Antibacterial activity of muscle wall extracts of sea cucumber (Stichopus horrens) from Chabahar coastal area, Iran, against pathogenic bacteria in rainbow trout (Oncorhynchus mykiss)

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ABSTRACT
The aim of this research was to evaluate the antibacterial activity of the muscle wall extracts of sea cucumber, Stichopus horrens, against Lactococcus garvieae, Streptococcus iniae, Aeromonas hydrophila, and Yersinia ruckeri bacteria in rainbow trout using the disk-diffusion and well-diffusion methods. In this study, nine sea cucumbers (with the mean weight of 1690 ± 12.18 g) were randomly obtained from two locations in Chabahar Bay, Iran in November 2018. The muscle wall of the body was extracted with each of the ethyl acetate, methanol, and acetone organic solvents. The antibacterial activities of extracts were determined. Only the ethyl acetate extracts of S. horrens in the concentrations at 8 and 12 mg/mL had an inhibitory effect on all the examined bacteria. Y. ruckeri and A. hydrophila bacteria were sensitive to the acetone extracts. The best property was recorded with the S.horrens ethyl acetate extracts, with the MIC value of 0.625 mg/mL against S. iniae and L. garvieae. The MIC values ranging from 0.626 to 1.25 mg/mL were also displayed with the acetone extracts against Y. ruckeri and A. hydrophila, respectively. In conclusion, the ethyl acetate extracts of S. horrens displayed the best spectrum of bactericidal effect obtained from four bacteria strains examined.

1. Introduction
To achieve the sustainable development of rainbow trout (Oncorhynchus mykiss) culture in the aquaculture industry of Iran, maintaining its health status is of great importance (Soltani et al. 2012). Pathogenic organisms, particularly Lactococcus garvieae (Haqhighi Karsidani et al. 2010), Aeromonas hydrophila (John et al. 2011), Yersinia ruckeri (Tobback et al. 2007), and Streptococcus iniae (Akhlghi et al. 2011), have rapidly spread in rainbow trout culture farms and have suffered serious economic losses. Today, the increasing failure of chemotherapy and antibiotic resistance displayed by pathogenic bacteria infectious agents have caused the screening of new secondary metabolites with various chemical structures of marine crustaceans, molluscs and echinoderms with desirable antibacterial activity (Kijjoa and Pichan 2004).

Recent years, the role of natural compounds of marine origin has become utmost important (Mancini et al. 2007). Natural compounds are usually used to refer to natural chemicals with medicinal properties (Hassanshahian et al. 2020). This is typically used for the secondary metabolites produced by living organisms (Sipkema et al. 2005). Natural compounds found in marine animals can be accordingly used as a rich source of compounds in food, medicine, pigments, and perfumes (Farjami et al. 2013). So far, researchers have studied a variety of antibacterial compounds, including polyhydroxylated sterols (Isaac Dhinakaran and Lipton 2014), napthoquinone pigments lysozymes, peptide antibiotics (Cusimano et al. 2019), and steroidal glycosides (Claereboudt et al. 2019) from echinoderms.

Bioactive compounds can be also isolated from various animal groups such as corals, crabs, tonics, fish thorns, and sponges (Kijjoa and Pichan 2004). Studies on the biological properties of the marine invertebrates have further demonstrated that most of the chemical compounds with biological properties belong to sea cucumbers (Farjami et al. 2013). As well, the presence of a variety of bioactive compounds, such as essential fatty acids, lectins, glycosaminoglycans (GAGs), phenolics, chondroitin sulphates, sulphated polysaccharides, cerberosidespeptides, glycoproteins, glycosphinolipids, terpenoids, triterpene glycosides (saponins), and steroids (glycosides and sulphates) can be effective on pharmacological and therapeutical properties of sea cucumbers (Datta et al. 2015). Numerous research studies have been performed on the antibacterial activities of different body wall extracts of Parastichopus parvimensis (Villasin and Pomory 2000), Actinopyja echinites, Aularches miliaris, Holothuria arta, Holothuria scabra (Abraham et al. 2001), Bohadschia mammorata (Mokhlesi et al. 2012), and Holothuria leucospilota (Farjami et al. 2013) against various pathogenic bacteria.

Sea cucumbers are also a large group of aquatic animals scattered all over the oceans around the world (Barnes 1987). They usually live near coral reefs or sea-grasses in warm and...
shallow waters (Yatnita and Syamsudin 2014). In this respect, sea cucumbers have a high economic value as well as signifi-
cant economic applications in East Asia in traditional food
and pharmaceutical industries (Bordbar et al. 2011).

The aim of the present study was to evaluate the antibacte-
rial activities of the ethyl acetate, methanol, and acetone
extracts of the muscle wall of sea cucumber, *Stichopus
horrens*, from Chabahar coastal area, Iran to develop alternative
drugs for the prevention or the treatment of diseases especially
*Streptococcus iniae*, *Lactococcus garvieae*, (Haghighi Karsidani
et al. 2010), *Aeromonas hydrophila*, and *Yersinia ruckeri*
in rainbow trout culture farms using both disk-/well-diffusion
methods.

2. Materials and methods

2.1. Sample collection

A total of nine live samples of sea cucumber, *S. horrens*, with the
mean weight of 1690 ± 12.18 g and the mean length of 16 ±
1.89 cm were randomly obtained from two locations in Chaba-
har Bay, in south-eastern Iran (25° 16' N, 60° 40' E and 25° 21'
N, 60° 35' E) in November 2018, and then transferred to the
laboratory. Identification of the collected sea cucumbers was
performed by applying the Food and Agricultural Organization
(FAO) authentication keys. The visceral organs and the coelomic
fluids were subsequently discarded (Figure 1). Afterwards, the
muscle wall of the body was cut into pieces of 1 cm², packed,
and then kept at −20°C until extraction (Haug et al. 2002).

2.2. Extraction and phytochemical analysis of
*S. horrens*

The *S. horrens* extracts were prepared using the maceration
method (Ridzwan et al. 1995). Briefly, 75 g of the frozen speci-
men was dried in an oven at 45°C for two days, ground, and
extracted with 200 mL of each of the ethyl acetate, methanol,
and acetone organic solvents for 72 h. After shaking for one
week at 150 rpm, the mixtures were passed through a 45 µm
nylon membrane filter and evaporated under vacuum con-
ditions at 40°C by a rotary evaporator (Heidolph Hei VAP Core,
Germany) and then the collected supernatant of each
sample was kept at 4°C for further analysis.

The extracts obtained were quantitatively examined for their
phytochemical constituents using various analytical tests and
reagents. The phytochemicals examined were sterol (Liu et al.
2007), flavonoid (Mahmoudi et al. 2010), phenol (Ebrahimzadeh
et al. 2009), and saponin (Sun and Pan 2005). In the most com-
monly used approach for sterol analysis requires many steps
including grinding, acid hydrolysis using HCl, alkaline saponifi-
cation using KOH, solvent extraction using hexane and deriva-
tization Gas chromatography (GC) analysis. And, 50 mg of
grounded *S. horrens* was mixed with 200 µg cholestane
(Sigma Aldrich, Steinheim, Germany) and 5 mL ethanolic HCl
solution (4 mol/L). Then the sample was strongly shaken and
refluxed for 1 h at 80°C. After cooling at room temperature,
10 mL ethanolic KOH solution (4 mol/L) was added into the
mixture (50 mg sea cucumber sample containing 200 µg cho-
estane). Again, the sample was shaken and refluxed at 70°C for
1 h. The sample was cooled at room temperature before 5 mL
deionized water, 1 mL potassium chloride and 10 mL hexane
were added into the mixture for 5 min and then placed into a
100 mL separator funnel. After adding hexane into the
mixture three times, hexane phase per samples was collected.
Then hexane phase was washed with 3 mL KOH solution
(0.25 mol/L) three times and then adjusted to pH 6. The
hexane extracts were taken into a 100 mL flat-bottom flask and
anhydrous potassium sulphates were added and finally,
using vacuum rotator evaporator (Heidolph Hei VAP Core,
Germany) were evaporated to 1 mL and shifted into a
septum-capped vial. To determine the free sterols, 100 mg of
dried *S. horrens* taken into conical flask, was mixed with
200 µg cholestane and 20 mL dichloromethane. After vibrating
by a vibrator machine for one hour at 150 rpm, the mixtures
were passed through a 45 µm nylon membrane filter and evap-
orated to 1 mL using a vacuum rotator evaporator and shifted
to septum-capped vials. After drying the extract under nitrogen
steam, 50 µL of redistilled dry pyridine and 50 µL of BSTFA (N,
O-Bis(trimethylsilyl) trifluoroacetamide) containing 1% TMCS
(trimethylchlorosilane) were added to it. The mixtures were
held overnight at room temperature and 1 mL dichloro-
methane was added to them and finally analysed by gas chro-
matographic methods.

To prepare saponin, 500 g of powered sample was extracted
with 70% EtOH three times under reflux for 2 h, and then
concentrated in vacuum (40°C) to evaporate the solvent to give
a small volume. After extracting with ether (3 x 0.5 L), the water
layer portion was extracted with n-BuOH until the n-BuOH
layer became colourless. The n-BuOH solution was concen-
trated and dried in vacuum (60°C). The dried extract was sub-
jected to D101 resin column chromatography, washed with
H2O, and eluted with EtOH to give about 30.68 of *S. horrens*
solution (yield 6.14%, w/w). Quantitative determination of sterol
and saponin was also performed through a G (GC-2010, Shi-
madzu, Tokyo, Japan) equipped with an auto injector (AOC-
20i, Shimadzu, Tokyo, Japan) and the flame ionization detector were 270 and 300 °C, respect-
ively. The silica capillary column (Supelco SP-2560: 100,
0.25 mm, film thickness 0.20 l m) temperature was then
raised from 240°C to 260°C at a rate of 2°C per min. Afterwards,
20°C until extraction (Haug et al. 2002).

Figure 1. Transverse slicing of *S. horrens*.
and put aside for standing for 5 min at room temperature. 0.8 µL of 6% Na₂CO₃ (Sigma Aldrich, Steinheim, Germany) was added to the mixture and then put aside to incubate for 2 h at the room temperature in dark. The measurement of absorbing the resulting solution was done at 725 nm with a spectrophotometer (Shimadzu, uv-1800, Tokyo, Japan). Finally, the phenol content was calculated as the gallic acid equivalent in mg/g of the extract. For flavonoid assessment colorimetric aluminum chloride method was used. Resin (50 µL of 1:10 g/mL) in methanol was mixed with 150 µL of methanol, 10 µL of 10% AlCl₃, 10 µL of 1 M potassium acetate, and 20 µL of distilled water. The solution remained at room temperature for 90 min; the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer (Shimadzu, uv-1800, Tokyo, Japan). Finally, the flavonoid content was calculated as quercetin equivalent in mg/g of the extract (Table 1).

### 2.3. Bacterial strains

The bacterial strains used in the present study were the gram-positive bacteria, namely, *Lactococcus garvieae* PTCC1884 and *Streptococcus iniae* PTCC188 and the gram-negative ones, i.e. *Aeromonas hydrophila* ATCC7966 and *Yersinia ruckeri* PTCC1887. All culture stocks were also grown in the Mueller-Hinton broth (MHB) (Merck KGaA, Darmstadt, Germany) at room temperature.

### 2.4. Determination of antibacterial activity of *S. horrens* extracts by the disk-diffusion method

The antibacterial activities of *S. horrens* extracts on the tested bacteria were determined using the disk-diffusion method (Vilasín and Pomory 2000). For each extract, 4, 8, and 12 mg/mL concentrations were also used to provide three disks. Upon the preparation of the suspensions of the tested bacteria through the 0.5 McFarland standard under a hood, the bacterial strains were inoculated with a swab on the Mueller-Hinton broth (MHB). Then, the discs containing different concentrations were placed on bacterial culture media and kept in a 25°C incubator for two days. Antibiotics, namely, tetracycline and enrofloxacin (30 µg/mL) were further employed as a positive control. Also, dimethyl sulfoxide-impregnated discs (DMSO) were used as a negative control. The bacterial growth inhibition zone was finally measured with a calliper.

### 2.5. Determination of antibacterial activity of *S. horrens* extracts by the well-diffusion method

The minimum inhibition concentration (MIC) values of the *S. horrens* extracts on the above-mentioned bacteria were determined by the well-diffusion method with some modification (Thornsberry and McDougal 1983). After adjusting the overnight cultures of the tested bacteria to 1 × 10⁶ colony-forming units (CFU)/mL, 100 µL of two-fold serial dilutions of each *S. horrens* extract was added to the well of the sterile 96-well microtiter plates, containing 100 µL of each of the bacterial suspensions in the MHB, then incubated. In this study, the serial dilutions of each *S. horrens* extract ranged from 0.019 to 10 mg/mL. After incubation for two days at 25°C, the MIC values were evaluated by a microtitre plate reader (MRP4 plus, Hiperon Co. UK). The MIC value was further distinguished as the lowest concentration of the *S. horrens* extract inhibiting bacterial growth. To measure the minimum bactericidal concentration (MBC), 100 µL of the wells showing no bacterial growth was cultured on the MHB and incubated at 25°C for 24 h. The MBC value of *S. horrens* was then demonstrated as the lowest concentration that decreased the viability of the bacterium to ≥99.9% (Kang et al. 2011). The tests were performed in triplicate.

### 2.6. Statistical analysis

The data were analysed using the IBM SPSS Statistics (version 16.0) software (Armonk, NY, USA) by parametric tests. Significant differences in growth inhibition zones, MIC, and MBC values of bacterial strains, extract types, and different extract concentrations were determined using one-way analysis of variance (ANOVA) at the 5% confidence interval using the Duncan’s Multiple Range Test.

## 3. Results

### 3.1. The phytochemical constitutes of *S. horrens* extract

The phytochemical components of *S. horrens* crude extract *S. horrens* are presented in Table 1. The results confirmed that the predominant compounds of *S. horrens* extract were phenol compounds.

### 3.2. Designation of antibacterial activity of *S. horrens* extracts by the paper disc-diffusion method

The results given in Table 2 proved that only the ethyl acetate extracts of *S. horrens* in the concentrations at 8 and 12 mg/mL had an inhibitory effect on all the examined bacteria. The ethyl acetate extract displayed the most inhibition zones on *Y. ruckeri* with 12.03 and 12 mm, respectively. However, the methanol extracts did not show inhibitory effects on the tested bacteria except for *Y. ruckeri* and *A. hydrophila*. The methanol extracts were the most sensitive ones against tetracycline and enrofloxacin, summarised in Table 2, revealed that *A. hydrophila* and *S. iniae* were the most sensitive ones against tetracycline and enrofloxacin, respectively.
3.3. Designation of antibacterial activity of *S. horrens* extracts by the well-diffusion method

According to Figure 3, the optical density (OD) absorption of the tested bacterial strains decreased as the concentration of *S. horrens* extracts augmented. The ethyl acetate extract of *S. horrens* in different concentrations had the best OD absorption of the tested bacterial strains, while only OD absorption of *Y. ruckeri* and *A. hydrophila* bacteria decreased as the concentration of acetone extract of *S. horrens* augmented. The results presented in Table 3 demonstrated that *S. horrens* extracts exhibited selective antibacterial properties. The best property was recorded with the *S. horrens* ethyl acetate extracts, with the MIC value of 0.625 mg/mL against *S. iniae* and *L. garvieae*. The MIC values ranging from 0.626 to 1.25 mg/mL were also displayed with the acetone extracts against *Y. ruckeri* and *A. hydrophila*, respectively. Only the methanol extracts of *S. horrens* were effective in *Y. ruckeri* with the MIC value of 0.625 mg/mL. The ethyl acetate extracts of *S. horrens* also illustrated the best range of bactericidal effect with a ratio of MBC/MIC ≤ 4 gained on four bacterial strains examined in this study.

4. Discussions

The multi-resistant nature of pathogens to antibiotic is a significant challenge for pathogenic bacterial infections. Therefore, the search for novel antimicrobial agents from various natural sources has become an essential and urgent need (Hassanshahian et al. 2020). On the other hand, there is an essential and continuous demand to extract new secondary metabolites with a variety of chemical structures of sea cucumbers with potential antibacterial activities (Hamayeli et al. 2019). Also, it seems that sea cucumber species, extract types, as well as bacterial strains are effective in forming the growth inhibition zone (Shakouri et al. 2017).

In the present study, some of the antibacterial compounds of *S. horrens* were determined. The results confirmed that the dominant compounds of *S. horrens* extracts were phenol compounds. Similar to our results, Suleria et al. (2015) concluded that brown phenolic compounds, including eckol, dieckol and phloroglucinol contributed to the antibacterial activities. Also, Manal et al. (2015) explained that the phenolic contents of the *Nitraria retusam* extract had a positive relationship with the potential antimicrobial activity. Tamokou et al. (2011) and Hamayeli et al. (2019) evaluated the chemical components of *Brillantaisia lamium* and *Stichodactyla haddoni*, respectively. Their results detected that the major antibacterial agents in this plant and sea anemone were a mixture of sterols (Tamokou et al. 2011; Hamayeli et al. 2019). In our study, we recognised these components as antibacterial agents in sea cucumber. Likewise, the antimicrobial potential of sea cucumber extract can be attributed to the presence of antimicrobial agents such as steroidal saponins (Bordbar et al. 2011). Other metabolites, such as polyunsaturated fatty acids (PUFAs) (Svetashev et al. 1991), glycolipids (Vaskovsky et al. 1970), polyamines (Hamana et al. 1990), carotenoids (Bullock and Dawson 1970), and sterols (Makarieva et al. 1993), may additionally act as bioactive compounds. So, the antibacterial activities of sea cucumber extracts may be due to the accumulation of several bioactive compounds.

Numerous pharmacological and chemical studies on various species of sea cucumber have demonstrated that such invertebrates contain triterpene tetraglycosides with antibacterial,
antifungal, and cytotoxic properties (Kalinin et al. 2015; Cuong et al. 2017). The results obtained in our study indicated that ethyl acetate extracts were able to isolate the bioactive compounds present in S. horrens and had the most significant antibacterial effect on gram-positive bacteria such as L. garvieae and S. iniae. Moreover, the methanol extracts did not show inhibitory impacts on the tested bacteria except for Y. ruckeri. Our results are in agreement with those obtained by Hirimuthugoda et al. (2006) reported that the methanol–acetone extracts obtained from the body wall of sea cucumber, Parastichopus parvimensis, from Santa Catalina Island, in southern California, the United States, was found effective in gram-negative bacteria, i.e. Escherichia coli and Bacillus subtilis using the disk-diffusion method. Sedov et al. (1990) and Mulyndin and Kovalev (2001) showed that the presence of secondary metabolites such as triterpene-glycosides can also boost the production of antibodies, protective effect of vaccines, and stimulated antibacterial resistance in mice against conditional pathogenic gram-negative microorganisms. Likewise, the antimicrobial potential of these extracts can be attributed to the presence of antimicrobial agents such as steroidal saponins (Bordbar et al. 2011) and sterols (Makarieva et al. 1993) which may additionally act as bioactive compounds. The present study explained that only the ethyl acetate extracts of S. horrens in the concentrations at 8 and 12 mg/mL had an inhibitory effect on all the examined bacteria and displayed the most inhibition zones on Y. ruckeri and S. iniae, respectively. Contrary to our results, Mokhlesi et al. (2012) reported that aqueous ethyl acetate extracts had failed to form growth inhibition zones on the tested bacteria (e.g. E. coli, Salmonella aureus, and Pseudomonas aeruginosa). Accordingly, it seems that species of sea cucumbers, extract types, and different extract concentrations can be effective in antibacterial properties. The discrepancy in the effect of sea cucumbers on different bacterial strains is associated with different amino acid sequences extracted from them (Shakouri et al. 2017). These results are in agreement with earlier reports proved that different concentrations of extracts from sea cucumber species have been effective in antimicrobial properties. Furthermore, the diameter of the bacterial growth inhibition zone has often reduced as the extract concentrations have dropped (Mokhlesi et al. 2012; Omran and Allam 2013; Shakouri et al. 2017).

Based on the study results, the best property was recorded with the S. horrens ethyl acetate extracts, with the MIC value of 0.625 mg/ mL against S. iniae and L. garvieae. In addition, the

Table 3. MIC, MBC values (mg/mL), and MBC/MIC ratio of three S. horrens extracts on all bacteria.

| Bacterial strains | Methanol | Acetone | Ethyl acetate |
|------------------|----------|---------|--------------|
|                  | MIC      | MBC     | MBC/MIC      | MIC | MBC     | MBC/MIC | MIC     | MBC     | MBC/MIC |
| Y. ruckeri       | 0.625    | 5       | 8            |     |         |         | 1.25    | 1.25    | 1       |
| S. iniae         | –        | –       | –            |     |         |         | 0.625   | 1.25    | 2       |
| A. hydrophila    | –        | –       | –            |     |         |         | 2.5     | 2.5     | 1       |
| L. garvieae      | –        | –       | –            |     |         |         | 0.625   | 2.5     | 4       |

+: not determined.
ethyl acetate extracts established a bactericidal effect against *S. iniae* and *L. garvieae* at concentrations of 1.25 and 2.5 mg/mL, respectively. This indicated that ethyl acetate extract was able to isolate the bioactive compounds presented in *S. horrens* and had the most significant antibacterial effect on gram-positive bacteria such as *L. garvieae* and *S. iniae*, and they could lead to the death of *S. iniae* at the lowest concentration. Our results are in agreement with earlier studies (Villasin and Pomory 2000; Nazemi et al. 2014).

### 5. Conclusions

It seems that different concentrations of the extract, extract types, as well as bacterial strains are effective in forming the growth inhibition zone. This study revealed that the ethyl acetate extracts of *S. horrens* displayed the best spectrum of bactericidal effect with a ratio of MBC/MIC ≤ 4 obtained from four bacteria strains examined by both disk-diffusion and well-diffusion methods. Thus, the ethyl acetate extracts of *S. horrens* with a concentration of 12 mg/g can be used as bioactive compounds for growth inhibition and finally lead to the death of bacteria in rainbow trout breeding farms. Ultimately, the results of this study can provide the basis for further research in the future to isolate the effective ingredients of sea cucumber, *S. horrens*, and prepare appropriate drug formulations of the best active ingredients.

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### Disclosure statement

No potential conflict of interest was reported by the author(s).

### Ethical statement

This article does not contain any studies on animals performed by any of the authors.

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