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PHENOTYPIC AND MOLECULAR EVALUATION OF *PSEUDOMONAS AERUGINOSA* AND *STAPHYLOCOCCUS AUREUS* VIRULENCE PATTERNS IN THE PRESENCE OF SOME ESSENTIAL OILS AND THEIR MAJOR COMPOUNDS

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Abstract

This study reports the effect of some essential oils (EOs) and of some of their major fractions on soluble virulence factors and quorum sensing (QS) gene expression profiles of 15 Staphylococcus aureus and Pseudomonas aeruginosa clinical strains. EOs were extracted from various angiosperm and gymnosperm vegetal taxons by hydrodistillation in a Neo-Clevenger-type apparatus and characterized by measuring the density and refractive index, as well as by gas chromatographic analysis. EOs and their major components proved to inhibit the phenotypic expression of six soluble virulence determinants (haemolysins, gelatinase, DN-ase, lipase, amylase, esculin hydrolysis) when used in sub-inhibitory concentrations, in both P. aeruginosa and S. aureus strains. EOs extracted from Salvia officinalis, Rosmarinus officinalis, Abies alba and Eugenia caryophyllata as well as some of their major compounds (limonene, eugenol and eucalyptol) inhibited QS genes expression in S. aureus, while in P. aeruginosa only E. caryophyllata EO proved to inhibit both las and rhl QS genes expression. Our results demonstrate that essential oils are efficient candidates for developing novel ecological antimicrobial strategies aiming to attenuate the pathogenicity and virulence of opportunistic pathogens isolated from severe infections.

Keywords *pore forming toxins, enzymes, RT-qPCR, quorum sensing genes*

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Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that infects primarily immunocompromised individuals, such as patients with cystic fibrosis, cancer, AIDS, or patients with indwelling medical devices or burns [1,2] by harboring a diverse spectrum of extracellular and cell-associated virulence factors [3].The coordinated expression of different virulence factors in *P. aeruginosa* strains is regulated by at least two well-defined, interrelated quorum sensing systems (QS) *las* and *rhl* [3,4,5]. The emergence of extended- and pan-resistant strains poses severe difficulties in the treatment of serious infections, and prompted the search for new anti-infective alternative strategies. *S. aureus* is an extremely versatile human pathogen responsible for a large spectrum of nosocomial and community-acquired infections, often with a chronic evolution and high mortality rates, due to an extraordinary repertoire of virulence factors that allow it to survive extreme conditions within the human host, to form biofilms on tissues and implanted medical devices and to invade the healthy tissues [6,7]. The management of *S. aureus*

infections is even more difficult due to the increasing number of methicillin resistant *S. aureus* (MRSA) and of multidrug resistant strains. The coordinated expression of *S. aureus* virulence factors during different stages of infection is regulated by a complex network of QS systems, one of them being the accessory gene regulator (*agr*) [8,9].

Since QS has a key role in the pathogenesis of opportunistic pathogens, such as *P. aeruginosa* and *S. aureus*, the inhibition of QS mechanisms by natural and synthesized chemical compounds has been recently suggested as an alternative anti-infectious strategy that circumvents the growing problem of antibiotic resistance, because it attenuates bacterial pathogenicity, without interfering with bacterial growth [10]. The antimicrobial activity of vegetal essential oils (EOs), evaluated qualitatively and quantitatively, is often their firstly demonstrated pharmacological property [11], but the specificity and complexity of EOs interactions with different components at cellular and molecular level are not fully understood. While investigating the mechanisms of the EOs antimicrobial activity, various and specific effects, including interference with the QS communication mechanism (e.g. cinnamaldehyde) were reported [11]. The *Ocimum gratissimum* oil (African basil) has been proved to inhibit exoenzymes with protease activity and the LPS expression. The eugenol inhibited listeriolysine O and *Mentha piperita* EOs reduced staphylococcal enterotoxin B production. Fungal microorganisms interact similarly with the EOs, their action sites being diverse and the mechanisms complex [12]. During this study, we have used phenotypic assays and qRT-PCR, to demonstrate the inhibitory activity of some EOs and of their major components on *P. aeruginosa* and *S. aureus* soluble virulence factors and QS genes expression.

Experiment Details

Vegetal material. For the EOs extraction and bioassays there were selected representatives of the plant species belonging to the angiosperm (families *Apiaceae, Lamiaceae, Myrtaceae, Rutaceae*) and the gymnosperms (*Pinaceae* family) groups, already known as EOs producing plants. The selection was performed after a rigourous literature screening. Vegetal material was obtained from local suppliers (*Rosmarinus officinalis*, *Mentha piperita*, *Foeniculum vulgare*, *Salvia officinalis*, *Eugenia caryophyllata*, *Citrus maxima*), spontaneous flora (*Picea abies*) and organic cultures (*Anethum graveolens* and trees of the *Pinaceae* family, i.e. *Abies alba*, *Pseudotsuga menziesii*, *Larix decidua*, *Piceaa abies*, *Pinus nigra*).

Microbial strains. In this study were used 30 clinical bacterial strains (15 belonging to *P. aeruginosa* and 15 *S. aureus* strains), isolated from biofilm- related infections in the Bacteriology Laboratory of the "Prof. C. C. Iliescu" Institute of Cardiovascular Diseases, Bucharest, Romania, during 2009-2010. Strains identification was performed by using automatic identification system Vitek II and API32 Staph and API 20NE galleries. The isolation sources of the selected strains are presented in Fig. 1. Tested bacterial strains have been previously characterized for the soluble virulence factors expression, i.e. pore-forming toxins (lecithinases, hemolysins, lipases) and exoenzymes (caseinase, amylase, DN-ase), siderophore-like factors, but also for their ability to adhere to inert substrata.

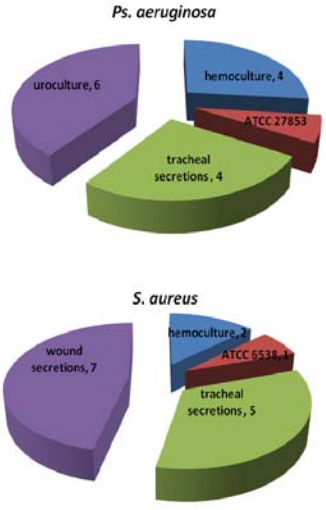

Figure 1: *Graphic representation of the isolation sources of the S. aureus and P. aeruginosa clinical strains*

Influence of the EOs on the expression of the soluble enzymatic virulence factors. Microbial strains were cultivated in nutritive broth, with and without the addition of subinhibitory concentrations of the tested EOs disolved in dimethyl sulphoxide (DMSO). The obtained overnight bacterial cultures were spotted onto special media for assessing the following virulence factors production: *haemolysins*: when the strains were streaked on blood agar plates containing 5% (vol/vol) sheep blood in order to obtain isolated colonies. After incubation at 37^{o}C for 24h the clear zone (total lysis of the red blood cells) around the colonies was registered as positive reaction. *Protease activity* was determined by using 3% gelatine/casein agar (Cantacuzino Institute Media Laboratory). After incubation at 37^{o}C up to 48h, a clear zone surrounding the growth area indicate gelatine/casein proteolysis (gelatinase/caseinase production). *DN-ase production* was studied using DNA agar medium. The strains were spott inoculated and after up to 48h incubation at 37^{o}C, a clear pink zone around the colonies was registered as positive reaction. *Lipase production*: the cultures were spotted on 1%Tween 80 agar and incubated at 37^{o}C up to 7 days. An opaque (precipitation) zone around the spot was registered as positive reaction. *Esculin hydrolysis* was highlighted on the esculin containing agar medium. In a positive reaction the presence of the iron salt, esculethol (an iron chelating agent) forms a brown-black complex that diffuses into the surrounding medium. *Amylase production* was studied on a starch agar medium and quantified as a colorless zone around the culture contrasting with the blue color resulted after adding Lugol solution.

The impact of EOs on the QS genes expression. The influence of extracted EOs and of some of their main fractions on QS genes expression in two selected strains (one *P. aeruginosa* and one *S. aureus*) was investigated by real time reverse transcriptase quantitative PCR (RT–qPCR). Total RNA was extracted from over night *S. aureus* and *P. aeruginosa* bacterial cultures treated and untreated with EOs (EO : DMSO, 1:1, v/v), by using an available comercial kit (GeneJet RNA Purification Kit Fermentas), following manufacturer s indications. For the *S. aureus* cultures additional treatments with lysozyme and lisostaphin which were added in TE (Tris/EDTA) (final concentration 10mg/mL) buffer have been performed. DNA traces from the RNA extracts were removed by treatment with DNase I (Fermentas). The RNA concentration was determined by using the fluorimetric Broad-Range RNA kit (Invitrogen). All RNA samples used in the RT (reverse transcription) reactions were brought to the same concentration (10 ng/µl). RT was performed by using RevertAid First Strand cDNA Synthesis Kit (Fermentas) with random primers. All reactions for relative quantification of QS genes expression by RT-qPCR were performed on qPCR system Mx3005P (Stratagene). The amplification of *agrI* gene was performed using Maxima Probe/ROX qPCR Master Mix (Fermentas), while Maxima SYBR Green /ROX qPCR Master Mix (Fermentas) was used for amplification of 16S rRNA gene, which was used as housekeeping gene for *S. aureus* [13]. The level of *agr*I gene expression in *S. aureus* strain was quantified by RT-qPCR using specific primers and probe for *agr*I gene detection (Table 1) [14]. The thermal cycling program for the amplification of *agr*I gene was: time 1 (*t*1),10 min at 95°C; *t*2, 15 s at 95°C; and *t*3, 1 min at 60°C (*t*2 and *t*3 were repeated 40 times). For housekeeping gene the amplification program consisted of: time 1 (*t*1),10 min at 95°C; *t*2, 15 s at 95°C; and *t*3, 1 min at 60°C (*t*2 and *t*3 were repeated 40 times), followed by a curve dissociation program specific for Mx3005P instrument. The relative quantification of *agr*I gene versus the housekeeping gene (16S rRNA) was performed in according with Pfaffl equation 1 [15].

In *P. aeruginosa* all qPCR assays were performed with TaqMan probes, primers specific for detection of QS and *rplU* genes using Maxima™ Probe/ROX qPCR Master Mix (Fermentas) (Table 2) [16,17]. The housekeeping gene *rplU* was used as the normalizing gene, being a ribosomal gene which is present in the bacterial cells in reasonably constant levels under normal growth conditions [16]. The amplification program used for QS and housekeeping genes consisted of 1 cycle for DNA-polymerase activation at 95 °C, 10 min, followed by 40 cycles, consisted from denaturation 95°C, 15 sec, primer annealing at 60°C, 1 min, and primer extension at 72°C, 30 sec. The

fluorescence data were acquired at the end of the primer extension step of each amplification cycle. Relative quantification of target gene (*lasI, lasR, rhlI, rhlR*) versus the reference gene (*rplU*) was performed according with Livak equation [18-20].

Table 1: *The primer and probe sequences used for amplification of agrI and 16S rRNA genes.*

Primer or Probe Name	Sequence (5′ - 3′)	Length (bp)	5′ Dye	conc. (nM)
agr type I				
F_*agr*	CCAGCTATAATTAGTGGTATTAAGTACAGTAAACT			200
R_*agr*	AGGACGCGCTATCAAACATTTT	106	FAM	200
P_*agr* [a]	ATAGGAATTTCGACATTATC			100
16SrRNA				
STPYF	ACGGTCTTGCTGTCACTTATA	257	-	200
STPYR2	TACACATATGTTCTTCCCTAATAA			200

[a] Minor groove binder probes with a nonfluorescent quencher bound to the 3′ end (Applied Biosystems).

Table 2: *The primer and probe sequences used for amplification of lasI, lasR, rhli, rhlR and rplU genes.*

Primer or Probe Name	Sequence (5′ - 3′)	Length (bp)	5′ Dye	conc. (nM)
lasI				
lasI-F	GCCCCTACATGCTGAAGAACA	62	FAM	200
lasI-R	CGAGCAAGGCGCTTCCT			200
lasI-P	CCCAACTGGTCTTGCCGATGG			100
lasR				
lasR-F	AAGGAAGTGTTGCAGTGGTG	68	FAM	200
lasR-R	GAGCAGTTGCAGATAACCGA			200
lasR-P	CCCAACTGGTCTTGCCGATGG			100
rhlI				
rhlI-F	CTTGGTCATGATCGAATTGCT	133	FAM	200
rhlI-R	ACGGCTGACGACCTCACAC			200
rhlI-P	AGGAAGTGCGGCGCCTGGG			100
rhlR				
rhlR-F	TGTTCGCCGTCCTGGAA	60	FAM	200
rhlR-R	CGCCATAGGCGTAGTAATCGA			200
rhlR-P	CCGACGACCGACGCCCGACCT			100
rplU				
rplU-F	TCACCGAAGGCGAATTCCT	103	VIC	200
rplU-R	TTCACGTCTTCGCCATTGG			200
rplU-P	ATTTCGACCGCGTCCTGCTGGTT			100

Results and Discussions

One of the practical purposes of the virulence research for a pathogen is to use the results to optimize the antimicrobial strategies. Virulence is correlated with the presence of bacterial structures and/ or biomolecules, and with certain physiological and synthesis features (i.e. the soluble virulence factors), responsible for the occurrence of specific lesions in the infected host [21]. In this study, *E. caryophyllata, R. officinalis, M. piperita* and *F. vulgare* EOs, in subinhibitory concentrations decreased the ability of all tested strains to produce soluble virulence factors. For *S. aureus* tested EOs inhibited the expression of most of the tested soluble factors, excepting lipase, which was stimulated, while in *P. aeruginosa*, only pore-forming toxins (i.e. lecithinase, haemolysins), involved in invasion and dissemination as well as amylase, were inhibited (Figure 2).

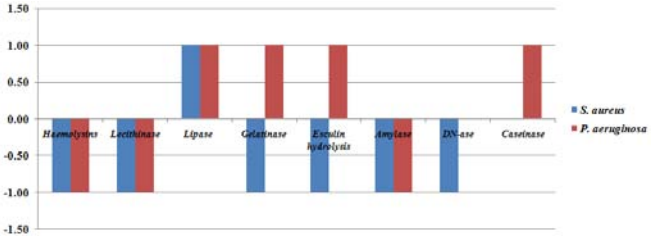

Figure 2*: Graphic representation of the influence of EOs on the expression of different soluble virulence factors in the analyzed P. aeruginosa and S. aureus strains.*

EOs modulatory effect on QS genes expression in *S. aureus* and *P. aeruginosa* strains. The expression of virulence factors in *S. aureus* is genetically controlled by complex systems, including *agr* (**a**ccessory **g**ene **r**egulator), *sar* (**s**taphylococcal **a**ccessory **r**egulator) and sae (**s**taphylococcal **a**ccessory **e**lement) loci. Qiu and collaborators [22], demonstrated that the treatment using sub-inhibitory concentrations of antibiotics interfere with the mRNA translation, which affects exoenzymes synthesis (i.e. virulence factors). For example, *S. aureus* strains grown in a culture medium supplemented with sub-inhibitory concentrations of clindamycin exhibited a dose-dependent inhibition of the genes expression encoding for the bacterial exoenzymes, effect demonstrated on *sar* genes. Supporting the anti-pathogenic effect hypothesis the same research group, reported that thymol induces significant inhibition of the *agrA* transcription, correlated with a lower virulence factors synthesis [23]. Similar effects have been observed against the *S. aureus* strains when using sub-inhibitory concentrations of eugenol and *Perilla frutescens* EO [24]. Bearing in mind the important role signaling systems in regulating virulence factors expression, the inhibition of these systems could lead to a decreased expression of the bacterial strains virulence. The relative quantification of the QS genes expression was achieved by the Comparative Quantitation program using Mx3005P instrument that compares the expression levels of a target gene in the test sample, represented by the bacterial culture treated with EOs/EOs fractions (the unknown) to a reference (control) sample (containing cDNA obtained by the reverse transcription of total RNA extracted from the same culture, treated with DMSO, the EOs solvent) (the calibrator). This type of experiment provides an effective method for comparing cDNA levels without necessarily knowing the absolute amount of target gene in each sample (Figure 3).

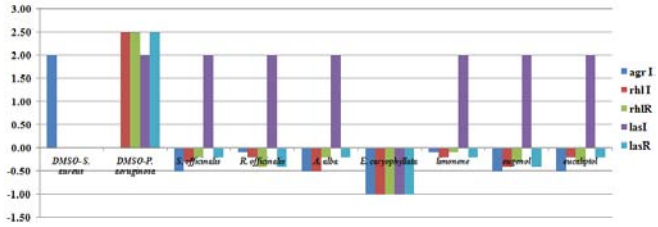

Figure 3: *Graphic representation of tested QS genes expression levels in P. aeruginosa and S. aureus strains cultivated in the presence of different EOs/major fractions.*

The modulatory effect on the QS gene expression for the *P. aeruginosa* strains has proved do be EO specific, all the four genes being repressed by the *E. caryophyllata* EO. EOs and their fractions revealed *agrI* repression for *S. aureus* samples treated with *S. officinalis*, *R. officinalis*, *A. alba*, *E. caryophyllata*, limonene, eugenol and eucalyptol, suggesting an inhibitory effect on the QS genes expression and indirectly on the strain virulence. The inhibition effects resulted in comparable values of the quantification parameter $2^{-\Delta\Delta Ct}$. The coniferous oils with a composition rich in terpenes, stimulated *agrI* gene expression in *S. aureus*, except *A. alba* EO, which exhibited an inhibitory effect, quantified as half of the limonene fraction inhibitory effect. *P. aeruginosa las* and *rhl* genes expression were differentially inhibited by EOs/ EOs fractions treatment. The expression of *rhl* gene was significantly downregulated by the EOs treatments, while *lasI* expression was upregulated. *E. caryophyllata* EO inhibited the expression of all tested QS genes.

Conclusions

Numerous studies describe antimicrobial activity of the plant extracts in terms of complex mechanisms of action of the pure substances or total extracts in attempting to provide a scientific foundation of the empirical observations in traditional medicine. During this study EOs extracted from the angiosperm group representatives of the families *Apiaceae*, *Lamiaceae*, *Myrtaceae*, *Rutaceae*, and plants belonging to the gymnosperms group, *Pinaceae* family, proved to inhibit the phenotypic expression of some of the tested soluble virulence factors at sub-inhibitory concentrations, as well as the expression of QS regulatory genes. A

diverse spectrum of EOs (*S. officinalis*, *R. officinalis*, *A. alba*, *E. caryophyllata*) as well as some of their major compounds (limonene, eugenol and eucalyptol) inhibited QS genes expression in *S. aureus*, while in *P. aeruginosa* analyzed strain, only *E. caryophyllata* EO proved to inhibit both *las* and *rhl* genes expression. The inhibition of this important regulatory mechanism of virulence factors expression represents a possible new strategy for the attenuation of pathogenicity and virulence of opportunistic pathogens isolated from severe infections.

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