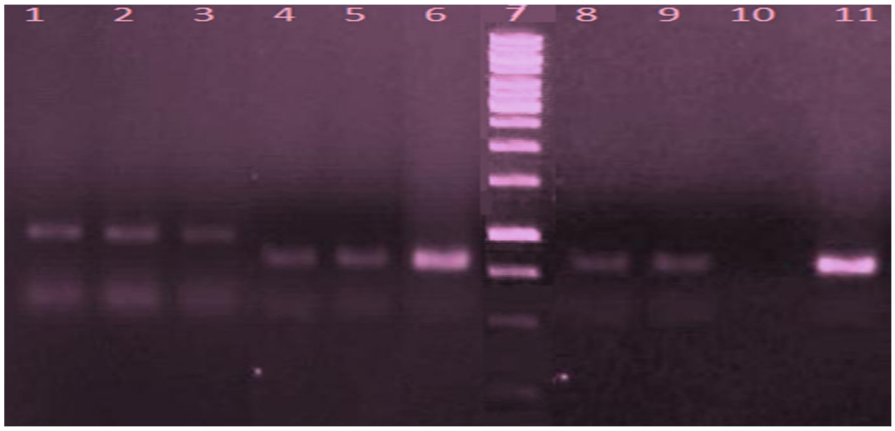

Figure 1. Analysis of PCR products of mecA (314 bp) and pvl (433 bp) amplification on 1% agarose gel. 1: positive control for pvl; 2 , 3: positive *pvl* clinical isolates; 4, 5, 6, 8 and 9: positive *mec*A in clinical isolates; 7:100bp molecular weight marker; 10: negative control (distilled water) and 11: positive control for mecA.

These isolates were subjected to susceptibility test against methanolic extract of *T. polium*. The results of growth inhibition are presented in Table 2.

Table 2. Antibacterial activity (inhibition zone) of *T. polium* methanolic extract against MRSA isolates

Isolate number	Effective dose mg/Disc			
	4	8	16	24
1	30.5*	26	28	29
8	24	22	24	23
9	24.5	24	28	28.5
12	28	28	27	27
15	29.5	29	32	31
18	34	34	34	34
21	23.5	22.5	27	29
23	26	29	30	28
24	23	23	24	28
28	25	24.5	25.5	26.5
30	23	25	26	24
33	34	28	29	32
36	20.5	24	25	23
42	18.5	18	22	22
45	34	30	32	31.5
46	30	31	30	29.5
47	25	29.5	28	28
48	25	26	29	27
49	31	28	35	33
50	28	28	29	30

* Inhibition zone diameter (mm), Disc 6.4 mm

As it can be found from the obtained results, in spite of methicillin resistance of the isolates, significant inhibition was found in different concentrations of *T. polium* methanolic extract; The inhibition zone was noticeable even at lowest concentration. Furthermore, while *mec*A and *pvl* negative strains mainly showed more sensitivity to methanolic extract, the presence of *mec*A did not have any significant effect on resistance to methanolic extract of *T. polium*. In contrast, *pvl* positive strains had less sensitivity (smaller inhibition zone) to this extract, i.e. the presence of this virulent factor made MRSA strains more resistant than *pvl* negative strains.

Discussion

Nowadays, increased resistance of pathogenic microorganisms to antibiotics has caused a challenge in public health and treatment of infectious diseases. It forces pharmaceutical industries to seek new antibacterial agents. Medicinal plants are good alternatives because these native resources have fewer side effects, are cheap and usually have a broad spectrum of activity against gram positive and gram negative bacteria. In many regions of the world, the extracts of medicinal plants are utilized owing to their antibacterial, antifungal and antiviral activities (12). *T. polium* is one of the most important medicinal plants that is mostly consumed in traditional medicine in Iran. The substances found in this plant include alkaloids, triterpens, flavonoids, glycosides, sterols, tannin and saponin. Flavonoids are one of the broadest groups of phenolic substances (13). Phenolic substances have the capacity to be chelated with metals; hence, reducing their reactivity by forming an inert metal-ligand chelation of transition metals (such as iron) and decreasing their bioavailability for bacteria (14). Plant tannin has antimicrobial activity, and the central wood of many trees, as a result of high concentrations of tannins, can prevent bacterial and fungal decay (13). Furthermore, , many compounds such as α-pinene, β-myrcene, cadinol, myrtenal,

limonene are found in the essential oil of this plant, and α-pinene can probably play an important role in the antibacterial activity of *T. polium* (15). In this study, the antibacterial activity of *T. polium* against MRSA was investigated. The results were hopeful owing to the significant inhibitory effect of methanolic extract of *T. polium* against methicillin resistant strains. This extract was even effective against *mec*A or *pvl* positive strains but with less inhibition zone in comparison with *mec*A or *pvl* negative strains. These data indicate that active constituents of this medicinal plant are good candidates for finding new anti-staphylococcal agents and infection control. Presently, MRSA infections are life-threatening infections that can be acquired from hospital or community. These strains are nearly resistant to the majority of available antibiotics, and finding new and effective antibacterial agents that can fight these infections is of great importance. The antibacterial properties of *T. polium* have also been investigated by other researchers. Tabatabaei Yazdi and Alizadebehbahani (2013) investigated the antimicrobial effects of aqueous and ethanolic extracts of *T. polium* (2000 µg/ml concentration) on *Streptococcus pyogenes* and *Staphylococcus epidermidis* (16). Mashreghi and Niknia (2012) surveyed the alcoholic extracts of *Peganum harmala* and *Teucrium polium* on the growth of *Escherichia coli* O157 and found that 0.3 mg/ml concentration had the optimum effect on E. coli O157 (17). The methanolic extract of *T. polium* was studied for its antioxidant activity by Belmekki et al. (2012). Their results showed that this plant is a source of polyphenols and flavonoids. They also confirmed their antioxidant activities and highlighted their potential to be used either as natural preservatives or as pharmaceuticals. The essential oil of Algerian *T. polium* L. was extracted and tested against some bacteria and fungi. This study showed that the essential oil of this medicinal plant exhibited moderate inhibitory effects on Bacillus cereus, *Enterococcus faecalis*, *E. coli* and *S.aureus* with minimum inhibitory effects at concentrations of 3 to 5 µg/ml (5). In spite of several researches on *T. polium*, no similar study was found that had investigated the antibacterial effects of this plant against MRSA and had assessed their relation to antibiotic resistance and virulent genes, i.e, *mec*A and *pvl*, respectively.

Resistance to methicillin is caused by *mec*A gene which encodes the low-affinity penicillin-binding protein PBP2a. The *mec*A gene is part of a 21- to 60-kb staphylococcal chromosome cassette mec (SCC mec), a mobile genetic element that also contains genetic structures such as Tn554, pUB110, and pT181, which encode resistance to non-β-lactam antibiotics (6).

PVL form pores in the membrane of host leukocytes, by synergistic action of 2 secretory proteins, indicated LukS-PV and LukF-PV, which are encoded by 2 co-transcribed genes of a prophage integrated in the S. aureus chromosome (18). PVL is mostly associated with community-acquired methicillin-resistant *S. aureus* (MRSA) infections and is recognizable from nosocomial MRSA by non-multidrug resistant and carriage of the type IV staphylococcal chromosome cassette element (SCC mec type IV) (19, 20).

Darabpour et al (2010) explored the antibacterial effect of the alcoholic extracts obtained from aerial parts of *T. polium* on some pathogenic bacteria by disc diffusion method. They found the minimal inhibitory concentration (MIC) of this extract against S. aureus as 40 mg/ml, and reported the methanolic extract of *T. polium* has synergistic effect with methicillin and vancomycin against *S. aureus* (2). Also many researchers have investigated the antibacterial activity of this plant against MRSA and other infectious bacteria such as E. coli, *Pseudomonas aeruginosa* and so on (21, 22, 23 and 24). Thus, *T. polium* extract can be effective for treatment of infectious caused by MRSA.

In conclusion, based on the obtained results in this study, it can be suggested that *T. polium* can be used as a source to discover new antibacterial agents, especially against MRSA strains. Furthermore, the presence of *pvl* gene can cause less sensitivity of test strains to antibacterial agents. So, it is preferred to screen all MRSA isolates for *mec*A and *pvl* genes for epidemiologic and infection control purposes.

Acknowledgement

The authors wish to thank the vice chancellor of research of Shahid Chamran University of Ahvaz for providing the research grant (Grant No. 874095).

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