Inhibitory Effect of the Brown Seaweed *Sargassum angustifolium* Extraction on Growth and Virulence Factors of *Staphylococcus aureus*

Afsaneh Mohkami[1*], Maziar Habibi-Pirkoohi[2]

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Abstract

Regarding the growing trend of multi-drug resistant strains of *Staphylococcus aureus*, the search for alternative and novel antimicrobial agent has been intensified. For this purpose, the present study was carried out to investigate the antagonistic effect of the brown seaweed *Sargassum angustifolium* on growth and virulence factors of *S. aureus*. A typical microdilution assay was performed to monitor inhibitory effect of *S. angustifolium* extraction on growth of the bacteria within a 6-h period. Furthermore, Real-Time PCR assay with specific primers was conducted to evaluate the impact of extraction on expression of genes encoding staphylococcal enterotoxins (sea) and capsule formation (*cap8*). The results revealed that *S. angustifolium* extract inhibited bacterial growth in a dose-dependent way; so that bacterial growth was fully stopped at the end of the assay. Antibacterial effect was enhanced by increasing in *S. angustifolium* extraction concentration. The results of Real-Time PCR assay indicated seaweed extraction was able to decrease virulence genes expression significantly. Again, by increasing extraction concentration, stronger inhibitory effect was observed. As a whole, the findings of this study suggest that *S. angustifolium* extract has potent antimicrobial effect that can be used for development of new generation of anti-staphylococcal medicines.

Keywords: Antimicrobial, Seaweed, *Sargassum angustifolium*, extraction, *Staphylococcus aureus*, Virulence factor.

Introduction

Medicines of natural origin have attracted much attention due to their useful properties such as minimizing environmental hazards, lack of drug resistance and availability. Natural products with antimicrobial effects are more easily degraded and, hence, are regarded as ideal alternative for synthetic antibiotics (Bhutiya et al., 2018). Meanwhile, the widespread use of antibiotics today has led to the emergence of resistant strains of microorganisms and increased antibiotic resistance. Therefore, research on natural antimicrobial agents is more interested obtaining new pharmaceutical sources (Hoseini et al.,

1- Research and Technology Institute of Plant Production, Shahid Bahonar University, Kerman, Iran
2- Zist Pajoohan Baran knowledge-based Inc., Shahid Bahonar University, Kerman, Iran
*email: amohkami@uk.ac.ir

2013). According to researches, seaweeds and macroalgae represent one of the sources with remarkable antimicrobial properties. All three major groups of algae *viz*. Rhodophytae (red algae), Chlorophytae (green algae) and Pheaophytae (brown algae) are naturally rich sources of active metabolites can be used in pharmaceutical industry (Thankaraj et al., 2019). There have been many reports on the considerable antimicrobial properties of seaweeds against various pathogenic bacteria belonging to both Gram positive and Gram negative strains (El Shafay et al., 2016). Widespread uses of marine algae in modern medicine stems and their ability in biosynthesis of great variety of secondary metabolites with biological properties including antiviral, antibacterial, antifungal activities (Moubayed et al., 2017).

Staphylococcus aureus represents a pathogenic bacterium residing as a common carrier in a wide range of hosts including livestock, poultry and, of course, human being (Lacey et al., 2016). The major residing sites of *S. aureus* are nares, skin, and the gastrointestinal tract and infection symptoms include endocarditis, bone and joint infections, bacteremia, and toxic shock (Graf et al., 2019). *Staphylococcus aureus* produces a wide range of infections ranging from simple skin infections such as boils, mumps, eyelashes and abscesses to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome and septicemia. *Staphylococcus aureus* is one of the five most common causes of nosocomial infections, especially post-operative wound infections. Every year, thousands of people in hospitals get *S. aureus* infections (Carpenter et al., 2016).

Pathogenicity of this organism depends on various virulence factors such as surface adhesion proteins, ability to produce toxins and enzymes, rapid drug resistance, and biofilm formation (Gao et al., 2018). Staphylococcal enterotoxins (sea, SEB, SEC1, and SED) represent a major class of *S. aureus* virulence factors that are main cause of gastroenteric syndrome and toxic shock syndrome by initiating the activation and proliferation of T cells with certain Vβ regions on their T-cell receptors (Klotz et al., 2003). Sea is a common enterotoxin of *S. aureus* which is considered a possible etiologic agent for some cases of antibiotic-associated diarrhea and toxic shock (balaban et al., 2000). Beside enterotoxins, other virulence factors contribute to staphylococcal infection. Indeed, virulence factors including surface-associated adhesions, cytotoxins, and capsular polysaccharides, are responsible for staphylococcal infections. Capsule represents a cell wall component that protects *S. aureus* from phagocytic uptake and hence, enhances microbial virulence (Verdier et al., 2007). Out of 11 capsular polysaccharide types discovered in *S. aureus*, Type 8 capsular polysaccharide (*CP8*) is the most prevalent capsule type in clinical isolates of the bacterium (Soleimani and Habibi, 2016).

Marine macroalgae have emerged as an abundant, natural and safe source of antimicrobial agents. Empirical studies suggest that various species of macroalgae possess

potent antimicrobial impact on different pathogenic bacteria. Martins et al (2018), for example, reported that the extract of the brown algae *Himantothallus grandifolius* on four bacteria namely *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli,* and *Pseudomonas aeruginosa*. In their study on phytochemical and biological evaluation of some *Sargassum* species, Mehdinezhad et al. (2016) found out that *S. angustifolium* had high antimicrobial properties due to high phenolic content. In another study, Telles et al (2018) reported that heterofucans extracted from *Sargassum filipendula* had high inhibitory effect on growth and biofilm formation of *Klebsiella pneumoniae* and Staphylococcus epidermidis. More recently, Javee et al (2010) isolated bioactive glycolipid from *Sargassum myriocystum* that had considerable antimicrobial impact on *Streptomyces* sp.. According to various reports on antimicrobial properties of marine macroalgae, the present study was conducted to investigate the inhibitory effect of the brown seaweed extraction (*Sargassum angustifolium*) on expression of Staphylococcal enterotoxin (sea) and CP8 genes at transcription level.

Materials and Methods

Seaweed collection and extraction

S. angustifolium was collected from Chabahar shores, south east Iran (Latitude / Longitude: 25.31749°/ 60.605475°). The sample was washed with tap water three times to remove debris, air-dried and shaded, then grounded by an electric mill. 30 gr of the *S. angustifolium* was mixed with 300 ml ethanol (70%) and shaked for 24h at room temperature. The extraction was dried using a rotary evaporator for complete removal of ethanol. The supernatant including the algae extraction was dried at 40° C. The dried pellet was then re-suspended in distilled deionized water and diluted to prepare 0.01 gr/ml concentration.

Physiological properties and mineral content

Proximate compounds including protein, carbohydrate, lipid and ash were determined by the method proposed by Pourashouri et al (2019). Briefly, moisture content was measured by drying the sample in oven (120oC) and calculating the weight before and after drying. For protein content measurement, seaweed sample was first digested by H_2SO_4 and Kjedahl catalyst and heated. Then the mixture was cooled and distillated water was added. Then reagents (6 drop of HCl) was added and the mixture was again heated until all NH_3^+ was distillated. Protein content was calculated according to the volume of HCl used for blank and sample titration. The mineral content was measured by atomic absorption spectrophotometer (Perkin Elmer Analyst 800).

Antimicrobial assay

Antimicrobial assay was conducted as proposed by Soleimani and Habibi (2016). *S. aureus* (PTTC 1431) was provided by Iranian Research Organization for Science and Technology (IROST). Minimum inhibitory concentration (MIC) of the *S. angustifolium* extraction (called SE hereafter) was determined using broth microdilution method in

a 96-well standard ELISA plate by overnight incubation. Luria Bertani (LB) broth containing *S. aureus* was used as a culture medium. Five concentrations of SE *viz*. 0 (control), 12.5, 25, 50, and 100 µg/ml were analysed. No SE was added to negative control well. The lowest concentration of SE inhibiting bacterial growth was assigned as MIC. Moreover, the *growth kinetics of S. aureus* in a 6 h period was monitored by drawing the growth curve at aforementioned concentrations of SE via measuring optical density (600nm) against three 2 h time intervals.

Real time PCR analysis

Real-Time PCR assay was conducted to assess expression of sea (Staphylococcal enterotoxin) and *cap8* gene after two hours of SE treatment. RNA isolation was performed by total RNA isolation kit (Denazist Co., Iran) and cDNA synthesis was carried out Hyperscript TM One-step RT-PCR Mastermix kit (Gene All Co.). Primer pair sequences for Real-Time PCR of the virulence factor were designed by AllelID 6.0 software (Table 1). The 16s RNA was used as house keeping (reference) gene and internal control in Real-Time PCR assay. Expression of the virulence gene (*cap8*) was quantitatively analyzed using a Real-Time PCR system (BioRad). Re-al-Time PCR was carried out in a 20 µl reaction volume containing 0.5µM of each primer and 10 µl of SYBR Green Real-time PCR Master mix (Genet Bio, South Korea). Quantitative Real-Time PCR experiments were performed in duplicate for each sample. The Real-Time RT-PCR data were analyzed by REST software.

Statistical analysis

This experiment was carried out as a completely randomized design (CRD) at three replications. The obtained data were analyzed using SPSS 18 software.

Results

Physiological characterization of extraction

The various physical properties together with mineral content of the SE are presented in Table 2. The results show carbohydrates form the dominant ingredient of the extract whose content is about 3.5 times more than proteins. Protein content is 12.45 (%DW) which is fairly high. Moisture and ash values were 10.55 (%DW) and 35.01 (%DW). As expected, lipid content was less than 1% which is a normal value usually seen in *S. angustifolium* extractions.

Table 1. Primer sequences of virulence factors and housekeeping genes

Gene	Forward sequence	Reverse sequence
sea	5'AAAATACAGTACCTTTGGAAACGGTT	5'TTTCCTGTAAATAACGTCTTGCTTGA
cap8	5'AGCCGTTAATCCGCCTAATGTC	5'ACTGGACCACCCGCTTCG
16s RNA	5'CGTGCTACAATGGACAATACAAA	5'ATCTACGATTACTAGCGATTCCA

Growth kinetics of S. aureus

Results of serial dilution assay showed that 50 µg/ml SE concentration could inhibit the growth of the pathogen; the MIC of the SE was therefore determined as 50 µg/ml. The changes in bacterial growth through a 6 h interval are represented in Figure 1. As seen in Figure, control group (not-treated bacteria) showed a normal growth pattern so that the highest growth rate was observed at the end of the assay. However, growth rate was decreased in all treatments drastically so that at the end of the assay, bacterial growth was near zero in all treatments. As expected, growth decrease was much sharp in higher concentrations of the SE; meaning that a drastic decrease of bacterial growth was observed in 100 µg/ml concentration of the extraction. Findings imply dose-dependent negative impact of SE on growth of *S. aureus*. The results of mean comparison test revealed that the difference between concentrations of 12.5 and 25 µg/ml was not significant ($P<0.05$); however, there was significant difference in terms of growth inhibition between these two concentrations and 50 and 100 µg/ml ($P<0.05$).

The results of SE treatment on expression of virulence factors are presented in Figures 2 and 3. Regarding *cap8* gene, SE treatment decreased gene expression compared to control and by increasing SE concentration, *cap8* expression reduction was more severe. In the first concentration of SE (12.5 µg/ml), *cap8* expression showed a 5 fold decrease; while gene expression decreased by about 21-fold at SE concentration of 100 µg/ml (Figure 2). Similar trend was observed in expression of Staphylococcal enterotoxin (sea) under treatment of various concentrations of S. angustifolium. Compared to *cap8*, sea expression was more vulnerable to seaweed treatment; meaning that higher decrease of this virulence factor was observed when the bacteria

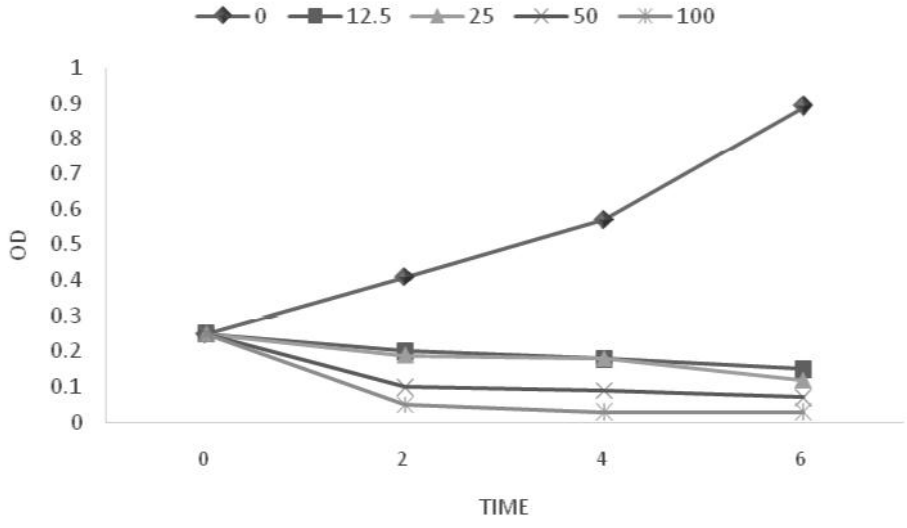

Fig. 1. The growth kinetics pattern of *S. aureus* under treatment with different concentrations of SE.

were incubated with 100 µg/ml extract of S. angustifolium. For both virulence factors, a relatively linear relation was observed between SE and decrease of gene expression. In this regard, the highest degree of gene expression reduction was observed at SE concentration of 100 µg/ml.

Table 2. Physiological properties of *S. angustifolium* extraction.

Compositions	value
Proximate composition	
Ash (% DW)	35.01±0.42
Crude protein (% DW)	12.45±1.37
Crude lipid (% DW)	0.50±0.17
Moisture (% DW)	10.55±0.61
Total carbohydrate (%DW)	41.49±4.63
Mineral composition	
Calcium (%)	2.26±0.34
Magnesium (%)	1.01±0.072
Iron (%)	0.06±0.01
Manganese (ppm)	13.33±3.5
Copper (ppm)	4.33±0.58
Zinc (ppm)	11.67±1.15

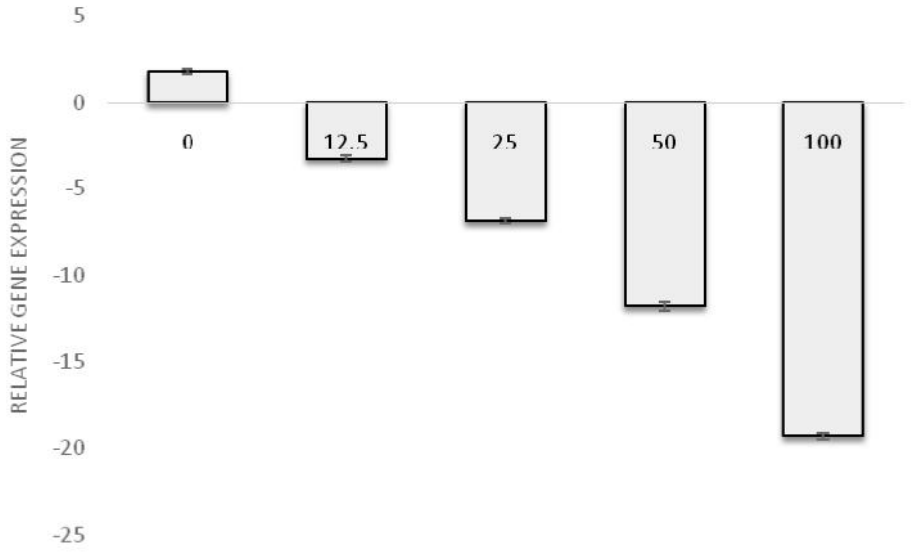

Fig. 2. Down-regulation of cap8 virulence factor at various concentrations of SE.

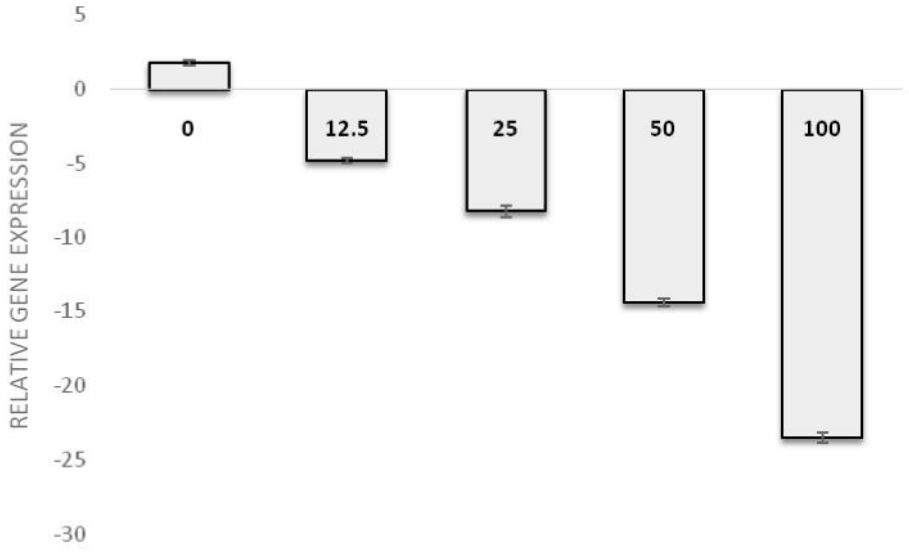

Fig. 3. Negative Impact of SE on expression of Staphylococcal enterotoxin (sea).

Discussion

Decreased antimicrobial efficiency has become a global public health issue and the paucity of new antibacterial drugs is evident. In search for new antimicrobial medicines with natural origin, the present study was conducted to investigate the inhibitory effect of *S. angustifolium* on growth and virulence factors of *S. aureus*. The results obtained in this study revealed that various concentrations of SE inhibited growth of the pathogenic bacteria *S. aureus*. Evaluation of bacterial growth kinetics apparently showed that *S. angustifolium* extraction has such a potent antimicrobial effect that within 6 hours completely inhibited bacterial growth. High potential of *Sargassum* species in preventing bacterial growth has been reported by many authors (Pourashouri et al., 2019; Pérez et al., 2016; El Shafay et al., 2016; Patra et al., 2008). The antimicrobial properties of *Sargassum* species on various gram positive and gram negative bacteria has been attributed to presence of bioactive compounds contained in extract of this seaweed. Oxylipins represents a major antimicrobial agent in seaweed extraction that may be cause of quick inhibition of bacterial growth (Perez et al., 2016). Other classes of bioactive compounds may be regarded as antimicrobial agent in *S. angustifolium* extraction inhibit growth of *S. aureus* in our experiment. For example, phenolic compounds with various molecular weights found in seaweeds have been reported to have considerable antimicrobial effect (Moorthi and Balasubramanian, 2015). Brown seaweeds have higher contents of phenolic compounds than green and red macroalgae, it may justify high antimicrobial impacts of these algae in preventing bacterial species (Ścieszka and Klewicka, 2019). β-carotene, violaxanthin and fucoxanthin in brown algae represent other classes of bioactive compounds that possess

high antimicrobial activities may contribute in inhibition of *S. aureus* growth (Ibañez and Cifuentes, 2013). Our results reconfirm previous reports on considerable antimicrobial impact of *Sargassum* (Yu et al., 2019; Telles et al., 2018). These findings have practical implications developing novel, non-chemical and cost-effective antimicrobial medicines as ideal alternative for current synthetic antimicrobial drugs.

Another part of this research was evaluation of inhibiting effect of SE on two virulence factors of *S. aureus* namely staphylococcal enterotoxin coding gene (sea) and capsule polysaccharide formation gene (*Cap8*). The results presented in this research showed that SE at various concentrations suppressed expression of the virulence factors. Staphylococcal enterotoxins are major virulence factors whose expression and secretion of this array of toxins and enzymes are tightly controlled by a number of regulatory systems; thus suppressing their expression can be regarded as an effective way to prevent from occurrence and dissemination of staphylococcal diseases (Kong et al., 2016). In a recent study, Kim (2019) remarked that suppression of the enterotoxins can prevent formation of toxic shock syndrome and even mortality caused by some strain of *S. aureus*. Capsule is another virulence factors of *S. aureus* whose underpinning gene expression was inhibited by SE. This antagonistic effect of SE suggests its potentiality for developing effective anti-staphylococcal medicine. It has been reported by many authors that inhibition of capsule formation is a considerable approach to fight staphylococcal infections (Ansari et al., 2019; Rausch et al., 2019; Nanra et al., 2013).

In general, the finding of this research considering antagonistic effect of SE on virulence factors of *S. aureus* is of great importance. It shows that SE not only disrupts bacterial growth but also imposes a specific effect on virulence of *S. aureus*. To the best of our knowledge, it is the first time that inhibitory effect of *Sargassum angustifolium* on virulence factors of *S. aureus* is reported. Targeting virulence factors of pathogenic bacteria represents a new approach in developing new generation of antimicrobial medicines and there have been increasing number of studies conducted in this field (Allen et al., 2014; Heras et al., 2015). The main advantage of this approach is avoiding general antibiotics that have caused occurrence of drug-resistant strains of bacteria. In the other words, targeting microbial virulence rather than survival seems to be an exciting strategy, since the modulation of virulence factors might lead to a milder evolutionary pressure for the development of resistance (Silva et al., 2016). As remarked by Soleimani and Habibi (2016), neutralization of the main virulence factors is an effective way to find effective preventative and therapeutic agents to combat staphylococcal infection. Our results reconfirmed that bioactive compounds found in seaweeds are potentially effective antimicrobial agents that can be used in development of novel antimicrobial medicines.

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