Isolation and identification of antibacterial steroid compounds from *Ulva fasciata* in the Persian Gulf

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Abstract

Due to numerous reasons, marine-obtained resources have attracted the interest of researchers. One of the subjects attracted the interest of researchers in recent years in the field of biologist marine algae due to their nutritional value, their benefits for health and their biological activities. In this study, the powder was extracted using acetone, then the extract purified by silica gel column chromatography with n-hexane and ethyl acetate combination to isolate the steroid compounds. Isolated compound was run through TLC and sprayed with vanillin-sulphuric acid reagent to detect the steroid compounds and profile of isolated compounds obtained by gas chromatography (GC). Antibacterial activity of steroids extracted of green algae *Ulva fasciata* from Qeshm Island in the Persian Gulf were assessed (in vitro). The antibacterial activity of the fractions was determined by Broth Dilution Methods against clinical Gram-negative bacteria: Escherichia coli, *Pseudomonas aeruginosa*, *Salmonella* typhi and Gram-positive bacteria: *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*. The steroids; cholest-7-en-3ol and cholestan-3-one, cyclic 1, 2-ethanediyl acetal compounds in column fraction were identified by GC. The results showed the minimum bactericidal concentration (MBC) of the cholest-7-en-3ol and cholestan-3-one, cyclic 1,2-ethanediyl acetal for S. aureus and *Bacillus cereus* were 500 μg mL⁻¹, the MBC of the cholest-7-en-3ol for B. subtilis was 500 μg mL⁻¹ and 1000 μg mL⁻¹ detected for cholestan-3-one, cyclic 1,2-ethanediyl acetal. The compounds had neither antibacterial activity on gram negative bacteria. Based on our results, extracted steroids from *U. fasciata* can be considered as a source of novel antibiotics.

Keywords: Green algae, Antibacterial activities, Natural products, Persian Gulf

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Introduction
Marine organisms are potential sources of bioactive secondary metabolites for use in development of new pharmaceutical agents (Abedin and Taha, 2008; EL-Gamal, 2010) and many of these substances have been demonstrated to possess interesting biological activities (Faulkner, 2002; Abdel-Raouf et al., 2008). Marine algae have been reported to produce a wide variety of bioactive secondary metabolites as antibacterial, and cytotoxic agents and the bioactive substances included alkaloids, polyketides, polysaccharide, phlorotannins, triterpenoids, sterols, Quinone’s, lipids and glycerol (Cabrita et al., 2010).

Secondary metabolites derivatives of algae offer as a potentially useful source of natural antibacterial agents for food and medical applications. Algae contain various inorganic and organic substances which have benefit for human. Antibacterial activity of algae extracts is generally assayed using various organic solvents which always provide a higher efficacy in extracting compounds for antimicrobial activity (Cordeiro et al., 2006; Tüney et al., 2006). Isolation of the natural compounds from algal could produce useful therapeutics, the harnessing and bioengineering of recently characterized allelochemicals represents a potential area of new marine antibacterial (Saritha et al., 2013).

Ulva species are the most abundant algae in coastal benthic communities around the world, Ulvacean are considered bio indicators species with increased importance in coastal ecosystem management, mainly related to green tides associated with eutrophication processes in shallow environments (Wichard, 2015). Exploring bacteria induced growth and morphogenesis in the green macro algae in order Ulvaes (Chlorophyta) (Basham et al., 2014). Ulva fasciata is one of the most common green algae and accrues almost every season (Makaremi et al., 2011) also known as sea lettuce and is a common algae used in parts of the world for consumption. Due to the increased resistance to antibiotics, the study of new antibiotic is very important from the perspective of medical treatment (Daneshvar et al., 2017). Many authors had found antimicrobial activities of macroalgae due to fatty acids contents (Daneshvar et al., 2017). Antimicrobial activity depends on both algal species and solvents used for their extraction (Prakash et al., 2011; Radika et al., 2012). Various algae have shown to have antibacterial activity in vitro against gram positive and gram negative bacteria (Ostensvik et al., 1998).

Due to the antimicrobial activity of steroids, this study investigates the isolation and identification of bioactive steroids and antibacterial activities of isolated compounds of U. fasciata from the Qeshm Island in the Persian Gulf.
Materials and methods

Sampling and identification

*Ulva fasciata* has been collected in February 2018 from the areas of Shibderaz in Qeshm Island in the Persian Gulf (Fig. 1). After identification of species, the samples were dried in the shadow.

![Geographical situation of sampling location in Qeshm Island.](image_url)

**Figure 1:** Geographical situation of sampling location in Qeshm Island.

Extraction

The powder of dried *Ulva fasciata* (100 g dry weight) was extracted with acetone solvent. After 72 hours of soaking in acetone, the solvent filtered and acetone evaporated to dryness, at low pressure at 30-35°C by using Rota vapor (Çitoğlu and Acıkara, 2012).

Isolation and identification of steroid compounds

Silica gel column chromatography with 70 cm height and 2 cm diameter used for purification of *Ulva fasciata* acetone extract (3/2 g). The packed column washed up by different combination (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100) of hexane: ethyl acetate solvent and all fractions were collected in 10 ml tube (108 fractions) (Çitoğlu and Acıkara, 2012).

Obtained fractions from the chromatography columns were performed by thin layer chromatography with a mobile phase including methanol-chloroform- n-butanol solvents with a ratio of 70:20:10. To identify the steroid compounds, vanillin-sulphuric acid reagent was used as a 1% solution of vanillin in ethanol and 5% sulphuric acid solution in ethanol by spraying on a thin-layer chromatography plate. After spraying, thin-layer chromatography plates were placed in the oven at 110°C for 10 minutes; changes in visible light were observed; the portions of the steroids were changed to blue color (Attaway et al., 1965). The gas chromatography-mass
spectrometry (GC-MS) spectrum was taken on GC-MS (Agilent7000 Series Triple Quad GC/MS MainFrame). The column part number 19091s-436 (60 m x 0.25 mm, film thickness 0.25 μm) was used for GC-MS. Helium was used as a carrier gas at a flow rate of 1.0 ml min⁻¹; the electron ionization mode with ionization energy of 70 eV was used for MS detection, with a mass range of 50–650 m/z. The compound was recognized using standard information in the Wiley 7.0 library using its retention time and mass fragmentation pattern.

**Antibacterial assay**

Antibacterial activity was determined against *Escherichia coli* ATCC 15224, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 1609, *Staphylococcus aureus* ATCC 1764, *Bacillus cereus* ATCC 11778 and *Bacillus subtilis* PTCC 1715 by performing the classic broth dilution susceptibility test. The amount of 1.5×10⁵ microorganisms in colony forming units [CFU] ml⁻¹, a 1:100 dilution of a suspension of turbidity equal to a McFarland standard 0.5, was added to an equal volume (1 ml) of each concentration (5, 10, 20, 30, 50, 100, 200, 300, 400, 500, 1000, 2000 µg ml⁻¹) of steroid components agent and to a tube of the growth control. After overnight, the tubes were examined for turbidity, indicating growth control of the microorganism. The lowest concentration of the agent that inhibits growth of the organism, as detected by lack of visual turbidity, was designated as the minimum inhibitory concentration (MIC). Later, the MIC has been determined; a known quantity (0.1 ml) of inoculums from each tube of broth that showed on visible turbidity after 22 to 24 hours’ incubation is subculture to solid agar plates. The number of colonies which grow up on the subculture after overnight incubation is then counted and compared to the number of CFU ml⁻¹ in the original inoculum. Since even bacterial extracts do not always totally sterilize a bacterial population, the lowest concentration of antimicrobial agent that allowed less than 0.1% of the original inoculums to survive is considered as the minimum bacterial concentrate (MBC) (Rosenblatt, 1991).

Kruskal-Wallis test was used to compare the mean concentration of each extracted material as well as tetracycline.

**Results**

*Isolation of fractions containing steroids*

In column chromatography, the acetone extract was packed and eluted with the distinct solvent system. Based on the TLC profile, 108 fractions have been gathered from column chromatography. The fractions C44 (Yield 121 mg) and B33 (Yield 92 mg) that gave a positive response for steroids by Vanillin-sulphuric acid reagent were selected and separated into eleven and ten sub fractions, by silica gel. The fraction of C44 E and C33F was subjected to TLC for isolation of active principle. After TLC run (Fig. 2), the band that gave a
positive response for steroids producing blue color with Aniline test, punched and dissolved in acetone, filtered and brown crystals of compound were obtained (2.3 mg) and then comparing GC-MS with Wiley 7.0 library verified the compound’s identity.

Figure 2: Thin layer chromatography of acetone extracts of Ulva fasciata.

Identification of steroids compounds
The fractions C44E and C33 F that gave a positive response for steroids by Vanillin-sulphuric acid reagent selected for GC–MS detection use. The cholest-7-en-3ol with the chemical formula of C_{27}H_{46}O (Fig. 3), molecular weight of 386.6535 g mol^{-1} belonging to the steroids group identified with 92% purity in fraction C44 E (ethyl acetate-hexane 40: 60) at 32-34 min retention time of gas chromatography.

Figure 3: GCMS analysis of cholest-7-en-3ol.
The cholestan-3-one, cyclic 1,2-ethanediyl acetal with the chemical formula of C_{29}H_{50}O_{2} (Fig. 4), molecular weight of 430.7 g mol\(^{-1}\) belonging to the steroids group identified with 94% purity in fraction C33 F (ethyl acetate-hexane 30: 70) at 34-38 min retention time of gas chromatography.

Figure 4: GCMS analysis of cholestan-3-one, cyclic 1, 2-ethanediyl acetal.

Antibacterial activities of fraction containing steroid compounds

As indicated in Table 1, the minimum inhibitory concentration of bacterial growth (MIC) of cholest-7-en-3ol and cholestan-3-one, cyclic 1, 2-ethanediyl acetal for B. subtilis was 500 \(\mu\)g ml\(^{-1}\) and for E. coli equal to 1000 \(\mu\)g ml\(^{-1}\). The MIC of cholest-7-en-3ol for S. aureus and B. cereus were 300 and 500 \(\mu\)g ml\(^{-1}\), the MIC of cholestan-3-one, cyclic 1, 2-ethanediyl acetal for S. aureus and B. cereus were 200 and 300 \(\mu\)g ml\(^{-1}\). The cholest-7-en-3ol and cholestan-3-one, cyclic 1, 2-ethanediyl acetal compounds have not shown any inhibitory effects on growth of S. typhi and P. aeruginosa.

Table 1: Minimum inhibitory concentration of fraction containing steroid compounds from U. fasciata.

<table>
<thead>
<tr>
<th>Tetracycline ((\mu)g ml(^{-1}))</th>
<th>cholest-7-en-3ol concentration ((\mu)g ml(^{-1}))</th>
<th>cholestan-3-one, cyclic 1,2-ethanediyl acetal concentration ((\mu)g ml(^{-1}))</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>500</td>
<td>500</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>100</td>
<td>300</td>
<td>200</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>100</td>
<td>500</td>
<td>300</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>200</td>
<td>1000</td>
<td>1000</td>
<td>Escherichia coli</td>
</tr>
</tbody>
</table>

As shown in Table 2, the minimum bactericidal concentration (MBC) of the cholest-7-en-3ol and cholestan-3-one, cyclic 1,2-ethanediyl acetal for S. aureus and Bacillus cereus was 500 \(\mu\)g ml\(^{-1}\), the MBC of the cholest-7-en-3ol for B. subtilis was 500 \(\mu\)g ml\(^{-1}\) and 1000 \(\mu\)g ml\(^{-1}\) detected for cholestan-3-one, cyclic 1,2-ethanediyl acetal.
Table 2: Minimum bactericidal concentration (MBC) of fraction containing steroid compounds extracted from U. fasciata.

<table>
<thead>
<tr>
<th>Tetracycline concentration (µg ml(^{-1}))</th>
<th>cholestan-3-one, cyclic 1,2-ethanediyl acetal concentration (µg ml(^{-1}))</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>500</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>200</td>
<td>500</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>200</td>
<td>500</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>200</td>
<td>-</td>
<td>Escherichia coli</td>
</tr>
</tbody>
</table>

The results of Kruskal-Wallis test showed that there was no significant difference between the concentration of extracts and tetracycline in inhibiting the growth of each four strains \((p>0.05)\). Similar results were observed \((p>0.05)\) among these compounds in their lethality, indicating that the extracted compounds had a similar effect to tetracycline on bacterial growth and lethality.

**Discussion**

The marine secondary metabolites have received considerable attention over the last decades. There are large numbers of marine natural products which have been targeted by pharmaceutical industry (Saberi et al., 2016). Due to the increase of resistance to antibiotics, the study on new antibacterial activities of steroid derivatives is very important for human health (Doğan et al., 2017; Cheng et al., 2016). The aim of this study was to investigate the antibacterial activities of the green algae (U. fasciata) steroids; cholestan-7-en-3-ol and cholestan-3-one, cyclic 1,2-ethanediyl acetal.

In the present study, the cholestan-7-en-3-ol and cholestan-3-one, cyclic 1,2-ethanediyl acetal, which are the steroid compounds, extracted from U. fasciata using acetone solvent (Veeranan et al., 2018; Fasya et al., 2019). The sterol as same as steroid compounds which is found in our study has been identified from green algae Ulva prolifera named as cholestan-5-en-3-ol (Wang et al., 2016), 7-oxo-cholestan-5(6)-en-3-ol from red algae Jania rubens (Ahmed et al., 2011), 3alpha, 6alpha-dihydroxy-5beta-cholestan-12-one and 6β-hydroxycholestan-4-en-3-one from red alga Laurencia papillosa (Alarif et al., 2011).

In this study, the antibacterial activities of cholestan-7-en-3-ol and cholestan-3-one, cyclic 1, 2-ethanediyl acetal has been examined. This research determined that fractions containing cholestan-7-en-3-ol and cholestan-3-one, cyclic 1, 2-ethanediyl acetal compounds extracted from U. Fasciata in a concentration of 500-1000 µg ml\(^{-1}\) has antimicrobial activity on gram-positive bacteria of B. subtilis, S. aureus and B. cereus and they have not shown any inhibitory effects on growth of antimicrobial activity on gram-negative bacteria of S. typhi and P. aeruginosa. In this case, Chandrasekaran et al. (2014) reported that ethyl acetate extract of U. fasciata shows strong antibacterial activates; MIC (125 µg ml\(^{-1}\)) and MBC (250 µg ml\(^{-1}\)) against B.
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subtilis. Chloroform and ethyl acetate fractions of U. lactuca have shown antibacterial activates against Staphylococcus aureus (1.6 µg ml^{-1}) and Enterococcus faecalis (0.2 µg ml^{-1}) (Habbu et al., 2016). In another study, the fractions containing cholesterol derivative, 24-propylidene cholest-5-en-3β-ol extracted from red seaweed Laurencia papillosa, showed the minimum inhibitory concentration ranged from 1.2 to 1.7 µg against Staphylococcus aureus, Bacillus subtilis and Escherichia coli (Kavita et al., 2014).

The results of these studies are in accordance with the studies on the steroid compounds extracted from U. fasciata of Qeshm Island which has antimicrobial activities against gram-positive bacteria. The study of the new antibacterial of steroids; cholesterol derivatives have the potential of being used as antibacterial drugs. A steroid is a biologically active organic compound and they have two principal biological functions: cell membranes and signaling molecules. Since steroids resemble cholesterol, it is thought that they compete with this for the receptors and it can also disrupt cell integrity and permeability. It is thought that this is the reason why steroids and cholesterol derivatives can have antibacterial activities (Doğan et al., 2017). Based on our results, the steroids extracted from green algae U. fasciata have shown a relatively strong antibacterial activity on gram positive bacteria and it could be considered as a source of novel antibiotic.

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