Inhibitory potential of some selected essential oils and their main components on the growth and quorum-sensing based pigment production of *Serratia marcescens*

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Abstract. In this study the antibacterial potential of ten essential oils (EOs) and their main compounds against the development and quorum sensing (QS) mechanisms of the opportunistic bacterium *Serratia marcescens* was determined. The growth and QS inhibitory effect was evaluated by paper disc diffusion assay. The effect of EOs and components on QS-regulated prodigiosin biosynthesis was also studied.

The results of our study indicated that some of the investigated EOs influenced the development and the QS-based activity of *S. marcescens*. Oregano and thyme oils showed the most pronounced antibacterial effect and had the strongest anti-QS potential. From the main oil compounds the phenolics, eugenol, carvacrol and thymol, proved to be efficient growth inhibitors. While eugenol and carvacrol had also a strong negative influence on AHL-mediated QS-systems in low concentrations, thymol was not effective in QS inhibition.

Keywords: essential oils, quorum-sensing, prodigiosin.

Introduction

The bacteria are capable to socially interact by quorum-sensing (QS) mechanism, which is a density-dependent cell-to-cell communication. This unique bacterial communication system involves the synthesis of diffusible

autoinducer molecules, which permit unicellular bacteria to regulate gene expression in order to synchronize their behaviour in accordance with population density. In Gram-negative bacteria the most common QS signal molecules are acylated homoserine lactones (AHLs) (de Kievit and Iglewski, 2000; Waters and Bassler, 2005; Coulthurst *et al.*, 2006; Khanafari *et al.*, 2006; Van Houdt *et al.*, 2007; Szabó *et al.*, 2010; Robson *et al.*, 2014).

The density-dependent QS signaling systems control diverse physiological functions in bacteria, such as biofilm formation, virulence factor and extracellular polysaccharides synthesis, secondary metabolite synthesis, swimming motility and bacterial resistance. Therefore, by controlling or inhibiting QS processes it could be possible to influence the microbial processes regulated by this mechanism (Brown and Johnstone, 2001; Annous *et al.*, 2009).

Serratia marcescens is a ubiquitous environmental Gram-negative bacterium which is well known for the production of a characteristic red pigment, prodigiosin. The biosynthesis of prodigiosin is directed by the *pig* gene cluster and is regulated by AHL-mediated QS mechanism (Thomson *et al.*, 2000; Haddix *et al.*, 2008).

Nowadays the growing interest in study of *S. marcescens* is related to some important findings. First, prodigiosin produced by many Serratia *marcescens* strains has been identified as having extremely broad antimicrobial activities and potent immunosuppresive, proapoptic and anticancer properties (Khanafari et al., 2006; Samrot et al., 2011; Kamble and Hiwarale, 2012; Do and Nguyen, 2014). Due to the various applications of prodigiosin it would be important to understand the effect of different factors interfering with QSregulated prodigiosin production. On the other hand, S. marcescens is an opportunistic pathogen, which is responsible for an increasing number of nosocomial infections and was repeatedly isolated from respiratory and urinary tract clinical samples; S. marcescens is also the causative agent of conjunctivitis in contact lens wearers (Robson et al., 2014). Nosocomial infections due to these clinical isolates are frequently problematic because the pathogens are commonly multidrug resistant (Morohoshi et al., 2007). Serratia strains have been also reported to be one of the causative agents of food spoilage (Turgis *et al.*, 2012). Thus, it is necessary to develop new treatment or control possibilities for Serratia infections.

Essential oils (EOs) are aromatic and volatile hydrophobic mixtures, originated from the plant secondary metabolism. EOs are obtained from different plant parts (leaves, flowers, seeds) mainly by steam distillation and contain more than 50 active components in different proportions (Bakkali *et al.*, 2008).

Due to their well known antimicrobial potential, these vegetal constituents get an increasing attention in medicine and pharmaceutical industry, in aroma therapy and cosmetic industry, as well as in biocontrol techniques and food industry.

Natural EOs have the advantage that they are more ecofriendly, inhibiting not only the growth, but also the microbial density-dependent cell-to-cell communication systems, so the development of the microbial resistance to their active compounds is much less likely (Kerekes *et al.*, 2013).

The aim of this study was to investigate the effect of some selected essential oils and their main components on the development of *Serratia marcescens* as well as on the QS-controlled pigment formation processes.

Materials and methods

Bacterial Strains, Media, and Culture Conditions

The *Serratia marcescens* SZMC 0567 strain used in the experiments was from Szeged Microbiological Collection, maintained by the Department of Microbiology of the University of Szeged, Hungary. The bacterium strain was grown on peptone glycerol agar and peptone glycerol broth, respectively.

Essential Oils and Major Components

The investigated essential oils were marjoram (*Origanum majorana*), lemon (*Citrus lemon*), clary sage (*Salvia sclarea*), juniper (*Juniperus communis*), cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*), lavender (*Lavandula officinalis*), oregano (*Origanum vulgare*), clove (*Syzygium aromaticum*) and summer savory (*Satureja hortensis*). The main components of the tested essential oils (eugenol, thymol, carvacrol, limonene, β -o-cimene, linalool, α -thujone, α -pinene) were also investigated.

Detection of Growth and Quorum-Sensing Inhibition by Diffusion Paper Assay

The liquid culture of *S. marcescens* with a density of 10⁸ cfu ml⁻¹ was spread in Petri dishes on the surface of peptone glycerol agar. EOs and their main components were applied in different quantities (1, 3, 5 and 8 μ l) on sterile paper discs placed on the surface of the inoculated media. After incubation at 37 °C for 24-48 h the effect of EOs and compounds against the

development of bacteria was determined by measuring the inhibition zone around the paper discs, whereas QS-inhibition effect was determined by estimating the colourless zone developed behind the inhibition zone.

Determination of Prodigiosin Synthesis in the Presence of EOs and Major Compounds

From the *S. marcescens* suspension (OD 0,506 at 620 nm) 100 μ l ml⁻¹ was inoculated in peptone glycerol broth, containing different quantities of essential oils or their main compounds (1, 3, 5, 8, 10, 13, 15, 18, 20, 23, 26 μ l ml⁻¹ culture medium). For the extraction of prodigiosin, bacterial suspensions incubated for 1 week were used, where there was an obvious evidence of prodigiosin production because the colour of the suspension turned from yellow to red. The cells were harvested by centrifugation at 10 000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in acidified ethanol (4% of 1M HCl in 96 ml ethanol). The mixture was centrifuged again, and the absorbance values of the supernatant containing the extracted prodigiosin was determined at 499 nm, where the prodigiosin absorbs maximally (Slater *et al.*, 2003).

Isolated prodigiosin was estimated using the following formula (Mekhael and Yousif, 2009):

$$Prodigiosin unit/cell = \frac{[OD499 - (1.381 * OD620)] * 1000}{OD620}$$

OD499 – pigment absorbance; OD620 – bacterial cell absorbance; 1.381 – constant

Six parallel measurements were made for each variant. The control underwent the whole procedure except for the oil/component treatment.

Statistical Analysis and Data Processing

For experimental data evaluation, R statistical analysis program was used. The comparison of different treatment groups was performed using one-way ANOVA and Tukey test, by evaluating the significant differences (P < 0.01) within the groups. For some data the Kruskal-Wallis non-parametric test was used.

Results

Growth and QS Inhibitory Effect of EOs and their Main Components

The results regarding growth and QS inhibition of essential oils are shown in Table 1. Five of the EOs tested (lemon, juniper, clary sage, marjoram and lavender) had no influence on the growth and QS communication mechanisms of *S. marcescens*, even if we have applied them in increasing concentrations. Their major components (limonene, α - pinene, α - thujone, linalool, β - ocimene) were also ineffective in the control of *S. marcescens* (Tabs. 1 and 2).

Table 1. The effect of essential oils on the growth and QS of *S. marcescens* (paper disc diffusion assay)

	Growth inhibition zone (mm)				Quorum-sensing inhibition zone (mm)					
Essential oils	s 1 μl	2 µl	3 µl	5 µl	8 µl	1 µl	2 µl	3 µl	5 µl	8 µl
cinnamon	20	24.6	26	27.3	29	5.6	4.6	4	-	-
clove	15.6	20	16.3	29.6	31	-	-	-	9.6	10.3
thyme	17.3	18	22.3	40.3	38.6	, -	-	1.6	14.3	14.7
oregano	18	23	21.6	40	41.6	. –	-	-	11.3	14.8
savory	12.3	16.6	18	23.4	31	-	-	-	-	-
lemon	-	-	-	-	-	-	-	-	-	-
juniper	-	-	-	-	-	-	-	-	-	-
clary sage	-	-	-	-	-	-	-	-	-	-
marjoram	-	-	-	-	-	-	-	-	-	-
lavander	-	-	-	-	-	-	-	-	-	-

Table 2. The effect of the essential oil major components on the growth and QS of*S. marcescens* (paper disc diffusion assay)

Aromatic compounds	Gro	wth in	hibitio	n halo	(mm)	Quorur	n-sensii	ng inhib	ition ha	lo (mm)
	1 µl	2 µl	3 µl	5 µl	8 µl	1 µl	2 µl	3 µl	5 µl	8 µl
eugenol	16	27.6	30.3	28.6	29	3	11.6	12.6	10	10
carvacrol	20.6	27.6	34	38.6	36.6	4	7.3	10.6	10.6	10
thymol	15.3	16.3	19.3	19.3	20.3	-	-	-	-	-
limonene	-	-	-	-	-	-	-	-	-	-
α-pinene	-	-	-	-	-	-	-	-	-	-
α-thujone	-	-	-	-	-	-	-	-	-	-
linalool	-	-	-	-	-	-	-	-	-	-
β-ocimene	-	-	-	-	-	-	-	-	-	-

One can observe the inhibitory capacity of cinnamon, clove, thyme, oregano and summer savory oils on the growth of *S. marcescens*. Cinnamon oil had a good inhibitory potential on the growth of *S. marcescens* even in the lowest concentration; by increasing the oil concentrations there were no significant differences between the samples (Fig.1).

In the case of some oils tested (clove, thyme, oregano and summer savory) the inhibitory effect against the bacterial growth was significantly pronounced as the concentration increased. At the highest quantity (8μ l) the diameter of growth inhibition halo produced by all of these oils exceeded the halo due to cinnamon oil, where the inhibitory effect was not concentration-dependent (Fig. 1). The most pronounced inhibition was detected in the cases of oregano and thyme oils.

As Tab. 1 shows, the cinnamon oil slightly interfere with QS communication systems, as the inhibitory potential of QS was detected only at lower quantities, while clove, thyme and oregano EOs had anti-QS potential only in higher concentrations. The most pronounced inhibition of QS was observed in the case of oregano at 8 μ l. Even if the savory EO proved to be a good inhibitor of the bacterial growth, it did not show inhibitory effect on QS-systems in the concentrations used (Tab. 1).

Some of the major aromatic compounds tested (eugenol, carvacrol and thymol) had negative effect against the development of *S. marcescens* (Tab. 2). Comparing the effect of components on the growth of *S. marcescens* no significant differences have been observed at low amounts, but as the applied quantities increased the inhibitory potential differ considerably (Fig. 2).

Eugenol, which is the main component of cinnamon and clove oils, had a strong quantity-dependent antibacterial effect, interfering not only with the growth, but also with the QS regulation systems of *S. marcescens*. Carvacrol, found in oregano, thyme and savory oils, proved to be the best growth inhibitor and interfere with QS-based communication systems, the inhibitory potential increased significantly at higher quantities. Surprisingly, in case of thymol, the another aromatic compound of the mentioned EOs, a good anti-growth effect was observed, but we could not detect any anti-QS potential (Tab. 2).

These findings suggest that the growth-inhibitory effect of oregano, thyme and savory oils is due to carvacrol and thymol, while the negative effect of oregano and thyme EOs on QS systems is related to carvacrol, found in considerable amounts (up to 60%) in these EOs. Comparing the growth-reducing effect of parent EOs with their main components in the case of eugenol we have found a same antimicrobial potential, obtaining a significantly stronger effect only between eugenol and clove oil at the concentration of 3 μ l (Fig. 3).

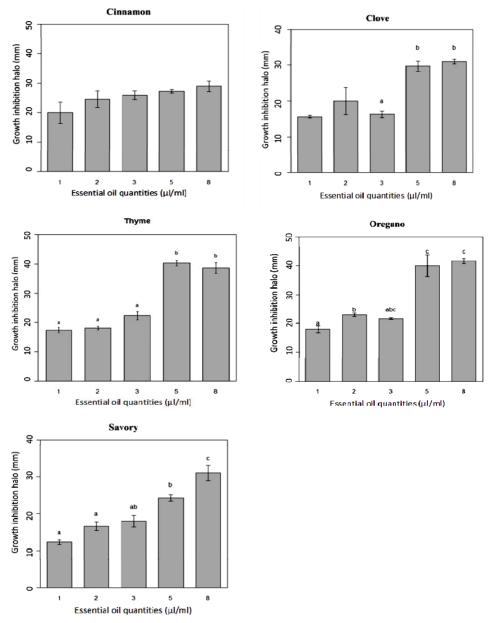


Figure 1. The inhibitory potential of the EOs studied. Significant changes are represented by different letters, while the same letters indicate no significance. Double or triple letters indicate that the given variant does not differ significantly from the variants marked with one or the other letters.

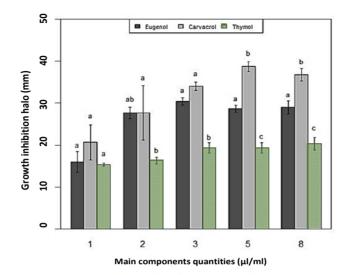


Figure 2. The growth inhibitory potential of some oil components. Different letters represent significant changes, while the same letters indicate no significance. Double letters indicate that the given variant does not differ significantly from the variants marked with one or the other letter.

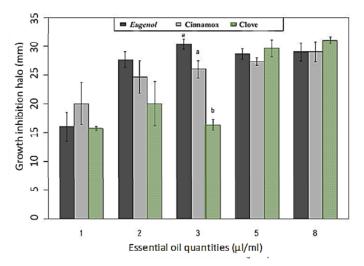
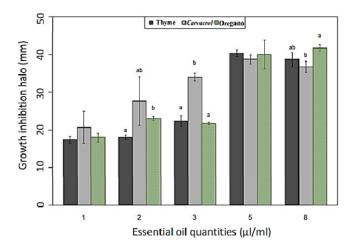
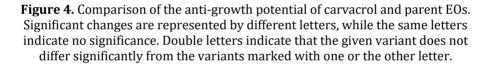


Figure 3. Comparison of the anti-growth potential of eugenol and parent EOs. Significant changes are represented by different letters, while the same letters indicate no significance. Double letters indicate that the given variant does not differ significantly from the variants marked with one or the other letter.

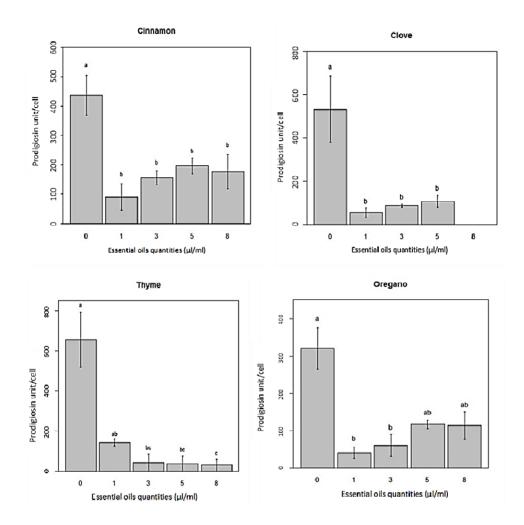
At large, the carvacrol presented also the same antimicrobial effectiveness than the parent EOs, having a considerably better inhibitory effect than thyme and oregano oils only when was applied in a quantity of 3 μ l (Fig. 4).

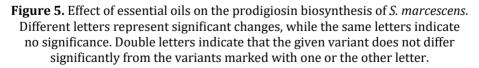




Effect of EOs and their Components on QS-regulated Prodigiosin Synthesis

As we have expected, the EOs which had inhibitory potential on the growth and QS regulation systems of *S. marcescens*, demonstrated by diffusion methods, significantly decreased the rate of prodigiosin production. In the case of cinnamon and oregano oils we could observe the same tendency in the inhibition of pigment synthesis, both oils had a strong negative influence even in the smallest quantities. There were no significant differences between the samples treated with increased oil concentrations. In contrast, thyme and clove EOs had a significant inhibitory effect against QS based prodigiosin production, the effectiveness of oils increased in higher concentrations (Fig. 5).





The major aromatic compounds of the EOs tested also influenced the QS-regulated prodigiosin biosynthesis of *S. marcescens*. As shown in Fig. 6, in the presence of eugenol the pigment production decreased significantly, but the increase of the applied quantities has not correlated with the intensification

of the prodigiosin biosynthesis inhibitory effect. The carvacrol had a pronounced quantity independent negative effect on the QS-mediated pigment production of *S. marcescens*, so it proved to be a very strong QS inhibitor. Generally, these results are in agreement with that found by paper disc assay.

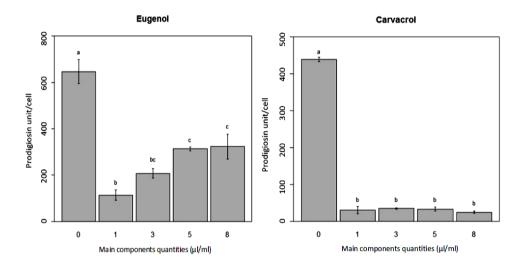


Figure 6. Effect of eugenol and carvacrol on the rate of prodigiosin biosynthesis. Different letters represent significant changes, while the same letters indicate no significance. Double letters indicate that the given variant does not differ significantly from the variants marked with one or the other letter.

Discussion

Quorum sensing systems of *S. marcescens* act as a global regulator of almost all the virulence factors, sliding motility and biofilm formation (Annous *et al.*, 2009; Kumar *et al.*, 2016). The biosynthesis of the red prodigiosin pigment is also under QS regulation, therefore, this pigment-producing bacterium can be used as a biosensor in QS regulation investigations. Since the QS regulation directly accords to its pathogeny, targeting QS systems could provide a new strategy to control the virulence of this bacterium (Bakkiyaray *et al.*, 2012).

Essential oils and their aromatic compounds are assumed to not result in the development of the resistance in bacterial strains. Instead, this natural products attenuate the expression of genes responsible for pathogenesis by interferring with bacterial communication systems (Packiavathy *et al.*, 2012). In general terms, the essential oils influence the integrity of the cell wall and cytoplasmic membrane, determining the loss of some cell compounds, that finally leads to death of the microbial cells.

Our results suggest that EOs containing phenolic compounds such as carvacrol, thymol and eugenol proved to be the strongest inhibitors of *Serratia marcescens*. These data are in agreement with other studies, that confirm that these compounds have a dose dependent inhibitory effect on enteroinvasive *Escherichia coli* and other Gram-negative bacteria (Kim *et al.*, 1995; Cosentino *et al.*, 1999; Dušan *et al.*, 2006; Gill and Holley, 2006). The phenolic components determine the release of lipopolysaccharides from the outer membrane and cause the disintegration of the cell wall. The inhibitory action is also explained by the increase of the cell membrane permeability to ATP and by the ATPase inhibiting activity of these compounds. In addition eugenol has a role in the inhibition of specific cellular processes and enzymes (Burt, 2004; Gill and Holley, 2006; Seydim and Sarikus, 2006; Kerekes *et al.*, 2013).

The EOs containing cyclic terpenes and terpene alcohols as main constituents have no effect on growth and QS-based prodigiosin biosynthesis of *S. marcescens*.

The antimicrobial effect of the EOs usually is due to the interaction between all the constituents and not only to a single component (Lis-Balchin and Deans, 1997; Mourey and Canillac, 2002), however there are some evidences that in some cases the components are better inhibitors than EOs and the use of a single compound is sufficient to treat bacterial biofilms (Kerekes *et al.*, 2013). According to our findings, the effect of the main compounds on the growth of *S. marcescens* differ not significantly from those of essential oils, suggesting that the EOs with phenolic compounds or only their major bioactive components applied in suitable quantities could be efficient biological alternatives in controlling the growth and virulence determining processes of *S. marcescens*.

Conclusions

Some of the investigated essential oils influenced significantly the development and the QS-based pigment production of opportunistic *S. marcescens*. Oregano and thyme oils showed the most pronounced antibacterial effect and had the strongest anti-QS potential. The phenolic oil components, such as eugenol, carvacrol and thymol, proved to be efficient growth inhibitors.

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J. PAPP, M. IACOB

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INHIBITORY POTENTIAL OF SOME SELECTED ESSENTIAL OILS ON SERRATIA MARCESCENS

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