Comparing inhibitory potential of *Eugenia caryophyllus* and *Origanum compactum* against the growth and gene expression of enterotoxins in *Staphylococcus aureus* ATCC 29213

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Abstract:

BACKGROUND: Bacterial resistance to antibiotics is a crucial public health problem. Essential oils (EOs) possess antimicrobial effects and have been screened as potential natural antimicrobial compounds. **OBJECTIVES:** This study was conducted to compare the effects of *Eugenia caryophyllus* (clove) and *Origanum compactum* (oregano) EOs on the growth of *Staphylococcus aureus* and the expression of the SEA, SEC and SEE genes. **METHODS:** The minimum inhibitory concentrations (MIC) of EOs and growth of bacterium at subMIC levels of EOs were determined. Enterotoxin detection was done using a commercial SE visual immunoassay kit after 18, 24, 48 and 72 h. Gene expression of enterotoxins was evaluated through RNA extraction, DNA synthesis and performing real time-PCR using specific primers for each SE. **RESULTS:** MIC of clove and oregano were 2 µl/ml and 1µ l/ml, respectively. Colony counts at 48 and 72h of cultures grown at 75% MIC of clove oil showed the growth rate was reduced 1.67 and 1.83 log10 cfu/ml compared to the control, and in the case of oregano at 75% MIC the decreases in growth rate were 2.25 and 2.68 log10 cfu/ml, respectively. When the target bacterium is cultured in the presence 75% subMIC of EOs, the transcript levels of sea, sec, see and the regulatory gene (agrA) were decreased 8.81, 9.13, 9.08 and 8.32 fold in the case of clove, and 11.56, 9.96, 11.07 and 11.15 fold in the case of oregano, compared to the control. **CONCLUSIONS:** The growth, gene expression and as a result secretion of enterotoxins A, C and E by *S. aureus* were decreased significantly at subMIC levels of EOs, especially at 75% MIC.

Introduction

The importance of *Staphylococcus aureus* in diseases ranging from acute infections (localised or invasive) to acute toxaemia is well-known (Baird-Parker, 1990). Staphylococcal food-poisoning syndrome is an intoxication caused by ingestion of staphylococcal enterotoxins (SEs). These toxins are classified on the basis of their

immunological reactivities and have been designated SEA, SEB, SECl, SEC2, SEC3, SED, SEE and SEH (Omoe et al., 2002).

There is currently an impetus for the discovery of natural antimicrobial agents for use as alternatives to synthetic compounds in food preservation and human remedies. The excessive and inappropriate use of antibiotics in agriculture and in human health to treat infectious diseases is responsible for the emergence of resistant organisms (Farahnik and Murase, 2016; Zhao et al., 2017). Essential oils (EOs) obtained by steam distillation from aromatic plants have recently gained in popularity and scientific interest as natural preservative compounds. EOs are a potentially useful source of molecules of diverse biological activities, and numerous scientific reports have highlighted antimicrobial activities of them (Bajer et al., 2017; Oussalah et al., 2007). Some studies have evaluated the inhibitory effects of natural compounds and EOs on growth, toxin production and gene expression of enterotoxins in S.aureus (Azizkhani et al., 2013; Qiu et al., 2010).

Cloves are the aromatic dried flower buds of a tree (*Eugenia caryophyllus*) of the family Myrtaceae (Chaieb et al., 2007a). They exhibit anti-mutagenic (Miyazawa and Hisama, 2003), anti-inflammatory (Mektrirat et al., 2016), antioxidant (Chaieb et al., 2007b), anti-ulcerogenic (Li et al., 2005), anti-thrombotic (Srivastava and Malhotra, 1991) and anti-parasitic (Yang et al., 2003) properties.

Oregano, a plant belonging to the Laminaceae family, is mainly used as a culinary condiment and is largely employed in popular medicine for the treatment of ailments such as digestive and pulmonary disorders (Asadbeigi et al., 2014). In addition, it is used as a preservative in many kinds of food (Asensio et al., 2015; Bhargava et al., 2015). The EO of oregano also exhibits significant antimicrobial activity (De Falco et al., 2014) and various extracts of the oregano plant have been tested for their biological activities (Fratini et al., 2017; Dutra et al., 2016).

The present study is the first work that investigated the antimicrobial effect of clove and oregano EOs at molecular level. This work was conducted to determine the MICs value of clove and oregano EOs that would inhibit the growth of *S. aureus* ATCC 29213 on the one hand, and that required to kill this bacterium (minimum bactericidal concentration: MBC) on the other. In addition, the effect of subMIC levels of EOs on the growth of the microorganism and the gene expression of enterotoxins A, C and E has also been evaluated.

Materials and Methods

Essential oils: Commercially available oregano and clove essential oils supplied by Pranarôm International (Ghislenghien, Belgium) were used in this study. The EOs were analysed by gas chromatography, ThermoQuest Co. (Manchester, UK).

Bacterial strains and reagents: *S. aureus* ATCC 29213, which has the ability to secrete SEA, SEC and SEE, was obtained as a lyophilized culture from the Pasteur Research Institute, Tehran, Iran. All chemicals and culture media were purchased from Merck (Darmstadt, Germany).

Determination of MIC and MBC: In order to determine the lowest concentration (MIC) in which visible growth of the bacterium is inhibited, a broth microdilution assay was employed (NCCLS, 2000).

Table 1. Primers used for quantitative RT-PCR.

Primer	Sequence (5'à3')	Primer length (bp)	Tm (ºC)
sea-F	ATGGTGCTTATTATGGTTATC	120	54
sea-R	CGTTTCCAAAGGTACTGTATT		
sea-F	TTTTTGGCACATGATTTAATTT	257	55
sec-R	CAACCGTTTTATTGTCGTTG		
see-F	CAGTACCTATAGATAAAGTTAAAACAAGC	178	55
see-R	TAACTTACCGTGGACCCTTC		
16S rRNA-F	GCTGCCCTTTGTATTGTC	278	54
16S rRNA-R	AGATGTTGGGTTAAGTCCC		
agrA-F	TGATAATCCTTATGAGGTGCTT	274	56
agrA-R	CACTGTGACTCGTAACGAAAA		

The assay was carried out with Tryptic soy broth (TSB) culture medium. To obtain and maintain a stable oil-water emulsion in the broth substrate during the experiment, the method of Mann and Markham (1998) was used with some modifications. Briefly, 5 ml/100ml dimethylsulphoxide (DMSO) as an emulsifier and 0.05 g/100ml agar-agar as a stabilizer were added to the broth substrate. Dilutions of EOs were set up using a 96-well microtitre plate (180µl of TSB containing specified concentrations of EO and 20µl of inocula were transferred to each microwell). The final bacterial inoculation titre in each microwell was 10^5 cfu/ml. As a control, the same amount of DMSO and agar-agar were also added to broth lacking EOs to take into account any effects these additives might have on the growth and/or toxin production of the test organism. The plate was covered with a sterile plate sealer. The contents of each well were mixed on a plate shaker at 300 rpm for 20 s and then incubated at 35 ºC for 24 h. Bacterial growth was determined by measuring absorbance at 600 nm. In order to determine the minimum bactericidal concentration (MBC) as the lowest concentration that reduces the bacterial population 99.9% after incubation at 35-37 ºC for 24 h, 100 µl of those microtitre cultures with no visible growth in the MIC determination assay were spread on Tryptic soy agar (TSA) and incubated at 35 ºC for 24 h. The concentration of EOs in those wells that yielded plates with no visible colonies was considered to be the MBC.

Growth of bacterium: EOs were added at subMIC levels (25, 50 and 75%MIC; subMICs of clove: 0.5, 1 and 1.5 µl/ml; subMICs of oregano: 0.25, 0.5 and 0.75 µl/ml) to 10 ml liquid TSB culture media containing 5 ml/100ml DMSO and 0.05 g/100ml agar-agar. The inoculation dose of *S. aureus* was 10^5 cfu/ml. Bacteria were cultured at 35 °C with aeration. The control culture contained 5 ml/100ml DMSO and 0.05 g/100ml agar-agar only. For colony counting, serial dilutions were prepared from TSB cultures incubated for 0, 18, 24, 48 and 72 h and spread on TSA plates (Azizkhani et al., 2013).

Enterotoxin detection: The RIDASCREEN SET kit from R-Biopharm Co. (Darmstadt, Germany), a commercial SE visual immunoassay kit with a minimum detectable limit of 0.50 to 0.75 ng of SEs per ml or g of sample, was used for SE detection. Strain ATCC 29213 was cultured in TSB with subMIC levels of EOs at 35 ºC for 72 h and enterotoxin detection was done according to the manufacturer's instructions after 18, 24, 48 and 72 h.

RNA extraction and purification: Strain ATCC 29213 was cultured in TSB in the presence of subMIC levels of EOs at 35 ºC for 72 h. RNA was prepared after 18, 24, 48 and 72 h of culture using the Tripure isolation reagent (Roche Applied Science, Bavaria, Germany) according to the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260 nm and purity was assessed measuring the A260nm/A280nm ratio using a NanoDrop Spectrophotometer 2000 (Thermo Scientific, Illinois, USA). RNA quality and integrity was visualized by ethidium bromide staining after electrophoresis of RNA on a 1 g/100ml agarose gel. DNA-free RNA was dissolved in DEPC-water (diethyl pyrocarbonate treated double-distilled water) and stored at -70 ºC.

cDNA synthesis: RNA was reverse transcribed into cDNA using the Omniscript Reverse Transcription kit, Qiagen Co. (Hilden, Germany) according to the manufacturer's instructions. cDNA was stored at -20 ºC until needed.

Real-time PCR: PCR reactions of 20µl total volume and containing Power SYBR Green (Applied Biosystems Co., Courtaboeuf, France) as recommended by the manufacturer were performed using the ABI PRISM 7500 Sequence Detection System from Applied Biosystems Co. (Courtaboeuf, France). The primer pairs used are listed in Table 1. Cycling conditions were as follows: one cycle at 95 ºC for 10 min, 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. All samples were analyzed in triplicate and normalized against 16S rRNA expression (Azizkhani et al., 2013). Since sec and see are positively regulated by the agrA two-component system, the transcription of agrA was also investigated. For SYBR Green based amplicon detection it is important to run a dissociation curve following the RT- PCR. This is due to the fact that SYBR Green will detect any double-stranded DNA including primer dimers, contaminating DNA, and PCR products from misannealed primers. The derivative plot of the melting curve of each gene in the reaction was therefore evaluated. Relative expression levels were determined by the ΔΔCt method described in Applied Biosystems User Bulletin no. 2.

Statistical analysis: All experiments (MIC and MBC determination, growth experiments, enterotoxin detection and PCR experiments) were repeated three times. Data were expressed as the mean ± STD Dev. Statistical differences were calculated using the independent Student t-test. A p value less than 0.05 was considered to be statistically significant.

Results

Chemical composition of EOs: The main compounds present in oregano EO are carvacrol (46.88%), thymol (15.26%), p-cimene (13.10%) and g-terpinene (11.61%), and those in clove EO are eugenol (83.96%), eugenile acetate (10.75%) and b-caryophyllene (3.25%).

MIC and MBC results: The MIC and MBC values of clove EO against *S. aureus* ATCC 29213 were 2 ± 0.001 and 4 ± 0.05µ l/ml respectively. The MIC and MBC values obtained for oregano EO against *S. aureus* were 1 ± 0.004 and 1.2 ± 0.007µ l/ml respectively.

Growth of S. aureus: From the results given in Fig. 1A, after 24, 48 and 72 h of incubation at 35 ºC, 75% MIC of clove EO decreased the final cell density of *S. aureus*

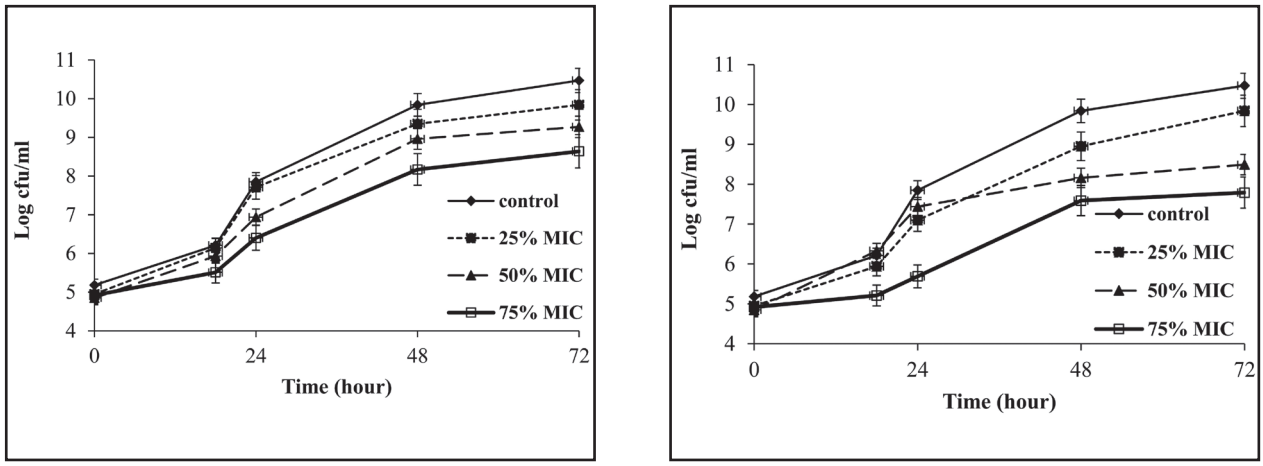

Figure 1. Colony counts of *S. aureus* cultured in the presence of subinhibitory levels of (1A) *Eugenia caryophyllus* and (1B) *Origanum compactum* EOs. The data are means and the associated error bars represent standard deviations. Deviation for three independent experiments ($p<0.05$).

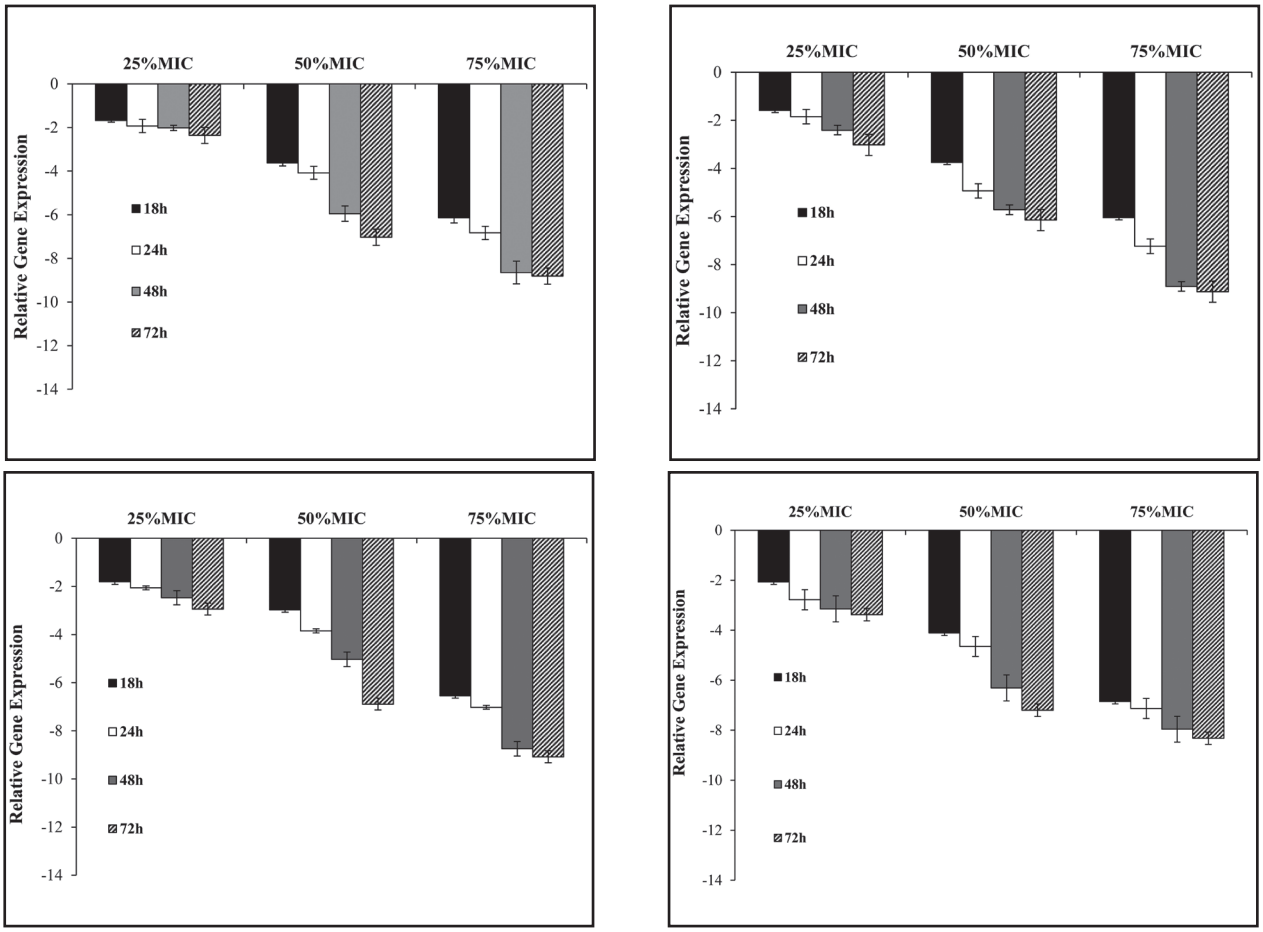

Figure 2. Relative expression of sea (2A), sec (2B), see (2C) and agrA (2D) in S. aureus. *S. aureus* ATCC 29213 was cultured with subinhibitory concentrations of *Eugenia caryophyllus* EO for 72 hours. Transcript levels were monitored by quantitative RT-PCR as described in the text. The fold reduction of gene expression in EO treatments is reported in comparison to the control culture lacking EO. The data are means and the associated error bars represent standard deviations. Deviation for three independent experiments ($p<0.05$).

by 1.45, 1.67 and 1.83 log10 (cfu/ml) respectively ($p<0.05$), compared to the control culture; colony counts of cultures grown in the presence of 75% MIC of oregano EO

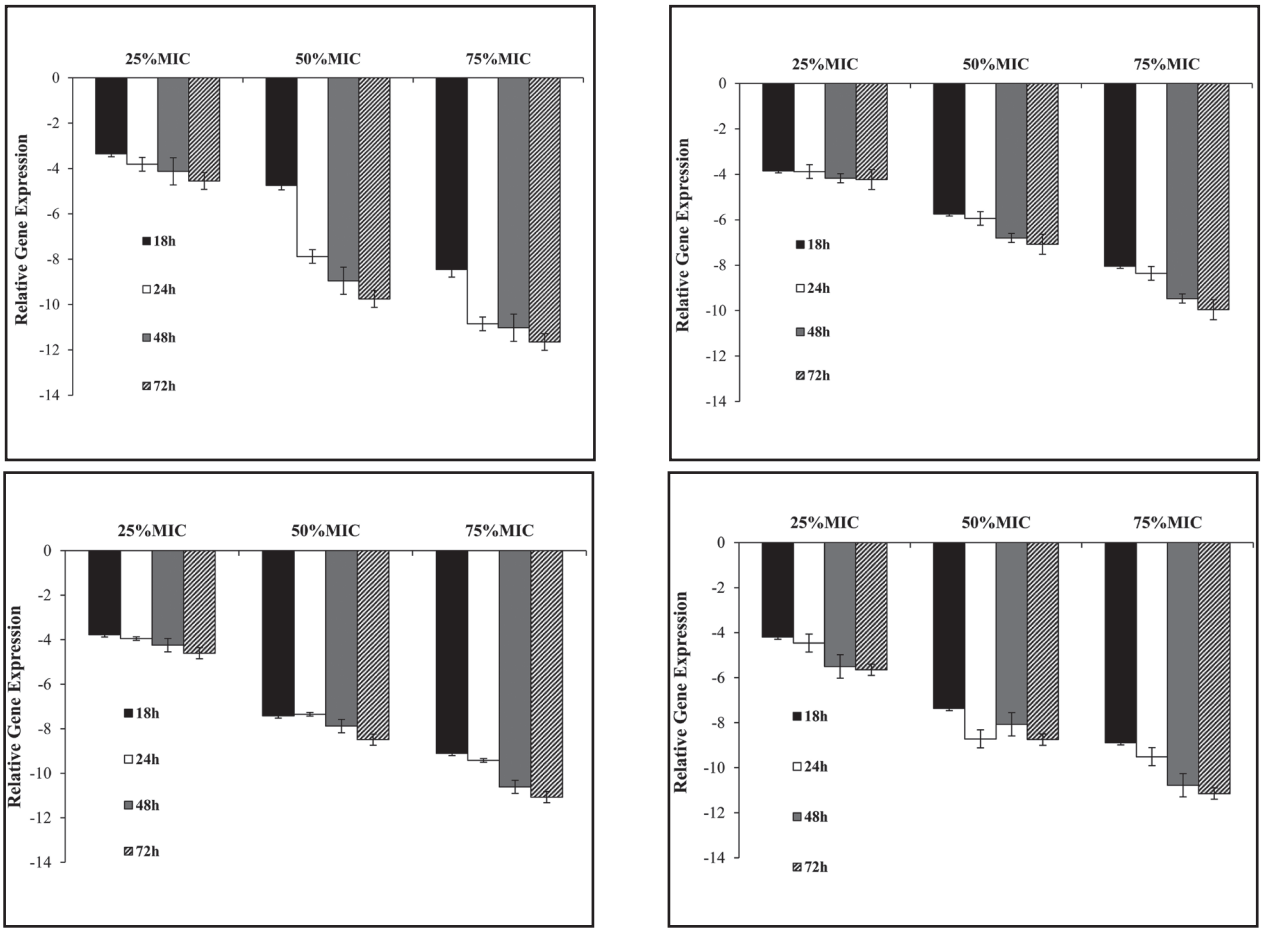

Figure 3. Relative expression of sea (3A), sec (3B), see (3C) and agrA (3D) in S. aureus. *S. aureus* ATCC 29213 was cultured with subinhibitory concentrations of *Origanum compactum* EO for 72 hours. Transcript levels were monitored by quantitative RT-PCR as described in the text. The fold reduction of gene expression in EO treatments is reported in comparison to the control culture lacking EO. The data are means and the associated error bars represent standard deviations. Deviation for three independent experiments ($p<0.05$).

for the same incubation periods (Fig. 1B) showed growth to be reduced 2.16, 2.25 and 2.68 log10 cfu/ml respectively ($p<0.05$). These reductions were found to be statistically significant compared to the controls for both EOs ($p<0.05$). There was also a statistically significant difference between the results for the different incubation periods ($p<0.001$).

ELISA results: In this study it can be seen that the EOs at 25% MIC had no inhibitory effect on enterotoxin production by *S. aureus* at any of the time periods analysed (18, 24, 48 and 72 h) compared to the control in the absence of EO. Increasing the concentration of EO to 50% and 75% MIC produced significant ($p<0.05$) inhibitory effects on enterotoxin production.

Transcription of the sea, sec, see and argA genes: It was apparent from the melting curve data (not shown) that no contaminating products were present in the reactions since contaminating DNA or primer dimers would have shown up as additional peaks separate from the desired amplicon peak. The melting temperatures (Tm) of the genes are presented in Table 1. A dose-dependent reduction in sea, sec, see and agrA transcription was observed in *S. aureus* upon treatment with the EOs (Fig. 2 & 3). For example, when cultured with 75% MIC of clove EO, the transcriptional

levels of sea, sec, see and agrA were decreased 6.13, 6.05, 6.54 and 6.85 fold after 18 h and 8.81, 9.13, 9.08 and 8.32 fold after 72 h in comparison to the control, respectively, ($p<0.05$). In the case of oregano EO, the expression of sea, sec, see and agrA was reduced 8.45, 8.06, 9.11 and 8.89 fold after 18 h and 11.65, 9.96, 11.07 and 11.15 fold after 72 h in comparison to control, respectively, ($p<0.05$).

Discussion

A number of reports have highlighted the potential that plant EOs (and their components) may, in food preservation, give the inhibitory effects they exert on microbial growth (Aumeeruddy-Elalfi et al., 2016; Kwon et al., 2017; Xiang et al., 2017). Several studies have focused specifically on the strongly antibacterial properties of clove EO (Cui et al., 2016; Fu et al., 2007; Khaleque et al., 2016; Li et al., 2005; Mulla et al., 2017), the high degree of its inhibitory activity probably derived from the antibacterial activities exhibited by a wide range of its constituent compounds: eugenol [2-methoxy-4- (2-propenyl) phenol], eugenyl acetate, beta-caryophyllene, 2-heptanone (Chaieb et al., 2007b), acetyl- eugenol, alpha-humulene, methyl salicylate, iso-eugenol, methyl-eugenol (Yang et al., 2003), phenyl propanoides, dehydrodieugenol, trans-coniferyl aldehyde, biflorin, kaempferol, rhamnocitrin, myricetin, gallic acid, ellagic acid and oleanolic acid (Khaleque et al., 2016).

It would appear that there are diverse mechanisms by which EOs - and their constituents - adversely affect microorganisms. It has been hypothesized that phenolic compounds are involved in microbial growth inhibition because these compounds sensitize the phospholipid bilayer of the microbial cytoplasmic membrane resulting in increased permeability, the absence of vital intracellular constituents and/or the impairment of bacterial enzyme systems (Juven et al., 1994). Phenolic components such as eugenol are highly active against microorganisms, and in the present study we found the principal constituent of clove oil to be eugenol (83.96%). It is known that the bactericidal or bacteriostatic activities of compounds of this type are determined by their concentration (Dorman and Deans, 2000).

The even stronger antibacterial activity of oregano EO compared to that of clove observed in this study correlates with the greater contents of carvacrol (46.88%) and thymol (15.26%) in *O. compactum* oil. These phenolics are among the most effective plant antibacterial agents known to date (Nazer et al., 2005), and several studies have demonstrated their ability to inactivate bacterial strains in synthetic media as well as in food systems (Knowles et al., 2005; Lambert et al., 2001; Valero and Frances, 2006). It has been proposed that the antibacterial activity of carvacrol derives from the physical distortion it induces in the membrane as a result of its accumulation in this hydrophobic environment, combined with the electron transporter activity conferred in part by its delocalised electron system resulting in further disruption of the transmembranal pH gradient. The consequent loss of the proton motive force results in ATP depletion and ultimately cell death (Ultee et al., 2002).

The MIC values obtained in the present study for clove and oregano EOs against *S. aureus* were 2µ l/Ml and 1µ l/ml, respectively, and are approximately in accordance

with those of Fu et al. (2007) and Nostro et al. (2006). Both EOs reduce the growth of *S. aureus* and its production of SE in a dose-dependent manner. The data (Fig. 1A, 1B) also reveal a greater inhibitory effect of oregano EO compared to that of clove. In this regard, a number of previous observations reported significant inhibitory effects of carvacrol-containing EOs on *Bacillus cereus*, *Salmonella typhimurium* and *S. aureus* (Azizkhani et al., 2013; Basti et al., 2007; Misaghi and Basti, 2007; Moosavy et al., 2008). The greater the carvacrol content of the EO, the greater the inhibitory activity observed.

Our data highlight the potential of oregano EO for significantly reducing and inhibiting enterotoxin production. Similar findings were made by de Souza et al. (2010) and Palmer et al. (2004) who reported the strong effects of subMIC levels of bay, cinnamon, clove and oregano EOs in decreasing the production of enterotoxins by S. aureus, as well as the antimicrobial activity of Z. multiflora Boiss. EO on enterotoxin C production (Azizkhani et al., 2013; Parsaeimehr et al., 2010). In their study, de Souza et al. (2010) observed total suppression of enterotoxin production in the broth to which *Origanum vulgare* L. EO had been added at subMIC levels (0.3 and 0.15 µl/ml). Qiu et al. (2010) evaluated the effect of subMIC levels of thymol (a phenolic fraction of some EOs such as that of oregano in this study) on methicillin sensitive and resistant isolates of S. aureus, revealing dose-dependent decreases in the growth of the microorganism and the production of SEA, SEB and α-hemolysin. We have made similar observations in the present study.

Inhibitors of protein synthesis at subMIC levels significantly reduce the production of virulence factors (including α-hemolysin, SEA, SEB and protein A) by *S. aureus* (Bernardo et al., 2004; Herbert et al., 2001) and many currently used synthetic preservatives affect the secretion of exotoxins, especially when used at suboptimal concentrations. Certain plant compounds (e.g. oleuropein and epicatechin gallate) and EOs (e.g. the oils of bay, cinnamon and cloves) can also influence the production of exotoxins when used at low concentrations (Palmer et al., 2004; Shah et al., 2008).

Previous reports have indicated that subMIC levels of antimicrobials may interfere with the translation of one or more regulatory gene products in *S. aureus* and by this means affect the transcription of exoprotein-encoding genes (Kuroda et al., 2007). Also, electron microscopy of EO-treated cells revealed the formation of holes in bacterial cell surfaces and the loss of cytoplasmic material (de Souza et al., 2010). In the current study, quantitative RT-PCR was used to investigate the influence of EOs on the expression of the agr locus of S. aureus. Our data show that the EOs tested significantly inhibit agrA transcription. However, the mechanisms by which *S. aureus* controls virulence gene expression are fairly intricate and involve an interactive, hierarchical regulatory cascade of the products of the agr gene along with other components (Chan and Foster, 1998). We therefore presume that the reduced production of virulence factors observed in our study may partially depend on EO-induced inhibition of the agr two-component system.

Oregano and clove EOs both show strong inhibitory effects against *S. aureus* in vitro, the most effective of the two being oregano. The ability of both EOs to inhibit the growth of this microorganism and its pro-

duction of enterotoxin indicates a potential for these oils as natural food preservatives. The activity observed is attributable to the phenolic compounds present in the oils. The results presented here may explain the traditional culinary and medicinal uses of these plants. Further work is necessary to assess the effectiveness of these EOs in food systems and to extend the molecular analysis of gene expression to other enterotoxin encoding genes and enterotoxigenic microorganisms.

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مقایسه قدرت بازدارندگی گیاهان میخک و پونه کوهی در برابر رشد و بیان ژنی انتروتوکسین‌ها در استافیلو کوکوس اورئوس ATCC ۲۹۲۱۳

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چکیده

زمینه مطالعه: مقاومت باکتریایی در برابر آنتی بیوتیک‌ها یکی از مشکلات اساسی در حوزه بهداشت عمومی به شمار می‌رود. اسانس‌های گیاهی واجد تاثیرات ضدمیکربی بوده و در زمره ترکیبات طبیعی ضدمیکربی بالقوه قرار گرفته‌اند. **هدف:** این مطالعه به منظور مقایسه تاثیر اسانس‌های میخک و پونه کوهی بر رشد استافیلو کوکوس اورئوس و بیان ژنی انتروتوکسین‌های A، C و E آن انجام شد. **روش کار:** کمینه غلظت بازدارندگی(MIC) اسانس ها و میزان رشد باکتری در غلظت‌های تحت بازدارنده اسانس ها تعیین گردید. جستجوی انترواتوکسین‌ها با استفاده از یک کیت تجاری ایمنواسی ویژه انتروتوکسین‌های استافیلوکوکی، پس از ۱۸، ۲۴، ۴۸ و ۷۲ ساعت، انجام شد. بیان ژن‌های کد کننده انتروتوکسین‌ها از طریق استخراج RNA، سنتزDNA و انجام واکنش زنجیره‌ای پلیمراز زمان واقعی با استفاده از توالی های آغازگر اختصاصی برای هر ژن کد کننده انتروتوکسین ارزیابی شد. **نتایج:** کمینه غلظت بازدارندگی اسانس‌های میخک و پونه کوهی، به ترتیب، برابر با ۲ µl/ml و ۱ بود. شمارش کلنی در محیط کشت حاوی ۷۵ ٪MIC اسانس میخک، پس از ۴۸ و ۷۲ ساعت، نشان داد که میزان رشد باکتری ۱/۶۷ و ۱/۸۳ log۱۰ cfu/ml نسبت به کنترل کاهش یافته است و در خصوص پونه کوهی، میزان کاهش رشد در ۷۵ ٪MIC، به ترتیب، معادل ۲/۲۵ و ۲/۶۸ log۱۰ cfu/ml بود. کشت باکتری هدف در غلظت ۷۵ ٪MIC اسانس ها، موجب کاهش سطح نسخه برداری ژن های see، sec، sea و ژن تنظیم کننده (agrA) به میزان ۸/۸۱، ۹/۱۳، ۹/۰۸ و ۸/۳۲ برابر در مورد اسانس میخک و ۱۱/۵۶، ۹/۹۶، ۱۱/۰۷ و ۱۱/۱۵ برابر در خصوص اسانس پونه کوهی، در مقایسه با کنترل، گردید. **نتیجه گیری نهایی:** میزان رشد، بیان ژنی و در نتیجه تولید و ترشح انتروتوکسین‌های A، C و E استافیلو کوکوس اورئوس به میزان قابل ملاحظه‌ای در سطوح تحت بازدارنده اسانس‌ها، به ویژه در غلظت ۷۵ ٪MIC کاهش می‌یابد.

واژه های کلیدی: انتروتوکسین، میخک، بیان ژنی، پونه کوهی، استافیلو کوکوس اورئوس

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