INTRODUCTION

Furunculosis (boils) is a common skin disease caused by *Staphylococcus aureus*. This infection is characterized by a deep inflammation of the hair follicle. It leads to abscess formation with accumulation of pus and necrotic tissue. Treatment with antibiotics is often ineffective and patients often suffer from recurrent episodes. The aim of the study was to determine the antibacterial activity of caraway essential oil (CEO) against *S. aureus* isolated from patients with furunculosis. *S. aureus* strains were characterized by a different virulence factors and resistance to antibiotics. Effect of CEO was evaluated against 15 strains of *S. aureus* isolated from patients and against 3 control strains. The susceptibility to antibiotics was determined using Kirby-Bauer disk diffusion method. The presence of genes encoding virulence factors was determined with PCR method. The presence of slime was examined with Congo red method. The composition of the CEO was evaluated by gas chromatography method with mass selective detector. The minimum inhibitory concentration (MIC) of CEO was determined by serial dilutions in tryptic soy broth containing 10% NaCl. Analysis of the oil composition showed that the predominant component was cuminaldehyde (46.7%). CEO showed inhibition activity against control strains and patients strains. The MIC values of essential oil ranged from 10 to 12 μL/mL and from 7 to 67 μL/mL, respectively. Lack of correlation between the antibiotic resistance of the bacterial strains, presence of genes, presence of slime and their sensitivity to essential oil was found. CEO can be used as alternative antibacterial agent in supportive treatment patients with furunculosis.

Keywords: *Staphylococcus aureus*, *Carum carvi*, caraway oil, essential oil, furunculosis
aerobic atmosphere for 24 h. \textit{S. aureus} strains were identified on the basis of colonies morphology, positive catalase test, positive Staph-Kit test (bioMérieux, Poland) and positive coagulase test (Institute of Biotechnology, Sera and Vaccines, Biomed, Poland).

\textit{S. aureus} ATCC 29213, \textit{S. aureus} ATCC 29213 and \textit{S. aureus} ATCC 43300 were used as a control strains, the property of Department of Microbiology and Diagnostic Immunology, Pomeranian Medical University in Szczecin.

Detection of slime production by light microscopy

Slime production of \textit{S. aureus} isolates was evaluated by Congo red method with modification (crystal violet as a positive dye), according to the protocol of Korres \textit{et al.} (2013). Cultures \textit{S. aureus} were incubated in trypticase soy broth (TSB) (Difco, USA) at 35±1°C for 24 h. After incubation, 2 drops of the bacterial suspension were applied onto a clean glass slide. Nystatine was smeared on a glass slide, washed with distilled water and air-dried. Slides were smeared with Congo red solution (1 min) and air-dried. Afterwards, slides were examined with optical microscope (Olympus, Japan) and photographed. As result positive was indicated by a colourless halo around the bacterial cells against a pink background.

DNA isolation

Total DNA of \textit{S. aureus} was isolated with a DNeasy Blood and Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. DNA was stored at 4°C.

Detection of \textit{S. aureus} virulence factors by PCR

Set of 5 Multiplex PCR was established to detect the genes as follows: a) sea, seh, sec and tst, b) see, seb, sem and sel and c) sep, efish, etd and eta sef d) sei, ser, seu and e) sen, seq, seq and seq2, as reported previously (Holtfreter \textit{et al.}, 2011). Single PCR was performed for the detection PVL ( luk-PV), methicillin-resistant \textit{S. aureus} (MRSA) (meca) and exfoliative toxin B (etb). Single and Multiplex PCRs were performed with the GoTaq Flexi DNA Polymerase System (Promega, USA), as described previously (Holtfreter \textit{et al.}, 2011). Amplification was performed in a Thermocycler Perkin Elmer Gene Amp System 9600 (Applied Biosystems, USA). DNA was amplified with the following thermal settings: the initial denaturation (2 min, 94°C); 35 cycles of annealing at 55°C (94°C, 15 s; 55°C, 20 s; 72°C, 40 s), final extension (74°C, 10 min). The amplified DNA was purified with QIAquick purification kit (Quagen, Germany). All PCR products were resolved by electrophoresis in 1.5% agarose gels (Sigma Aldrich, Germany) in 1xTris borate-EDTA buffer (BioRad, France), stained with ethidium bromide (Sigma Aldrich, Germany), and visualized under UV light. 100-1500 bp DNA Ladder (Promega, USA) was used for precise sizing of PCR products. \textit{ÁS} Positive controls used reference strains (Baba \textit{et al.}, 2002; Wu \textit{et al.}, 2010; Holtfreter \textit{et al.}, 2011).

Essential oil analysis

CEOs used in this study were obtained from Vera-Nord Company, Poland (commercial producer of plant essential oils and aromatic substances). The oil exhibited a strong and characteristic odor. It was intended to the production of cosmetics and household chemical products.

The analysis of CEO composition was performed by gas chromatography method with mass selective detector (GC-MS) using an Agilent 6890N gas chromatograph with a 5973N mass selective detector. The resolution of analytes was achieved using a HP-5MSi column (5% phenyl/95% dimethylpolysiloxane), 30 m o.25 mm id, and 0.25 µm film thickness. The column temperature was programmed as follows: initial temperature 60°C, ramp rate 8°C/min, final temperature 300°C (hold 5 min). Helium was used as carrier gas at a flow rate of 1.2 mL/min. The injector temperature was set at 250°C, MS quad: 150°C, MS source: 230°C. Mass spectra were obtained using electron impact ionization at 70 eV in full scan mode (mass range: 20–500 m/z).

Before the analysis 100 µL of tested essential oil was dissolved in 1 mL of acetone (p.a.). The identification of the CEO components was based on the comparison of their mass spectra with the reference spectra from NIST 02 library. The relative contents of the particular compounds in essential oil were their peak area percentages in a total ion chromatogram.

Screening susceptibility of bacteria to antibiotics

The antimicrobial susceptibility of \textit{S. aureus} isolates was performed in accordance with the European Committee on Antimicrobial Susceptibility Testing recommendations (EUCAST, 2013). Susceptibility to: clavulanic acid - CIP (5 µg disk), ciprofloxacin - G (10 µg disk), trimethoprim-sulfamethoxazole - SMX (25/25,75 µg disk), carbenicillin - M (10 µg disk) and cefoxitin - FOX (30 µg disk) (Becton Dickinson, USA) was evaluated with disk diffusion method performed with Mueller-Hinton agar (MHA) (bioMérieux, Poland) inoculated with a suspension (1.5x10^7 CFU/ml) of the \textit{S. aureus} isolates. The plates were incubated at 35±1°C for 18±2 h and inhibition zones were measured. Strains resistant to FOX were considered as MRSA. The D-test was performed with clindamycin - CC (2 µg disk) and erythromycin - E (15 µg disk) (Becton Dickinson, USA). These disks were placed 20 mm apart on the MHA plate seeded with the test strain. \textit{S. aureus} strains resistant to CC and E were considered to have constitutive macrolides, lincomides and streptogramins B resistance (cMLSb) phenotype. More resistant strains with flattening of the susceptible zone of inhibition to CC adjacent to the E disk (D-shape) were considered to exhibit resistance inducible phenotype to macrolides, lincomides and streptogramins B (iMLSb). Strains with circular zone around CC were considered to exhibit MLSb phenotype (macrolides and streptogramins B resistance) (Sadri \textit{et al.}, 2011).

Broth microdilution method - determination of the minimum inhibitory concentration (MIC)

The microdilution test was conducted in 96-well plates according to Urbanik \textit{et al.} (2014) with some modification. A dilution series of the CEO was obtained using 1% Tween 80 (Difco, USA) solution as the solvent. The final concentrations were 100-0.5 µL/mL. Each well received 100 µL of the specific concentrations of the CEO and TSB with addition 10% NaCl (Chempur, Poland) inoculated with 10 µL bacterial suspension (1.5x10^6 CFU/mL). The positive solvent control was completed with 100 µL of 1% Tween 80 solution. The final volume in each well was 110 µL. The microplates were covered with parafilm and incubated for 24 h at 35±1°C. Inhibition of bacterial growth was confirmed by cultivation preincubated plates on Columbia agar with addition 5% sheep blood. The MIC values were defined by the lowest concentration of the CEO that inhibits the growth of the microorganism. The control wells solvent does not affect the growth of all tested bacterial strains. Each MIC test was conducted with three replicates.

RESULTS AND DISCUSSION

Chemical composition of the CEO

The results of qualitative and quantitative analysis of CEO purchased from Vera-Nord Company are shown in Table 1. The main constituent of essential oil was cuminaldehyde (46.7%) followed by ß-pinene (10.3%), durene (9.7%), Ï-terpinene (8.9%) and limonene (4.7%). A total of 17 components were identified in the oil, accounting for 84.2% of the total oil (Table 1).

It has been proven that the main components of essential oil depends on many environmental and genetic factors and oil extraction method (Ačmiovic \textit{et al.}, 2014). A number of studies examining the essential oil content and composition of CEO have been performed. For example, research conducted by Dawidar \textit{et al.} (2010) showed that Egyptian commercial essential oil from \textit{C. carvi} L. contained D-carvone (42.61%) and D-limonene (33.53%) as its two main components. D-carvone and limonene were also found to be the main components of the commercial CEOS provided by three independent companies (Etol, Dragoco and Poliena-Aroma) (Silmie \textit{et al.}, 2008, Dimić \textit{et al.}, 2012; Gniewosz \textit{et al.}, 2013). Our studies demonstrated different results than these experiments in which essential oils did not contain cuminaldehyde – the main component identified in commercial CEO provided by Vera-Nord Company (Table 1). However, research conducted by Razzaghzai-Abyaneh \textit{et al.} (2009) showed that cuminaldehyde was present in essential oil extracted from \textit{C. carvi} seeds. The caraway oil from Iran contained cuminaldehyde (22.08%) and Ï-terpinene (17.86%) as its two main components, followed by p-cymene (7.99%).

Presence of slime

18 \textit{S. aureus} strains were examined with Congo red method. The slime production of \textit{S. aureus} was recorded in 12 (66.7%) isolates (Table 2). On the microscopic slides, a spherical, Gram-positive cocci were surrounded by a well-defined halo on a pink background (Figure 1). Biofilm formation is important ability of bacteria and plays essential role in increased resistance to antimicrobial agents (Podbieska \textit{et al.}, 2010). In addition, slime interferes with phagocytosis and enhances adhesion to host tissue and inanimate objects. According to Gündoğan \textit{et al.} (2006) there is lack of correlation between antibiotic resistance and slime production among \textit{S. aureus} observed. The results showed that not all of resistance strains produced an extracellular substances. In addition, we noticed that 6 susceptible strains also produced slime (Table 2).

In our study \textit{S. aureus} extracellular matrix did not affect on the MIC values. Both high and low concentration CEO did not impact on the presence of slime. It may indicate that slime did not play significant role in CEO attack on the bacteria surface.
In total, 80% (12/15) of the furunculosis strains were luk-PV positive and 13.3% (2/15) were mecA positive (Table 2). S. aureus produces variety of virulence factors, according to available data only PVL is associated with furunculosis formation (Demos et al., 2012). Research performed by Masnik et al. (2010) showed that most of methicillin-susceptible S. aureus (MSSA) isolates from patients with furunculosis harbored luk-PV genes. These same conclusions are reported in other reviews (Yamasaki et al., 2005; Cupane et al., 2012). The presence of genes of exfoliative toxins eta, etb, etd and toxic shock syndrome toxin gene (tst) was not confirmed (Table 2). Exfoliative toxins A and B (ETA and ETB) are exotoxins produced by S. aureus, which are involved in staphylococcal scalded-skin syndrome (SSSS) and bullous impetigo (Jursa-Kulesza et al., 2009). In our study no ETA and ETB was observed. Study conducted by Bukowski et al. (2010) shown that ETD-producing S. aureus strains are mainly isolated from furuncles or abscesses, but not from SSSS. However, in our research all isolates were etd negative. Among enterotoxins genes (SEs) (sea to seu) only seb and sel were detected. Among all tested strains, 33.3% (5/15) were seb positive and only 1 strain (6.7%) was sel positive (Figure 2, Table 2). These genes are located on a pathogenicity island such as SaIP3 (seb, sek, seq, in seb, sel and sek), SaPlm1/in1 (tst, sec and sel) and SAPI50 (tst, sec and sel) (Yamamoto et al., 2013). Enterotoxin B is mainly responsible for food poisoning outbreaks and can cause toxic shock syndrome (Pinchuk et al., 2010; Karauzum et al., 2012). A recent study performed by Sina et al. (2013) showed that S. aureus strains isolated from skin and soft tissue infections (STTI) such as furuncles, skin abscesses and cellulitis; and bone infections often harbored seb. Moreover, research conducted by Masnik et al. (2010) indicated that seb was present among about 25% strains isolated from furuncles.
According to Peter (2012) CEO extracted from C. carvi L. is generally safe for internal and has no toxic effect. Although it may cause skin irritation if used in high concentration. Moreover, CEO can irritate the eyes. Therefore, CEO should not be used directly on the skin. In addition, study performed by Morschedi et al. (2015) showed that cumuladhyde (the main component identified in commercial CEO provided by Vera-Neid Company) is a nontoxic compound. These authors demonstrated no toxic effect on the cells.

CONCLUSION

There were no significant differences in MIC values depending on the susceptible/resistance to antibiotics, virulence genes and presence of slime. Commercial CEO has inhibitory effects on growth of S. aureus strains isolated from patients with furunculosis, regardless of the degree of resistance to antibiotics and virulence of the strain. CEO can be used as an alternative antibacterial agent in the treatment of furunculosis (especially in persons suffer from recurrent episodes).

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REFERENCES


