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Antibacterial and anti-PmrA activity of plant essential oils against fluoroquinolone resistant *Streptococcus pneumoniae* clinical isolates

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Running head: Antibacterial and anti-PmrA activity

Significance and Impact of the study: The present study introduced *Thymus daenensis and Origanum vulgare* essential oil as new antibacterial and anti-efflux pump agents against fluoroquinolone resistant *S. pneumoniae* clinical isolates. These findings indicate that combination of these tow essential oils with fluoroquinolone antibiotics may provide alternative methods to overcome the fluoroquinolone resistant *S. pneumoniae*.

Abstract

Streptococcus pneumoniae (pneumococcus) is recognized as one of the major cause of infections in communities and hospitals. In this study, anti-pneumococcal and anti-efflux pump activity of two medicinal plants (*Thymus daenensis* and *Origanum vulgare*) essential oils were evaluated. Checkerboard assay test was performed for investigation of the effects of selected EOs on ciprofloxacin and ethidium bromide uptake in *pmrA* overexpressed fluoroquinolone resistant pneumococcus. Using quantitative real-time RT-PCR the PmrA efflux pump gene (*pmrA*) expression was evaluated following treatment with selected EOs. Gas Chromatography-Mass Spectrometry analysis was performed for identifying the major components of the tested EOs. The MIC values for pneumococcus isolates were 0.625-2.5 $\mu l.ml^{-1}$ for *T. daenensis* and 1.25-5 $\mu l.ml^{-1}$ for *O. vulgare* EOs. We confirmed that in all strains *T. daenensis* and *O. vulgare* have a total or partial synergistic effects with ciprofloxacin and ethidium bromide (FICI from 0.14 to 0.75). In other hand MIC/2 concentration of *T. daenensis and O. vulgare* EOs caused a significant downregulation of *pmrA* gene ($P<0.05$) in 7 of 8 strains. This study showed that *T.*

daenensis and O. vulgare EOs have strong antimicrobial and anti-efflux pump activity against clinical isolates of *S. pneumoniae* and might be useful in controlling pneumococcal infections.

Keywords: *Streptococcus pneumoniae*, efflux pump, plant essential oils, *Thymus daenensis*, *Origanum vulgare*

Introduction

Streptococcus pneumoniae (pneumococcus) is one of the most important causes of respiratory infections, including sinusitis, otitis media, pneumonia, and invasive infections such as septicemia and meningitis. Infections caused by this bacterium have been related with increased morbidity and mortality, especially in children under two years of age and elderly adults (AlonsoDeVelasco et al., 1995; Henriques-Normark and Tuomanen, 2013). Recently, antibiotic resistance in pneumococcus is increasing worldwide, especially relating to β-lactams and macrolides (El Garch et al., 2010). Epidemiological studies show that in countries with high level of antibacterial resistance and consumption, fluoroquinolone resistance has started to emerge (Takeuchi et al., 2017). Resistance to the fluoroquinolones in pneumococcus is known to occur by two mechanisms. Chromosomally mediated resistance may occur through mutation in the genes coding for both subunits of DNA gyrase (*gyrA* and *gyrB*) and/or topoisomerase IV (*parC* and *parE*). Resistance to this antibiotic may also occur through the action of efflux pumps (Gill et al., 1999).

Bacterial efflux pumps are proteinaceous transporter structures localized in the cytoplasmic membrane of almost all bacterial cells. These structures contribute to resistance to a wide range of antibiotics (Poole, 2007). The overexpression of multidrug efflux pumps in bacterial cells cause to resistance to various antibiotics, including fluoroquinolones, some dyes (e.g., ethidium bromide), detergents (e.g., sodium dodecyl sulfate [SDS]), and disinfectants (e.g., cetrimide) (Piddock, 2006). Efflux pumps not only can export a broad range of antibiotics relation to their specific poly-substrate, but also have an important role in the acquisition of additional resistance mechanisms by lowering intracellular antibiotic concentration and promoting mutation accumulation (Piddock, 2006; Poole, 2007).

PmrA (pneumococcal multidrug resistance protein) pump a member of the major facilitator superfamily, which encoded by *pmrA*, is associated with fluoroquinolone resistance in pneumococcus. (Gill et al., 1999).

The problems of bacterial resistance highlight the urgent need for discovery of new drugs with new modes of action and/or combination therapy to treatment of infections caused by resistant human and animal pathogens (Piddock, 2006). Relying on critical importance of efflux pumps in antibiotic resistance, it is clear that efflux pumps can be targets for new antimicrobial agents. Recently the use of efflux pump inhibitors (EPI) has been investigated to improve and potentiate the activity of exported antibiotics such as fluoroquinolones (Brenwald et al., 1997). Previous studies reveal that among various agents with EPI activity plant-derived compounds play a critical role. This potential is due to the enormous compound diversity, low toxicity and high tolerability (Stavri et al., 2006).

We hypothesized that essential oil from plants that are used as herbal medicines contain molecules that act as EPIs against the fluoroquinolone efflux pumps of pneumococcus bacteria. Accordingly, the present study phenotypically and genotypically evaluates the PmrA EPI activity of tow plant essential oil; *Thymus daenensis* and *Origanum vulgare* on fluoroquinolone resistant pneumococcus clinical isolates. These plants were chosen as they have a use in various systems of medicine and some are used as topical antimicrobials.

Results and discussion

GC-MS analysis

The yields of the EOs were 3.9% and 4.93% for *T. daenensis* and *O. vulgare*, respectively. The essential oil obtained from leaves of tested plants was analyzed by gas GC-MS and 33 compounds representing 99.5% of the essential oil were identified. Carvacrol (40.69%), γ-terpinene (30.28%) and α-terpinene (5.52%) were the most components of *T. daenensis* fresh leaves. For *O. vulgare* EO, the main compounds were pulegone (44.31%), 1,8-cineole (17.47%), and borneol (6.20%).

Antibacterial activity test, by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for essential oils

In vitro bacteriostatic and bactericidal activities of the tow selected essential oils were evaluated on pneumococcus strains by broth microdilution method. The results of antibacterial test showed that both *T. daenensis* and *O. vulgare* have good potential for pneumococcus growth inhibition and also cell disruption against clinical isolates and standard strain. The MIC values were 0.625-2.5 $\mu l.ml^{-1}$ for *T. daenensis* and 1.25-5 $\mu l.ml^{-1}$ for *O. vulgare* essential oils. The MBC values were 1.25-2.5 and 2.5-10 $\mu l.ml^{-1}$ for *T.*

daenensis and *O. vulgare* essential oil, respectively. *T. daenensis* and *O. vulgare* plants have been used in Iran medical systems since they possess potent antibacterial other medicinal properties. Despite some publications on the antimicrobial activity of these EOs (El Gendy et al., 2015; Fadli et al., 2012; Zarshenas and Krenn, 2015), to our knowledge, this is the first investigation of the antimicrobial and anti-efflux pump activity of the leaves oil of tested plant EOs against fluoroquinolone resistant pneumococcus clinical isolates. The results of microdilution test revealed that each tow tested EOs have a good antibacterial activity against pneumococcus clinical isolates and the essential oil of *T. daenensis* was found to be more effective (Table 1). This potential is due to presence of antimicrobial components such as carvacrol (40.69%), γ-terpinene (30.28%) and α-terpinene (5.52%) for *T. daenensis* and pulegone (44.31%), 1,8-cineole (17.47%), and borneol (6.20%) for *O. vulgare*. This was in agreement with the previous studies which reported that EOs containing these phenolic compounds have a strong antimicrobial activity (Alma et al., 2003; Dorman and Deans, 2000; Ertas et al., 2015). The main targets of these components to kill bacteria or inhibition their growth are cell wall, cytoplasmic membrane, and proteins embedded in the membrane (Dorman and Deans, 2000). This component can also disrupt bacterial lipids, RNA synthesis, ATPase activity and efflux pumps (Tapia-Rodriguez et al., 2017).

Checkerboard assay to efflux pump inhibitory activity

In this study we used 7 clinical isolates and one standard strain for study of potential synergistic effects of EO from *T. daenensis* and *O. vulgare* with ciprofloxacin (CIP) and ethidium bromide (EtBr). Using checkerboard method we confirmed that in all strain *T. daenensis* and *O. vulgare* have total or partial synergistic effect with CIP and EtBr. (FICI from 0.14 to 0.75). None of the strains showed "no effect" or "antagonistic effect" (Table 2). The checkerboard assay was used to study the combinations of antibiotics (CIP) or

EtBr and potential EPIs as a screening method. The significant reduction to MICs of CIP and EtBr in *pmrA* overexpressed strain by specific agents such as EO can be considered as *pmrA* efflux pump inhibitory (Stavri et al., 2006). In the present study the synergistic (total synergism or partial synergism) effect among all tow tested EOs with CIP and EtBr was observed in all strains, suggesting that these natural compound interacts with PmrA pump. Drug synergisms between chemical antibiotics and plant EOs have been published previously (Stavri et al., 2006). The plant alkaloid reserpine was first isolated from the roots of *Rauwolfia vomitoria* Afz. This compound inhibits tetracycline efflux in *Bacillus subtilis* (Neyfakh et al., 1991) and NorA efflux pump in multidrug resistance (MDR) *S. aureus* (Neyfakh et al., 1993). Negation of fluoroquinolone resistance in *S. pneumoniae* has also been reported in the presence of reserpine (Brenwald et al., 1997).

Quantification of gene expression by real-time RT-PCR

The effect of sub-MIC concentrations of *T. daenensis* and *O. vulgare* EOs on the expression of *pmrA* gene was measured by clinical isolates and reference strain. MIC/2 concentration of *T. daenensis and O. vulgare* EOs caused a significant down regulation of *pmrA* gene ($P<0.05$) in 7 of 8 strains (Figure 1). The real time PCR revealed that 1/2MIC concentration of the *T. daenensis* and *O. vulgare* were significant downregulated *pmrA* gene in 7 of 8 strains. This result confirmed the checkerboard finding, suggesting inhibitory activity of selected plant EOs on the PmrA efflux pump. According to the important role of this pump in resistance of pneumococcus to the fluoroquinolones antibiotics, these findings can be more valuable. In subject to expression of genes coding for efflux pumps, Chovanova´ et al. 2015, showed markedly repressed of *tet*(K) gene of *Staphylococcus epidermidis* upon treatment with *Salvia fruticosa* EO (Chovanová et al.,

2015). Since EPI are able to restore the effectiveness of antibiotics which are no longer available for therapy, these agent groups is a promising tool to combat bacterial resistance (Stavri et al., 2006).

In conclusion, the present study introduced *T. daenensis* and *O. vulgare* EOs as effective herbal agents for antibacterial and anti-PmrA efflux pump activity at sub-MIC concentrations and good candidate for combination therapy against pneumococcus. However, more efforts are required to conduct clinical trials of these EOs in the future.

Materials and methods

Bacteria strains and culture conditions

Seven pneumococcus clinical isolates which had previously been characterized as fluoroquinolone resistant were included in the present study (Azadegan et al., 2015). The pneumococcus isolates have been collected from patients admitted to the city hospitals and private laboratories in Tehran, Iran, over a period of 24 months, from 2011 to 2013. Fluoroquinolone resistance determination was carried out using disk diffusion method and microdilution broth for CIP antibiotics according to the guidelines of the Clinical and Laboratory Standard Institute and MIC $\geq$ 2 $\mu g.ml^{-1}$ was considered as fluoroquinolone resistance (Wayne, 2007). Determining of *pmrA* related efflux pump activity in tested strain was performed by real time PCR for *pmrA* expression according to section 2.5. *S. pneumoniae* NCTC 7466 was used as standard strain. The bacteria were routinely grown in tryptic soy broth (TSB; BD Difco; Merck, Germany) or on blood agar plates supplemented with 5% v/v sheep blood at 37°C in an atmosphere of 5% CO2.

Essential oils and GC-MS analysis

Thymus daenensis L. (herbarium code: MPH 2000), and *Origanum vulgare* L. (herbarium code: MPH 2215) were obtained from Shahid Beheshti Plant Institute, Tehran, Iran. The samples were dried in shadow at room temperature for 10 days and the essential oils were extracted from the dried leaves by the hydrodistillation method. Dried leaves of plants (50 g) were submitted to hydrodistillation, using Clevenger-type apparatus for 3 h, according to the standard procedure (El Gendy et al., 2015). The obtained EOs stored in a sealed dark vials, then kept at 4ºC prior to further analysis. These EOs were analyzed by GC-MS analysis using a Hewlett Packard 5972A mass selective detector coupled with a Hewlett Packard 6890 gas chromatograph, equipped with a cross-linked 5% PH ME siloxane HP-5MS capillary column (30m × 0.25 mm, film thickness 0.25 μm). The GC was done as below conditions: carrier gas, helium with a flow rate of 2 mL/min; column temperature, 60˚-275ºC at 4ºC/min; injector and detector temperatures, 280ºC; volume injected, 0.1 μL of the oil; split ratio, 1:25. The MS operating parameters were as follows: ionization potential, 70 ev; ion source temperature, 200ºC; resolution, 1000. Identification of components in the oil was based on GC retention indices relative to n-alkanes and computer matching with the Wiley 275.L library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature (Kabouche et al., 2009).

MIC and MBC for essential oils

The MICs of the plant essential oils were determined by broth microdilution method. First, two-fold serial dilutions of the EOs (0.312 to 40 $\mu l.ml^{-1}$) in TSB medium supplemented with 0.1% dimethyl sulfoxide (DMSO) were prepared to increase solubility of the EOs. Then, 100 μl of these solutions were distributed into each well of 96-well microtiter plates

(SPL, South Korea) and 100 µl of the bacterial inocula (corresponding to 0.5 of the McFarland) was added to each well. A negative control (spiked medium-DMSO and bacteria), and a positive control contained medium-DMSO, bacteria and vancomycin (0.1 mg/ml) were also applied. Plates were aerobically incubated at 37 °C in 5% CO_2 for 24 h. The MIC was defined as the lowest concentration of the EOs at which no visible growth was detected. For MBC determination, 5 µl of inoculums was incubated on Blood agar medium and incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration at which the original growth was reduced by ≥ 99.9% (Giweli et al., 2012).

Checkerboard method to efflux pump inhibitory activity

The checkerboard assay identifies synergic combinations of antimicrobial agents and has been used to screen for potential EPIs. The efflux pump inhibitory activity of plant EOs against pneumococcus *pmrA* overexpress was performed by this method. The MICs of CIP and EtBr in the absence or presence of plant EOs were determined by the microdilution method as described above. CIP and EtBr are substrates for the *pmrA* efflux pump and significant reduction in the MICs of these agents in *pmrA* overexpressed strains treatment with plant essential oils, considering as efflux pump inhibitory activity. For checkerboard assay twofold serial dilutions of CIP/EtBr prepared in horizontal rows of 96-well microtiter plate were subsequently cross-diluted vertically by twofold serial dilutions of each tested EOs (Figure 2). Microtiter plates were inoculated with pneumococcus strains and incubated for 24 h at 37∘C. MIC values of the combinations were determined as the lowest concentration that completely inhibited bacterial growth (Chovanová et al., 2016). The Fractional inhibitory concentration index (FIC_{index}) is defined as follows: MIC of substance A tested in combination/MIC of substance A tested alone + MIC of substance B

tested in combination/MIC of substance B tested alone. The FIC_I is interpreted as follows: FICI ≤ 0.5, total synergism; 0.5 < FIC ≤ 0.75, partial synergism; 0.75 < FICI ≤ 2, no effect; and FIC > 2, antagonistic effect (Fadli et al., 2012). Experiments were performed in triplicate.

Efflux pump gene expression in treatment with selected essential oils

RNA extraction and cDNA synthesis

To assess the effects of the EOs on the *pmrA* efflux pump, gene expression in the grown against 1/2MIC concentration of the EOs versus the grown without EOs were compared using a quantitative real-time RT-PCR assay. For RNA isolation, each bacterium was grown with and without of each EOs (MIC/2) in tubes contained TSB medium with DMSO (0.1%) and incubated at 37 °C for 24 h. The bacterial cells were harvested by centrifugation (5000 g for 5 min at 4°C) and immediately processed for RNA extraction using a commercial RNA extraction and purification kit (SinaClon, Iran) according to the manufacturer's instructions. The quality and quantity of the extracted RNA were determined by agarose gel electrophoresis and confirmed by measuring the absorbance at 260/280 nm using a Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific, USA). The extracted RNAs were stored at -70 °C until further experiments. Thereafter, the purified RNAs were reverse transcribed to cDNA using a commercial cDNA synthesis kit according to the manufacturer's manual (Takara, Japan) and the cDNA molecules were stored at -70 °C to use as DNA templates in the real time RT-PCR reactions.

Quantitative real-time RT-PCR

The real-time PCR assay was carried out using a commercial SYBR Green master mix (Amplicon, Denmark) and previously described pairs of primers. The sequences of the primers (BioNEER, Korea) are listed in Table 2. The reactions were conducted in a

Corbett Life Science Rotor-Gene 6000 Cycler (Qiagen, Germany) in 25 µL reaction mixtures containing 12.5 µL of SYBR Green Supermix (2×), 400 nM of forward and reverse primers and 5 µL of cDNA in RNase/DNase-free water. The *gyrA* housekeeping gene was considered as an internal control to normalize the expression levels of *pmrA*. The amplification proceeded as follows: denaturation at 95 °C for 10 min and then 40 cycles, including denaturation at 95 °C for 30 sec, annealing at 54 °C (for both *pmrA* and *gyrA*) for 30 sec, and 72 °C for 30 sec. A negative control was included in each run. The specificity of the real-time PCR was checked by a post-PCR melting-curve analysis performed under the following conditions: temperature starting at 60°C for 10 s followed by 0.5°C/10 s rises up to 95°C. All the samples were analyzed in triplicate and finally, relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Data are expressed as mean ± SD. The statistical calculations were performed using graphpad prism software. An unpaired Student's *t*-test was used to analyze the data. A P-value of 0.05 was considered statistically significant. All the experiments were repeated for three times.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Results of MICs of *T. daenensis* and *O. vulgare* EOs and FIC $_{index}$ for CIP and EtBr against *S. pneumoniae* strains.

		MICs of pneumococcus strains (µg.ml^{-1})				**FIC $_{index}$****			
Strain No.	Description	Thymus	Origanum	CIP	EtBr	(Thymus+ CIP)	(Thymus +EtBr)	(Origanum +CIP)	(Origanum +EtBr)
1	CIP resistance*	0.625	2.5	5	5	0.37^{a}	0.51^{b}	0.7^{b}	0.75^{b}
2	CIP resistance	1.25	5	5	10	0.14^{a}	0.32^{a}	0.62^{b}	0.75^{b}
3	CIP resistance	2.5	5	10	20	0.14^{a}	0.4^{a}	0.25^{a}	0.37^{a}
4	CIP resistance	1.25	2.5	10	20	0.22^{a}	0.37^{a}	0.25^{a}	0.7^{b}
5	CIP resistance	1.25	2.5	5	20	0.37^{a}	0.62^{b}	0.22^{a}	0.51^{b}
6	CIP resistance	0.625	1.25	2.5	5	0.37^{a}	0.75^{b}	0.56^{b}	0.75^{b}
7	CIP resistance	0.625	2.5	5	5	0.37^{b}	0.51^{b}	0.37^{a}	0.75^{b}
NCTC 7466	CIP susceptible	0.625	2.5	1.25	2.5	0.62^{b}	0.51^{b}	0.51^{b}	0.62^{b}

* MIC $\geq$ 2 µg.ml^{-1}. ** (a: total synergism, b: partial synergism, c: no effect, d: antagonism)

Table 2. Primers used in the present study

Genes	Sequence (5′-3′)	Reference
pmrA	TCCAGTATGGGCTTTTCCAG CCAATCCAAAGAGGAAACGA	(El Garch et al., 2010)
gyrA	CCTGTTCACCGTCGCATTCT AGTTGCTCCATTAACCA	(El Garch et al., 2010)

Figure 1. *PmrA* gene expression in bacterial treated with 1/2MIC of *T. daenensis and O. vulgare* essential oils; a: significant downregulation; b: non-significant downregulation.

Figure 2. The schematic checkerboard method showing the synergy of CIP/EtBr and tested EOs. (shaded cells indicate growth and unshaded cells no growth, PC: positive control and NC: negative control).