RESEARCH ARTICLE

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Anti-enterotoxic activity of *Areca*, *Origanum majorana*, and *Long pepper* on Staphylococcal enterotoxins: A mixture study

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ABSTRACT
The present paper studies antibacterial activity (AA) and anti-enterotoxigenic activity (AEA) of three Iranian medicinal plant extracts, *Origanum majorana*, *Areca*, and *Long pepper*. In addition, the anti-enterotoxigenic interactive effects of these extracts as herbal extract blend (HEB) were studied. Kirby-Bauer, agar dilution, and Real-time PCR methods were used to study the AA and AEA of the extracts. The statistical method of mixture design was used to optimize and find out the best HEB with the highest AEA on A and B enterotoxins of *S. aureus*. In individual AA study of extracts, *O. majorana* extract had the highest AA. Among the extracts evaluated in this study, the extract of *O. majorana* had much AA on *S. aureus* and its enterotoxins production. As an interesting result, there is an inhibitory synergistic effect between the extracts studied in this paper on gene expression of *sea* and *seb*. The maximum AEA was observed by treat with HEB including 47% *O. majorana*, 26% *Areca*, and 27% *Long pepper*.

Key words: *Staphylococcus aureus*, herbal extract, antimicrobial activity, anti-enterotoxigenic activity, real-time PCR

Introduction

Food-borne pathogens (FBP) directly threaten the health of consumers and caused food-borne diseases. Therefore, the importance of prevention of FBPs growth is increasing day-by-day. For this purpose, different antibiotics have been used in the food industry; however, the risks of using synthetic antibiotics have become an outcry worldwide (Josephs-Spaulding et al., 2016). Hence, introducing and using natural and safe preservatives that have no harmful effects on the health of consumers is crucial for the food industry (Preedy, 2015).

Staphylococcus aureus is one hazard FBP, which produces different enterotoxins that are known as staphylococcal enterotoxins (SE). SE generally target the intestine tracks and stimulate an enteric-vagus nerve reflex triggering the vomiting centers of the brain, and also act as superantigens, stimulate T-cell proliferation and release of cytokines from T-lymphocytes (Soleimanzadeh et al., 2018). It is believed that SE is stable against thermal processes and somewhat proteolytic enzymes, which leads to their activity in the digestive tract after consumption (Balaban and Rasooly, 2000).

Medicinal plants and their extracts have wide applications in cosmetics, foods, drugs, etc. Extracts of aromatic plants are containing different volatile compounds, which these compounds have several biological activities such as antimicrobial activity (AA) (Preedy, 2015). Busatta et al. (2008) studied the application of *Origanum majorana* L. essential oil as an antimicrobial agent in sausage and reported that the addition of marjoram essential oil to fresh sausage exerted a bacteriostatic effect at oil concentrations lower than the MIC, while a bactericidal effect was observed at higher oil concentrations. Vági et al. (2005) studied essential oil composition and AA of O. L. extracts obtained with ethyl alcohol and supercritical carbon dioxide and reported that this extract can be used as preservatives in the food and cosmetic systems. Ahmad et al. (2016) reported that long pepper had suitable AA against *S. aureus*, *S. aparophyticus*, *Salmonella typhi*, and *Shigella dysenteriae*.

AA of natural extracts against FBP microorganisms is considered important because of their safety, compatibility with nature, and especially their high efficiency against the microorganisms resistant to synthetic antimicrobial agents. However, it is required to high levels of these natural extracts to create an appropriate AA in a food system, which might

change the organoleptic characteristics of the food. Hence, researchers are seeking for a combination of natural extracts to enhance their AA, as the using dosages are low (Calo et al., 2015). As well as, unlike treatment with a synthetic antimicrobial agent, the possibility of creating antibiotic-resistant strain is very low in the treatment with herbal extract blend (HEB) (Ouedrhiri et al., 2016).

It is reported that various terpenic compounds have different AA in individual and mixed studies; as well as, there are different antimicrobial interactive effects in the mixed form. For example, synergistic effects between rosemary and marjoram extracts have been reported on Listeria monocytogenes and *Yersinia enterocolitica* (De Azeredo et al., 2011), and between Chinese cinnamon and cinnamon bark on FBP bacteria (Ghabraie et al., 2016). Therefore, finding an HEB with high AA is necessary.

Nowadays, statistical methods have been successfully used to find an optimum mixture in biotechnology and food technology (Azizpour et al., 2017a). Mixture design (MD) is one of these methods, which have been widely used in experimental studies. MD approach is appropriate to produce products that are composed of several components (Dutcosky et al., 2006). This statistical approach evaluates the relationship between components or factors on responses. As well, this approach can predict an optimal condition or formulation based on desirable range of responses (Flores et al., 2010). To date, MD has been successfully used in microbiology, such as study effects of different types of chloride salts on *Lactobacillus pentosus* growth parameters (Arroyo-López et al., 2009), optimization of medium for growth of yeast enriched with selenium (Yin et al., 2009), optimization of pharmaceutical formulations (Cafaggi et al., 2003), optimization of the medium of *Saccharomyces cerevisiae* for production of ethanol from sorghum juice (Yu et al., 2009), the introduction of an optimum cell-free supernatant (CFS) of multiple-strain mixture for *Lactobacillus* against FBPs (Yolmeh et al., 2017), and anti-enterotoxigenic activity of Lactobacillus on staphylococcal enterotoxins biosynthesis (Yolmeh et al., 2020). In addition, unlike microbial heat-inactivation that is widely studied mathematically, there are few studies on non-thermal inactivation particularly medicinal herb extracts in mixed form.

Finding the methods to eliminate the pathogenicity of pathogens by gentle treatments or processes, instead of extreme thermal processes, is important for pharmaceutical and food industries so that the bioactivities of compounds will not be damaged. The use of natural extracts is one of these gentle methods. Hence, the present paper aimed to find an optimum HEB with the highest anti-enterotoxic activity (AEA) against *S. aureus* using real-time PCR through the statistical method of MD.

Materials and Methods

Materials, microorganisms, and microbial growth conditions

Iranian traditional medicinal plants (*Origanum majorana*, *Areca*, and *Long pepper*) were purchased from the local market in Mashhad (Iran). All chemicals and mediums used in this study were analytical grades and obtained from Merck (Germany) and Liofilchem (Italy) Companies. *S. aureus* (ATCC 25923) was prepared from Ferdowsi University of Mashhad, Mashhad, Iran. These strains were plated on Mueller Hinton agar (MHA) and incubated at 37°C for an overnight to achieve a single colony. Inoculants used in this study were prepared by inoculating a single colony into Mueller Hinton broth (MHB) at 37 ºC for an overnight adjusting to 0.5 McFarland turbidity and then serially diluted to obtain the concentration of 1×10^6 CFU/mL in MHB.

Preparation of plant extract

The medicinal plants (*Areca*, *O. majorana*, and *Long pepper*) were crushed using a manual mortar and then dissolved in ethanol for 6 hr. After filtration through Whatman paper filter NO.1, the extracts were concentrated using a rotary evaporator (Preedy, 2015). Then, the concentrated extracts were completely dried at 45˚C and powdered.

Antimicrobial activity

Kirby-Bauer method

The AA of extracts was studied by the disk diffusion method according to the method described by Rostami et al. (2016). The studied concentrations of extract were 1, 2, 3, and 4 mg/mL. Briefly, the discs were soaked in the different extract solutions for 10 min, and after removing excess solution from the disk, used on plate cultured by the pathogen.

Determination of minimum inhibitory concentration (MIC)

MIC of medical plant extracts against common food-borne pathogenic bacteria was studied using the agar dilution method according to the method described by Rostami et al. (2016). The concentrations of extracts used in the section were 2, 4, 8, 16, and 32 mg/mL for MIC, respectively. The lowest concentration that inhibits bacterial growth was considered as MIC concentration.

Growth condition of S. aureus at a sub-MIC concentration

The extracts were added to tryptic soy broth (TSB) medium at 0.05, 0.1, and 0.2% as sub-MIC concentrations. *S. aureus* was inoculated at 1×10^5 cfu/mL, cultured, and incubated at 35°C for 24 h. As well as, the negative and positive control (the medium without CFS) cultures were used.

Enterotoxin detection

RIDASCREEN SET kit (R-Biopharm, Darmstadt, Germany) was used to detect A and B enterotoxins of the *S. aureus* grown in TSB including the sub-MIC concentration of

extracts. The minimum detectable limit of the kit was 0.50 to 0.75 ng of SEs per mL (Parsaeimehr et al., 2015).

RNA extraction and purification

The RNA of *S. aureus* was extracted by GeneAll hybrid isolation reagent (Korea) according to the Company instructions and the method described by Azizkhani et al. (2013). In order to purify RNA and remove DNA contamination from samples, RNase-free DNase I were treated. The purified RNA was assayed by spectrophotometer and the absorbance at A_{260nm}/A_{280nm} ratio, which must be between 1.8-2.1. The quality and integrity of RNA was checked by electrophoresis on 1% agarose gel by ethidium bromide staining, then stored at -70 ˚C.

Complementary DNA (cDNA) synthesis

cDNA was prepared by reverse transcriptase (HyperScript, GeneAll, Korea) and according to the method disclosed by Parsaeimehr et al. (2015).

Classic PCR

This section was done to ensure the remove of DNA from the RNA sample. Therefore, the PCR was performed on RNA samples, cDNA sample, and negative control by the primer of DNAgyrB gene. Table 1 shows the thermocycler program employed for PCR. After electrophoresis, the samples were without amplification band, which means there was no genomic DNA as contamination.

Table 1. *Thermocycler program used for PCR*

Program	Step	Temperature (˚C)	Time	
1	Initial denaturation	95	5 min	
	Denaturing	94	30 s	35 cycles
2	Annealing	60	20 s	
	Extending	72	20 s	
3	Final extending	72	2 min	

Real-time PCR

The total volume of ingredients in real-time PCR was considered 25 µL. Gene expression of *sea* and *seb* was evaluated using Power SYBR green PCR master mix (Applied Biosystems). The reaction medium was containing 12.5 µL of SYBR green, 2 µL of template of cDNA, 0.5 µL of each forward and reverse primers, 9.5 µL Rnase-free water.

The PCR cycling conditions: at first one cycle at 95 ˚C for 10 min, then 40 cycles at 95 ˚C for 15 s, 60 ˚C for 1 min and 72 ˚C for 30 s. The sequence of primers used for quantitative real-time PCR in this study were 5′-AAAATACAGTACCTTTGGAAACGGTT-3′ (sea-F, 92 bp, Yolmeh et al., 2020), 5′-TTTCCTGTAAATAACGTCTTGCTTGA-3′ (sea-R, 92 bp, Yolmeh et al., 2020), 5′-ACACCCAACGTTTTAGCAGAGAG-3′ (seb-F, 81 bp, Yolmeh et al., 2020), 5′-CCATCAAACCAGTGAATTTACTCG-3′ (seb-R, 81 bp, Yolmeh et al., 2020), 5′- CGCAGGCGATTTTACCATTA-3′ (gyr-F, 141 bp, Yolmeh et al., 2020), and 5′-GCTTTCGCTAGATCAAAGTCG-3′ (gyr-R, 141 bp, Yolmeh et al., 2020). The samples were normalized against *gyrB* expression. In order to detect any double-stranded DNA such as contaminating DNA, primer-dimer, and PCR product from misannealed primer, running a dissociation curve after real-time PCR is important in this type of amplicon detection. Therefore, the melt-curve of each gene was studied (Azizkhani et al., 2013). The ΔΔCt method was used to evaluate relative expression levels of *sea* and *seb* according to Applied Biosystems User Bulletin No. 2 (1997).

Experimental design

Three-component simplex-centroid MD was employed to find the best HEB against AEA of *S. aureus*. For this purpose, Mixture design 7 was employed. The components were namely *O. majorana* extract (A*), *Areca* extract (B*), and *Long pepper* extract (C*), which sum of them was one (A* + B* + C* = 1).

Statistical analysis

The least-square multiple regression method was used to investigate the relationship between the components and responses.

The accurate model for each response must be having R^2-adj and R^2-pred near to one. The statistical significance of the regression coefficients was studied using analysis of variance (ANOVA) and Duncan test at a confidence level of $P<0.05$. The mixture surface plots derived from the selected model were used to investigate the interactive effects of the components on each response (Azizpour et al., 2017b). The finding of the best blend of a medical plant extract with the highest AEA against *S. aureus* was the aim of the MD approach used in this paper.

Results

Antimicrobial activity in individual form

Kirby-Bauer method

S. aureus showed sensitivity to the extracts studied in this paper and the AA was increased by increasing extract concentration, as is shown in Table 2. Extract of *O. majorana* showed higher AA than the other two extracts.

Table 2. *Average diameter (mm) of microbial free zone area of S. aureus treated with extracts.*

Pathogen	Extract		
	O. majorana	*Areca*	*Long pepper*
S. aureus	$1.1\pm0.50^{b*}$	1.8 ± 0.4^{ab}	2.3 ± 0.5^{a}

** Different letters show significant difference.*

Table 3. *S. aureus and E. coli growth under different concentrations of extracts*

	Pathogen	0.05%	0.10%	0.20%	0.40%	0.80%	Negative control	Positive control
O. majorana	*S. aureus*	+	+	–	–	–	–	+
	E.coli	+	+	+	+	–	–	+
Areca	*S. aureus*	+	+	+	+	–	–	+
	E.coli	+	+	+	+	–	–	+
Long pepper	*S. aureus*	+	+	+	+	–	–	+
	E.coli	+	+	+	+	–	–	+

+: Grow *–: Not grow*

Determination of MIC

In the MIC and MBC study of three extracts, *Escherichia coli* was used as gram-negative bacteria and comparable bacteria. MIC of extracts gram-negative bacteria was remarkably more than gram-positive types. MIC of *O. majorana* for microorganisms was ≤ MIC concentrations of the other two extracts (Table 3).

Effect of components on the expression of genes

Table 4 shows the A and B enterotoxins produced by *S. aureus* grown on sub-MIC concentrations of the extracts. The lowest production of A and B enterotoxins was observed for *O. majorana*, so that both enterotoxins were not produced at 0.2% of *O. majorana*.

Table 4. *Production of A and B enterotoxins by S. aureus under sub-MIC concentrations of extracts*

		0.05%	0.10%	0.20%	Negative control	Positive control
O. majorana	A Enterotoxin production	+	–	–	–	+
	B Enterotoxin production	+	–	–	–	+
Areca	A Enterotoxin production	+	+	–	–	+
	B Enterotoxin production	+	+	–	–	+
Long pepper	A Enterotoxin production	+	+	–	–	+
	B Enterotoxin production	+	+	–	–	+

As shown in Table 5, the AEA of extracts in blend form was notably higher, compared to individual forms (#5, #6 and #8 runs).

Fitting the response surface models

Based on R^2, R^2-adj, and R^2-pred values, the quadratic model was the best model to predict genes expression of *sea* and *seb*:

Gene expression of *sea* = – 11.02 (A*) – 6.02 (B*) – 8.47 (C*) – 30.33 (A*) (B*) – 31.39 (A*) (C*) – 18.06 (B*) (C*)

Table 5. *Mixtures composition of HEB in a three-component constrained simplex lattice MD and experimental results for expression of sea and seb*

Run	Components			Relative gene expression	
	O. majorana (A*)	*Areca* (B*)	*Long pepper* (C*)	SEA	SEB
1	0.500	0.000	0.500	-15.8	-16.4
2	0.000	0.500	0.500	-11	-14.1
3	0.500	0.500	0.000	-11.8	-15.5
4	0.000	0.500	0.500	-10.8	-13.5
5	0.000	0.000	1.000	-8.5	-9.8
6	1.000	0.000	0.000	-11	-11.5
7	0.339	0.331	0.329	-18.5	-18.3
8	0.000	1.000	0.000	-6.5	-7.8
9	1.000	0.000	0.000	-10	-12
10	0.170	0.664	0.166	-14.8	-17
11	0.672	0.328	0.000	-17.5	-20.4
12	0.171	0.163	0.666	-15.4	-19.2
13	0.000	1.000	0.000	-6.1	-8.4
14	0.000	0.000	1.000	-9.1	-9
15	0.670	0.161	0.169	-21.6	-22.5
16	0.500	0.000	0.500	-16.5	-16.1

Gene expression of *seb* = – 12.25 (A*) – 7.78 (B*) – 9.52 (C*) – 35.59 (A*) (B*) – 26.77 (A*) (C*) – 23.66 (B*) (C*)

Based on the analysis of variance (ANOVA) and that whatever decrease in P-value and increase in F-value for each term in the models would have a higher effect on response. Significant effects ($P<0.05$) were observed on the linear mixture and interactive terms of (A*) (B*), and (A*) (C*) for expression of *sea*. However, there is no significance on the expression of *sea* for interactive terms of (B*) (C*), and (A*) (B*) (C*) ($P>0.05$) (Table 6). About expression of *seb*, the linear mixture and all interactive terms had a significant effect on it ($P<0.05$). The (A*) (C*) and (A*) (B*) interactions had the highest effect on the expression of *sea* and *seb*, respectively (Table 6). The lack-of-fit values of both selected models were insignificant ($P>0.05$), which shows the suitability of the models to predict the expression of *sea* and *seb* genes, as shown in Table 6.

Table 6. *ANOVA of the models for the responses*

Source	SEA				SEB			
	DF	MS*	F	P	DF	MS	F	P
Model	5	50.05	9.75	0.0013	5	50.59	10.84	0.001
Linear mixture	2	31.71	6.18	0.0179	2	20.98	4.50	0.040
AB	1	79.43	15.48	0.0028	1	109.30	23.42	0.001
AC	1	95.55	18.62	0.0015	1	69.51	14.89	0.003
BC	1	30.69	5.98	0.0345	1	52.68	11.29	0.001
Residual error	10	5.13	-	-	10	4.67	-	-
Lack of fit	5	10.06	5.58	0.060	5	9.16	3.62	0.052
Pure error	5	0.20	-	-	5	0.17	-	-
Total	15	-	-	-	15	-	-	-

** Mean of squares*

Discussion

Antimicrobial activity in individual form

Kirby-Bauer method

The average diameter of microbial free zone area for extracts examined in this study was comparable with penicillin and gentamicin antibiotics. The diameter of microbial-free zone area for each of three extracts against *S. aureus* was higher than antibiotic disks (Table 2). Josephs-Spaulding and Singh (2016) reported that the highest AA of *Areca catechu* L. was observed against *Vibrio cholera*. Deans and Svoboda (1990) reported that *Beneckea natriegens*, *Erwinia carotovora*, and *Moraxella* sp. were the most susceptible bacteria to marjoram oil, and of the fungi tested; *Aspergillus niger* showed the most susceptible. It is reported that *Long pepper* has appropriate fungicidal activity (Lee et al., 2001).

Determination of MIC

As is shown in Table 3, the bactericidal activity was observed for each of three extracts. However, the bactericidal effect was not observed for the extract of *Long pepper* at the studied concentrations. Like results of MIC assay, findings of MBC assay also revealed that the bactericidal activity was higher against gram-positive bacteria compared to gram-negative types. The finding of this study are in concurrence with researches on rosemary extract (Campo et al., 2000), essential oils of Turkish medicinal plants (Digrak et al., 2001), and extract of various onions (Benkeblia, 2004), extracts obtained from inflorescences of *Cirsium canum* L. (Kozyra et al., 2015), the extract of *Mentha piperita* L. (Singh et al., 2015), carotenoids extracted from *Micrococcus roseus* and *Rhodotorula glutinis* (Rostami et al., 2016), which observed that resistance of gram-negative bacteria to natural antimicrobial substances is more compared to gram-negative types.

Effect of components on the expression of genes

Among treatments, the maximum downregulation of both genes was achieved by run #15 (0.67% of component *O. majorana*, 0.161% of component B*, and 0.169% of component C*). The synergistic activity among antimicrobial agents of extracts is the main reason for higher AEA than individual forms. Similarly, a synergistic activity between nisin and *Zataria multiflora boiss*. essential oil on the production of enterotoxin C and α-hemolysin from *S. aureus* is reported by Parsaeimehr et al. (2010). As well, several cases of inhibitory synergistic activities have been reported, for example among high-pressure treatment and bacteriocin-producing lactic acid bacteria on *S. aureus*, nisin, and monolaurin on vegetative cells of *Bacillus* sp. in milk, and Nisin A and polymyxin B on *Escherichia coli* and *Listeria innocua* by Arqués et al. (2005), Mansour and Millière (2001), and Naghmouchi et al. (2010), respectively.

The findings of the percent study are in agreement with the result of Qiu et al. (2010), who studied the effect of sub-MIC concentrations of thymol against α-hemolysin and A and B enterotoxins production from methicillin-resistant and methicillin-sensitive *S. aureus* isolates. Their results have been shown that the bacterial growth and the production of SEA, SEB, and α-hemolysin decreased by an increase in concentrations of thymol.

Expression of sea

Figure 1 shows a mixture surface plot of *sea* expression of *S. aureus* treated with HEB at sub-inhibitory concentrations. Among the components, *O. majorana* and *Long pepper* extracts had the lowest and highest effects on the gene expression, respectively, of course at high concentrations. *sea* expression was increased initially as concentrations of all three components increased, but decreased at high levels of components. The lowest *sea* expression was achieved by treatment with the blend including 0.7 of A* component, 0.1 B* component, and 0.2 of C* component (Figure 1).

Expression of seb

AEA of the HEB on *seb* expression was higher than their individual forms. At the high concentrations of each component, the lowest and highest effects on *seb* expression were achieved by A* and B* components. However, the

maximum downregulation of *seb* expression was achieved by lower concentrations of C* component (Figure 1). Similarly *sea*, *seb* expression was decreased initially by increasing concentrations of all factors but decreased at high levels of each factor.

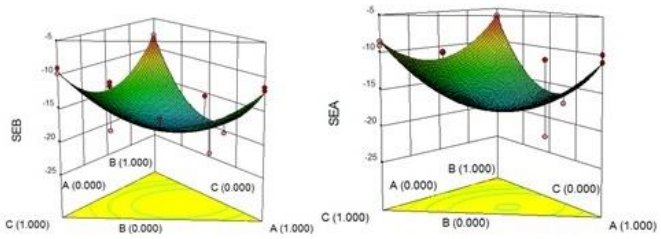

Figure 1. *Mixture surface plots for gene expression of SEA and SEB in S. aureus treated with sub-MIC concentrations of HEB (A: O. majorana extract, B: Areca extract, C: Long pepper extract)*

Optimization

In order to find a HEB with the highest AEA, a numerical optimization technique was used. So the importance and weight values of each factor were considered the same. At this condition, an HEB including 47% A*, 26% B*, and 27% C*, was found as the optimum HEB against gene expression of *sea* and *seb*, which composite desirability of this HEB was 0.79. Gene expression for *sea* and *seb* of the strain treated with the optimum HEB were predicted -17.99 and -19.75, respectively by software. The observed expression for *sea* and *seb* were -17.05 and -18.86, respectively, which were close to predicted types.

Conclusions

The extracts studied in this research showed a suitable AEA against the expression of *sea* and *seb* of *S. aureus*, in addition to inhibiting effect on the growth of *S. aureus*. At the presence of *Areca*; *O. majorana*; *Long pepper* extracts, *sea* had higher sensitivity compared to *seb*. Among the *extracts*, the extract of *O. majorana* had the highest AEA. The highest AEA was observed by treatment with an HEB including 47% A* (*O. majorana*), 26% B* (*Areca*), and 27% C* (*Long pepper*). The results of this paper revealed that the relative prevention of pathogenicity of *S. aureus* is possible by decreasing gene expression of SEs at sub-inhibitory concentrations of medical extracts.

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