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Original Article

Inhibition of the *sea* Gene Expression in *Staphylococcus aureus* Using the Aqueous and Alcoholic Extracts of the Grapevine (*Vitis vinifera L.)* Seeds

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Abstract

Staphylococcus aureus is an important etiological agent for causing food poisoning leading to high mortality in the world. The *sea* gene is encoded in a polymorphic family of temperate bacteriophage chromosomes and became a prophage, and the transcription of this gene is associated with the life cycle of this prophage. It has been suggested that the grape polyphenols can eradicate the enterotoxin production of food-borne bacteria. This study aimed to evaluate the activity of the aqueous and alcoholic extracts of the grape seeds in inhibiting the expression of the *sea* gene encoding staphylococcal enterotoxin type A in *S. aureus* isolated from different sources. This study used five enterotoxin A producing isolates belonging to *S. aureus.* The results showed that minimum inhibition concentration and sub-minimum inhibition concentration of the aqueous extract were 32 and 16 µg/mL for all isolates, respectively. However, in the case of the alcoholic extract, these concentrations were 16 and 8 µg/mL for all isolates, respectively, and the results of the chemical analysis of the aqueous and alcoholic extracts confirmed that they contain active chemical compounds, such as flavonoids, alkaloids, tannins, and glycosides; moreover, they contain many functional groups according to the analysis of the infrared spectrum. Both extracts were shown to be active in inhibiting the expression of the *sea* gene in the isolates under study. As the results indicated, the gene expression of these isolates was inhibited by approximately 0.31-0.63 fold, and all pathogenic and environmental isolates showed a decrease in the expression of this gene. These results practically open the door to the possibility of using these extracts to inhibit the ability of *S. aureus* to produce these dangerous enterotoxins; thereby decreasing or preventing their pathogenicity, especially their food poisoning infections.

Keywords: Expression, Food poisoning, Grape seeds, *Sea* gene, *Staphylococcus aureus*

1. Introduction

Staphylococcus aureus is an important etiological agent for causing food poisoning leading to high mortality in the world (1), and more than half of food poisoning outbreaks were caused by this bacteria (2). The most important symptoms contain vomiting, fever, and nausea (3, 4). More than 23 types of Staphylococcal enterotoxins were discovered, and more variants had been identified based on their antigenic structure that called from Staphylococcal Enterotoxin type A (SEA) to Staphylococcal Enterotoxin type 1Y (5). These enterotoxins were characterized by heat-stable, as well as pH- and proteases-resistant properties that share many common features. They are non-glycosylated and single-chain proteins with a homologous and globular structure, as well as low molecular weight (19-30 kDa) (6). These enterotoxins can also be classified in two sets, namely the true SEs

which contain the toxins demonstrating emetic potency and the enterotoxins-like toxins SELs which lacked the emetic ability (7). SEA is one of the most commonly involved enterotoxins in outbreaks of food poisoning (8). The *sea* gene is encoded in a polymorphic family of temperate bacteriophage chromosomes and became a prophage, and the transcription of this gene is associated with the life cycle of this prophage (9). This gene had 84% nucleotide homology with the *sea* gene. SEA is a superantigen that stimulates immune T cell production to secret transduction signals, such as interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), IL-2, and IL-6 (10). There were many attempts to inhibit the pathogenic effects of SEA, and the Food and Drug Administration proved the cytotoxic T-lymphocyte Antigen-4 Immunoglobulin $CTLA_4$ -Ig and dexamethasone in order to inhibit cell death and reduce cytokine levels in various animal models (11).

Grapevine (*Vitis vinifera L.*) had been considered a major grape species distributed in the Caucasus toward the Mediterranean region, and it has been always used in some industries and for eating (12). Furthermore, studies were attempting to add some compounds, such as tea and grape polyphenols to eradicate the enterotoxins production of food-borne bacteria (13, 14). Therefore, the current study aimed to evaluate the inhibition of the *sea* gene expression in *S. aureus* using aqueous and alcoholic extracts of the seeds in *Vitis vinifera L.*

2. Materials and Methods

2.1. Bacterial Isolates

This study used five enterotoxin A producing isolates belonging to *S. aureus*. The *S. aureus* was isolated from different sources of tape water, pathogenic (pus), food, normal flora (nasal tract), and soil. They were then given symbols, such as SA_1, SA_2, SA_3, SA_4, and SA_5, respectively. These bacteria had been isolated, diagnosed, and previously categorized by their ability to produce enterotoxin A in previous studies.

2.2. Plant Extracts

The grapevine (*Vitis vinifera L.*) was used to prepare two types of extracts (aqueous and alcoholic). These fruits were obtained from the local markets and then washed well with double distilled water. The seeds were then isolated from the remaining parts of fruits manually, fractured, and cracked using ceramic jars to extract what was inside the seeds. The aqueous and 95% alcoholic extracts were prepared according to Khanzada, Iqbal (15) and filtered through several layers of medical gauze. Finally, the extracts were sterilized by Millipore filter of 0.45µm and concentrated by a rotary evaporator at 50°C. The extracts were then preserved in a sterile dark and sealed bottles in a refrigerator at 4°C to be used in subsequent steps.

2.3. Qualitative Phytochemical Screening and Functional Group Detection in the Prepared Plant Extracts

Qualitative phytochemical screening tests in prepared plant extracts, such as tannins, saponins, resins, flavonoids, volatile oils, glycosides, and alkaloids were detected according to Adeloye, Akinpelu (16), and the detection of the functional groups was made using a Fourier Transform Infra-Red (FTIR) Spectrophotometer (Shimadzu Co, Japan).

2.4. Detection of the Minimum Inhibition Concentration and Sub-Minimum Inhibition Concentration of the Prepared Plant Extracts

The minimum inhibition concentration (MIC) and sub-minimum inhibition concentration (SMIC) of the prepared plant extracts on bacterial isolates under study were detected using a microdilution method in the tissue culture microplate according to Wayne (17), where the prepared nine concentrations of 256, 128, 64, 32, 16, 8, 4, 2, and 1 µg/mL from each extract well in the microplate were inoculated with 100 µL of muller Hinton broth, 100 µL of prepared concentration of each extract alone, and 50 µL of bacterial suspension (for each isolate alone). The positive control well contains bacterial suspension and muller Hinton broth, while the

negative control well contains the prepared concentration of extract and muller Hinton broth. The microplate was incubated at 37˚C for 24 h, and the results were then read by observing the growth in wells. The least concentrations of the extract that have been able to inhibit and permit bacterial growth had been considered MIC and SMIC, respectively. The results were finally recorded.

2.5. Inhibition of the *sea* Gene Expression Test

2.5.1. RNA Extraction

For RNA extraction of the bacterial isolates, all bacterial isolates were cultured on tubes containing 5 ml of tryptic soy broth as controls without treatment. These isolates were cultured on the tubes containing the SMIC of each alcoholic and aqueous extract alone, and all tubes were incubated at 37˚C for 24 h, then the RNA of each isolate from each tube was extracted using the Quick RNA Bacterial Miniprep™ kit provided from Zymo Research Co., USA. The procedure was conducted according to the instruction manual of the kit.

2.5.2. cDNA Synthesis

The RNA samples which were extracted in the previous step were used to be transformed to cDNA using the prime Script™ RT reagent kit provided from Takara Bio INC., USA, and all the kit components were placed at room temperature before use. The master mix solutions were prepared according to the instructions of the company that provided the kit, and 8 µl of the prepared Master Kit solution were taken and placed with 2 µl of the RNA sample for each isolate with and without treating with alcoholic and aqueous extracts of each grape seed alone. Afterward, the mixed well and the tubes were incubated at 37˚C for 15 min in the thermal cycler type TC-pro (BOECO Co., Germany).

2.5.3. Real-Time PCR

The obtained samples from the previous step (cDNA) were used for the real-time polymerase chain reaction (RT-PCR) to measure the expression of the *sea* gene with and without the alcoholic and aqueous extracts of the grape seeds, compared to the reference gene. This experiment was conducted using the KAPA SYBR^{R} Fast $_{q}$PCR Master Mix kit/Promega/USA according to the instruction manual of the kit using the RT-PCR instrument/Sacace, Italy. The primers and cycling program conditions are shown in tables 1 and 2.

Table 1. Primers used in this study

Primer	Sequence	Ref.
sea-F	5'-TTGGAAACGGTTAAAACGAA-3'	(18)
sea-R	5'- GAACCTTCCCATCAAAAACA-3'	
(Ref. gene) 16srRNA-F	5' – TACACACCGCCC GTCACA-3'	(19)
16srRNA-R	5'– TTCGACGGGCTAGCTCCAAAT-3'	

Table 2. RT-PCR cycling program conditions

Step	Temperature ˚C	Time	No. of Cycle
Denaturation	95	5 min	1
Amplification	95	30 sec	
Annealing	53	30 sec	40
Extension	78.7	30 sec	
Termination	72	2 min	1

2.6. Statistical Analysis

The statistical analysis of the results was performed using the SAS program and LSD at $P \leq 0.05$ (20); moreover, the AnalyStat program was used to obtain the mean±SD and SEM values.

3. Results and Discussion

3.1. Detection of the Minimum Inhibition Concentration and Sub-Minimum Inhibition Concentration of the Prepared Extracts

The results of this study showed that aqueous and alcoholic extracts of the grape seeds had antimicrobial effects on all *S. aureus* isolates. This is due to the phenolic compounds that were high in both fresh grapes and grape seed extract. The percentages of the phenolic compounds were measured from 22% to 60%, respectively. In fresh grapes, as well as the extracts of the grape seed, high oxygen radical absorbance

capacity was exhibited, and it was revealed that the anthocyanin pigment, malvidin-3,5-diglucoside, and phenolics were major compounds isolated from grapes (21). Wangensteen, Miron (22) tested the activity of many bioactive compounds by releasing them from grape type *pomace* and demonstrated that the bioactive compounds had the ability to significantly inhibit LDL oxidation in the human body. Moreover, this reflects the fact that these extracts possess the active compounds and groups necessary to influence the growth of these bacterial isolates. As it has been confirmed in the subsequent results of the current study, the MIC is the lowest concentration of the materials that inhibits the growth of microorganisms, while SMIC is the concentration below MIC that permits the growth of microorganisms and does not inhibit it (23). The results in table 3 showed that the MIC of aqueous was 32 µg/mL, while its SMIC was 16 µg/mL; in addition, the MIC and SMIC of the alcoholic extract were 16 and 8 µg/mL, respectively. It is noticed that the alcoholic extract required lower concentrations than the aqueous extract, which confirms the results of other research on the preference of the alcoholic extract in the effect of antimicrobial extracts since organic solvents, such as methanol, ethanol, and acetone work to dissolve and extract all the active compounds from their raw materials, which do not dissolve in water. Therefore, it was observed that the alcoholic extract affected the isolates under study at a concentration lower than its aqueous counterpart (24, 25).

Table 3. MIC and SMIC of the aqueous and alcoholic extracts of the grape seeds under the study of the *S. aureus* isolates

Extract type	MIC µg/ml	SMIC µg/ml
Aqueous	32	16
Alcoholic	16	8

Furthermore, our results were not consistent with the findings of a study by Shrestha, Theerathavaj (26) who showed the potent effects of the grape seeds extract with MIC of 0.625 mg/mL and minimum bactericidal concentration of 1.250 mg/ml for both strains of *S. aureus*. The antimicrobial effects of the grape seed extracts belong to the phenolic contents found in grape seeds are partially hydrophobic and are considered to interact with the bacterial cell wall and lipopolysaccharide interfaces by decreasing membrane stability. In addition to the amount of phenolic content in the grape seed extracts measured in gallic acid equivalent, it has been directly correlated with the antibacterial properties (27). Moreover, the study of the bacteriostatic and bactericidal effects of the grape seed, especially the pharmacodynamics of gallic acid on *Escherichia coli*, *Salmonella enteritidis*, and *S. aureus*, as well as structure-activity correlation assays showed that three hydroxyl groups of the compound are effective against *E. coli* and *S. enteritidis*; furthermore, all of the substituents of the benzene ring were effective against *S. aureus* (18).

3.2. Qualitative Phytochemical Screening and Functional Groups Detection in Prepared Extracts

The recorded data showed that the aqueous extract of the grape seeds contained active compounds, such as tannins, saponins, flavonoids, volatile oil, glycosides, and alkaloids, while alcoholic extract consisted of tannins, flavonoids, volatile oils, resins, glycosides, and alkaloids as active compounds (Table 4). These results were in line with the findings of a study conducted by Gorodyska, Grevtseva (19) who concluded that grape seed powder consisted of many active materials as well as glycerin, xanthosine, methyl ester, carciol, and linolenic acid. In addition, the results of the FTIR analysis had confirmed that the aqueous extract of the grape seed had many functional categories, such as hydroxyl phenol, aldehyde, and alkanes, while the alcoholic extract of the grape seeds had secondary amine, methyl, and alkanes as functional groups (Table 5 and Figures 1, 2). These results are consistent with the findings of a study performed by Ananga, Obuya (28) who concluded that grape seeds are an amazing source of polyphenol compounds including monomeric, such as catechin, epicatechin, and gallic acid, as well as polymeric (e.g., procyanidins).

Table 4. Qualitative phytochemical screening results of the extracts prepared from the grape seeds

Extract	Active compounds						
	Tannins	**Saponins**	**Resins**	**Flavonoids**	**Volatile oils**	**Glycosides**	**Alkaloides**
Aqueous	+	+	-	+	+	+	+
Alcoholic	+	-	+	+	+	+	+

Table 5. Active groups found in the extracts prepared from the grape seeds

Extract	Active groups	Symbol	Fr.cm^{-1}
Aqueous	Hydroxyl phenol	R-OH	3367
	Aldehyde	R-CooH	2941,1741
	Alkane	C-N	1365
	Keton	C=O	1614
	Alkane	C-O	1056
Alcoholic	Secondary amine	NH	3394
	Methyl	-C-H	2943
	Alkene	C=C	1614
	Alkane	C-C	1400
	Alkane	C-O	1058

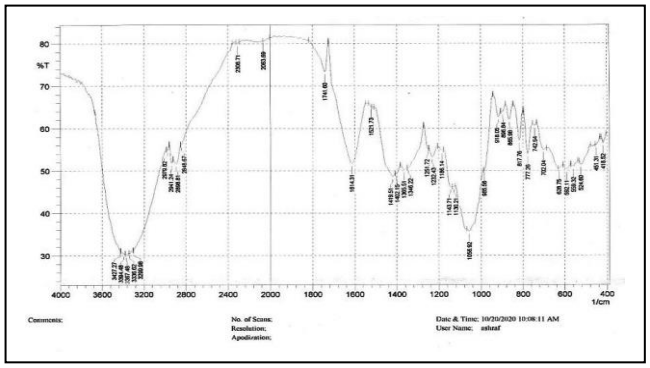

Figure 1. FTIR analysis of the aqueous extract of the grape seeds

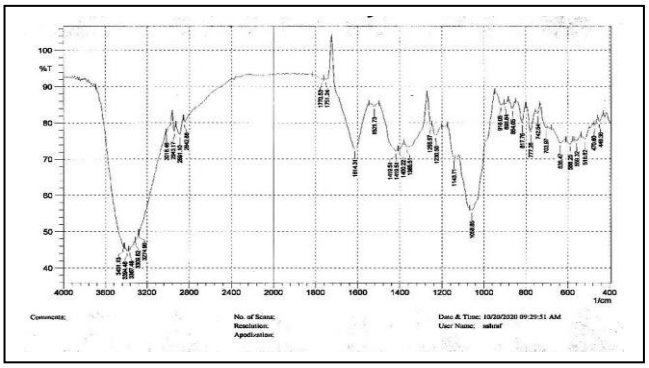

Figure 2. FTIR analysis of the alcoholic extract of the grape seeds

3.3. Gene Expression Experiment

The results revealed that the aqueous extract of the grape seeds inhibited the expression of the *sea* gene, compared to controls in the change of folding from 1 to 0.31. There were significant differences among the isolates according to their isolation source from 0.31 to 0.63 at $P \leq 0.05$, and the isolate (SA_4) from normal flora was the most affected, followed by isolate (SA_2) from pathogenic source, (SA_3) from food, (SA_1) from tape water, and (SA_5) from the soil in descending order (Table 6). Accordingly, it can be concluded that the aqueous extract of the grape seeds can inhibit the expression of the *sea* gene due to containing many functional groups, such as hydroxyl phenol, RCOOH, C-N, and C-O. Furthermore, the results revealed that the alcoholic extract of the grape seeds inhibited the expression of the *sea* gene, compared to controls, in the change of folding from 1 to 0.34, and there were significant differences among the isolates according to their isolation source from 0.34 to 0.63 at $P \leq 0.05$ (Table 7), which may be due to the chemical compound found in the alcoholic extract of the grape seeds, such as phenolic acid, gallic acid, ellagic acid, catechin, and monoglucuronide (29).

Table 6. Changes in the *sea* gene expression for isolates under study when treated with the aqueous extract of the grape seeds

Isolates	Relative expression		Changes in folding
	Control	Treated	
SA_1	4	5	0.5*
SA_2	4.1	5.5	0.38*
SA_3	2.6	3.71	0.46*
SA_4	4.2	5.9	0.31*
SA_5	3.3	3.95	0.63*
Mean	3.6	4.812	0.456
SD	0.6804	0.9552	0.1218
SEM	0.3043	0.4272	0.0545
	LSD		0.336

*Mean differences are significant at $P \leq 0.05$

Table 7. Changes in the *sea* gene expression for isolates under study when treated with the alcoholic extract of the grape seeds

Isolates	Relative expression		Changes in folding
	Control	Treated	
SA_1	1.9	3	0.47*
SA_2	3.75	5.1	0.4*
SA_3	4.35	5.9	0.34*
SA_4	3.8	4.5	0.63*
SA_5	2	3.37	0.38*
Mean	3.1	4.374	0.444
SD	1.1299	1.2008	0.1141
SEM	0.5053	0.537	0.051
	LSD		0.702

*Mean differences are significant at $P \leq 0.05$

This is due to the fact that the grape seed extract, whether aqueous or alcoholic, contains many active compounds, such as polyphenols, tannins, and acids, which made it effective in inhibiting the gene expression of the enterotoxin A gene since these active compounds contain effective groups as they are shown in table 5 which have the ability to bind to the operator of the gene and inhibit its encoding at the transcriptional level (30).

It also has the property of chelating, flocculation, and redox phenomenon as it binds to the regulatory proteins and enzymes participating in producing this toxin and blocking its role (31). Similarly, some studies had concluded that the phenolic compounds extracted from plants, such as ortho-phenyl phenol, inhibit the anabolisms of many amino acids and highly down-regulate the genes that encode the enzymes involved in diaminopimelate pathways and some proteinous virulence factors, such as a toxin. As a result, the action of these compounds was similar to the mechanism of some antibiotics (32). In addition, the structure-activity of these compounds demonstrated that the presence of electron-donating groups is essential for exhibiting its antivirulence properties, such as the inhibition of the production toxins (33). Accordingly, in our study, these results revealed that the aqueous and alcoholic extracts of the grape seeds, with their active compounds and groups, have the ability to inhibit the production of enterotoxins by *S.aureus* isolates from different isolation sources, which opens the door for additional studies of these extracts in preventing the pathogenicity of these bacteria, especially their food poisoning infections.

Authors' Contribution

Study concept and design: K. A. B.
Acquisition of data: K. A. B.
Analysis and interpretation of data: K. A. B.
Drafting of the manuscript: K. A. B.
Critical revision of the manuscript for important intellectual content: K. A. B.
Statistical analysis: K. A. B.
Administrative, technical, and material support: K. A. B.

Conflict of Interest

The authors declare that they have no conflict of interest.

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