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Research Article

Isolation and semi purification of steroid compounds from *Colpomenia sinuosa* (Derbès & Solier, 1851) algae of the Persian Gulf and *in vitro* screening of antimicrobial effects

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Abstract

One of the subjects attracted the interest of researchers in recent years in the field of biology is marine algae, due to their nutritional value, their benefits for health and their biological activities. In this study, steroid fractions of acetone extract of *Colpomenia sinuosa* (Phaeophyceae alga) from the Persian Gulf and Oman sea (Iran) have studied and evaluated for their antibacterial activity against pathogenic bacteria: one gram-positive bacterium (*Staphylococcus aureus*) and two gram-negative bacteria (*Escherichia coli, Pseudomonas aeroginosa*). Antimicrobial susceptibility tests have expressed in terms of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) of the test organisms with respect to the acetone extracts of *Colpomenia sinuosa.* Extracts of all tested marine algae have showed inhibition against all of the pathogenic microbes. In addition, the highest inhibition activity among all the extracts was shown from Cholestane fraction to *S.aoreus* and lower inhibition activity was shown from Oleic acid fraction to *P. aeruginosa.*

Keywords: Brown algae, *Colpomenia sinousa*, Persian Gulf, Biological activity, Steroids

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Introduction

For numerous reasons, marine-obtained resources have attracted the interest of researchers. One of the reasons might be the fact that more than 70% of the Earth's surface is cover by oceans and 36 classes known living fauna and flora live in there. Among these 36 classes of creatures, 34 of them live in the oceans and they are host for more than 300,000 known species. Marine algae, popularly known as seaweeds, are considered very important, because they are excellent sources of single cell protein, hydrocarbons, biogas, polysaccharides such as agar-agar, alginic acid, carrageenan, antibiotics, color pigments, important medicines (Vishnu and Murugesan, 2014). Seaweeds are one of the most promising and the richest sources of primary and secondary metabolites. These compounds play different simultaneous roles for the seaweeds and they can act as; herbivore impediments, antifouling, antimicrobial and allelopathic, or as agents screening ultraviolet rays (Taheri, 2020). Based on recent research, these compounds could be used by the pharmaceutical industry to come up with drugs to cure diseases such as cancer, Acquired Immune-Deficiency Syndrome (AIDS), viral, bacterial and fungal infections, inflammation, pain, arthritis, and etc. (Al-Haj *et al*., 2009). Nowadays, algae comprise approximately 9% of biomedical compounds obtained from the oceans. (Jirge and Chaudhari, 2010). Marine algae affected by seasonal changes that would have different compounds in metabolic responses (photosynthesis and growth) and different levels of compounds (Orduña-Rojas *et al*., 2002). The previous studies have been revealed the effects of seasonal changes in the chemical combination and nutritional value of marine algae (Kumar, 1993; Mercer *et al*., 1993; Kaehler and Kennish, 1996). *C. sinuosa* is a brown algae that belongs to the class of Pheophyceae, the order of Scytosiphonales, and the family of Scytosiphonaceae (El Asri *et al*., 2017). The present study was performed to evaluate the isolation, purification and characterization of steroid compounds from marine brown algae *C. sinuosa* and further investigation for their antimicrobial activity.

Materials and methods

Sample collection and preparation of seaweed extracts

The brown algae *C. sinuosa* was collected from various places of the Persian Gulf and Oman Sea in February and March 2014 and have transferred to biotechnology laboratory of the Persian Gulf and Oman Sea ecology institute. The algae were thoroughly washed up with tap water to remove sand, debris, epiphytes, and animal waste. Each of algae thalluses which were morphologically distinct, were placed in new polyethylene bags and kept in the ice box. The samples were shade dried or placing in the oven at 45°^{C}. The dried leaves have pulverized in a mechanical grinder and the coarse powder has been used for further studies (Nazemi et al., 2014 a). 100 g of powdered samples have been extracted

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and pooled using solvent such as acetone for 72 h (Çitoğlu and Acıkara, 2012). The extracts have evaporated to dryness under vacuum on a rotary evaporator (Heidolph, Laborata 400) at 40°C. The dried extracts have collected and stored at 4°C for further studies.

Phytochemical screening

The acetone extract of *C. sinousa* has been used for qualitative phytochemical studies. Screening of steroids was carried out according to the standard methods (Harborne, 1998; Trease, 1983).

Bacterial strains

The bacterial strains of *Escherichia coli* ATCC15244, *Pseudomonas aeruginosa* ATCC1053, and *Staphylococcus aureus* ATCC1764 have procured from Center of Iranian Collection Fungi and Bacteria.

Antibiotic susceptibility test

Antibiotic sensitivity tests of the bacterial strains have determined by standard Clinical and Laboratory Standards Institute disc diffusion method (Clinical and Laboratory Standards Institute, 2012). Antibacterial agents from different classes of antibiotics ampicillin and tetracycline (5 µg $disc^{-1}$).

Extract preparation and antimicrobial assay

Isolation of steroid compounds from seaweed

One hundred grams of dried acetonic extract was subjected by silica gel column chromatography for fractionation. A glass column (70 cm×2cm dia.) has been filled by chromatography silica gel with the size of 0.2-0.6 mm. The columns were equilibrated thoroughly by passing selected mobile phase repeatedly. The acetonic extract has loaded on to the packed glass column. Flow rate has been set to 10 ml min^{-1}. Washing acetonic extract carried out using n-hexane-ethyl acetate solvents as a mobile phase with following ratios: 100: 0-90: 10-80: 20- 70: 30-60: 40-50: 50-40: 60-30: 70-20: 80-10: 90-0: 100. The fractions have been separated every 10 cc (112 fractions). Ethyl acetate-methanol also used as a mobile phase with these ratios: 100: 0-90: 10-80: 20-70: 30-60: 40-50: 50-40: 60-30: 70-20: 80-10: 90- 0: 100. The fractions were separated every 10 cc. The fractions were analyzed for presence of steroids. (Çitoğlu and Acıkara, 2012).

Identification of steroid compounds

Fractions obtained from the glass chromatography column have stained by the hairpin tubes on the plates of Thin-layer chromatography (TLC). They were exposed to the air for 20 minutes to be dried and placed into TLC tank, containing methanol-chloroform-butanol solvents with ratios 70:20:10. To prove the presence of the steroid compound, Vanillin-Sulfuric Acid reagent was used as 1% solution of vanillin in ethanol and 5% solution of sulfuric acid in ethanol, in form of spray on TLC. Samples were placed in the oven at 110 °C for 10 minutes. The steroid samples changed to blue color

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which was investigated in visible light (Attaway *et al*., 1965).

The area of blue spot in TLC plate without spray of identifier should use for injection to the GC MASS machine (Agilent 7000 Series Triple Quad GC/ MS MainFrame; Carrier helium gas 99/99 percent, Dr C5975, Column: Part number 19091s-436, Length of 60 m, the internal diameter of 0.25 mm, internal layer of 0.25 micrometers) at the research laboratory of Shahid Beheshti University for identification of steroid type.

Testing antibacterial properties

A modified resazurin microliter plate assay has been used in order to determine the MIC of the crude algal extracts, according to methods suggested by Sarker *et al*. (2007). Antibacterial properties were carried out for strains of *E. coli*, *P. aeruginosa*, and *S. aureusa* by using the Bacterial Broth Dilution Methods. Each strain was cultured and placed in the incubator for 24-72 h at 37 °C in order to use single colonies to carry out the test.

The obtained single colonies were entered into the test tubes containing broth media in 1 cc volume; it is worthwhile pointing out that the number of tested bacteria is 1.5×10^6. Among the fractions containing icosapent/oleic acid, eicosyl ester/ Eicosatetraenoic acid, methyl ester/ cholestane-3-one-cyclic-1, 2-ethanediyl acetal/1, 2-benzenedicarboxylic acid, diisooctyl ester, only oleic acid and cholestane fraction have been investigated for antimicrobial effects. These two substances dissolved in broth environment with following concentrations: 1, 2, 4, 10, 20, 30, 50, 100, 200, 300, 500, 1000, 2000 micrograms per ml. then 1 cc of them has been added to the above test tubes. For considering a positive control, tetracycline and ampicillin antibiotics were used with above concentrations, and for a negative control, the bioactive substance has not added to one of the tubes. A medium without effective ingredient and the bacteria has placed inside a tube to determine the test error in case of environmental pollution. Next, all the tubes have been sealed with cotton wool and placed in an incubator at 37 °C for 24 hours.

After 24 hours, test tubes were taken out of the incubator and their opacity has examined. A control tube with no bioactive compounds was very opaque because the bacteria had the opportunity to grow at that time. Other test tubes were compared with the above test tube visually, which was an average of 3 times test for any bacteria. Any tube that was opaque excluded from the experiment and those tubes which no opacity separated for further study. The Minimum Inhibitory Concentration (MIC) has calculated based on the lowest concentration of desired steroids mixture that inhibit the bacterial growth. The minimum bactericidal concentration (MBC) was determined by the method of Rosenblatt (1991). All the tubes that observed no microbial growth (no turbidity) after 24 h of incubation have been sub-cultured onto the surfaces of freshly prepared Mueller-Hinton agar medium and

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incubated at 37 °C for another 24 h. After 24 hours, the number of colony-forming units (CFU) has studied. The MBC has indicated as the lowest concentration of the extract that did not allow a visible bacterial growth on agar medium after 24 h incubation.

Results

The aim of this study was to evaluate different oxygenated steroids of *C. sinuosa* extract for *in vitro* antimicrobial activity against all the steroids, which were characterized by Gas Chromatography Mass-Spectrometry (GC-MS). GC-MS analysis of steroids compounds clearly showed the presence of five compounds. Chromatogram GC-MS analysis of *C. sinuosa* extract showed the presence of five major peaks and the components corresponding to the peaks determined as followed respectively: 1,2-Benzenedicarboxylic acid, diisooctyle ester ($C_{24}H_{38}O_4$) (Fig. 1), icosapent ($C_{20}H_{30}O_2$) (Fig. 2) , Oleic acid,eicosyl ester ($C_{38}H_{74}O_2$) (Fig. 3), Eicosatetraenoic acid, methyl ester ($C_{21}H_{34}O_2$) (Fig. 4), cholestane-3-one-cyclic-1,2 ethanediyl acetal ($C_{23}H_{50}O_2$) (Fig. 5). The obtained peaks from GC-MS analysis represent approximate molecule structure. Our further investigations have been organized based on these semi-purified steroids compounds.

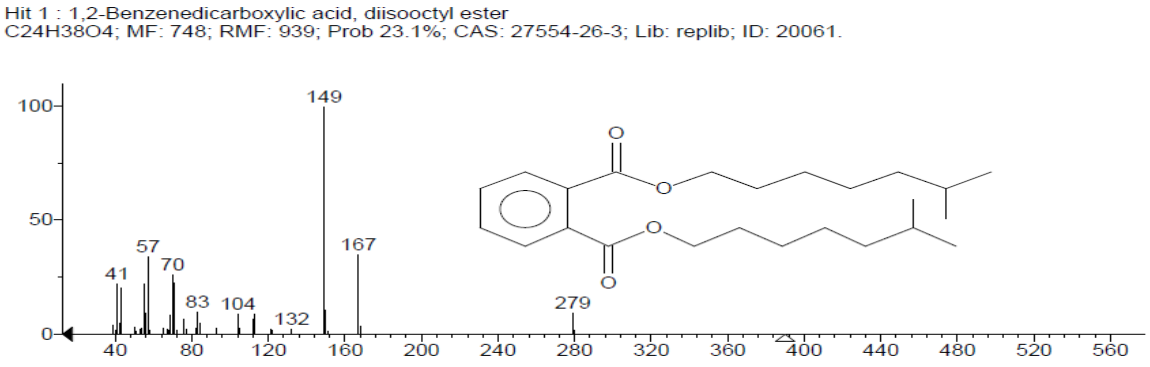


Figure 1: Structure of 1,2-Benzenedicarboxylic acid, diisooctyle ester ($C_{24}H_{38}O_4$) present in *Colpomenia sinuosa* extract using GC-MS analysis. This peak represents approximate

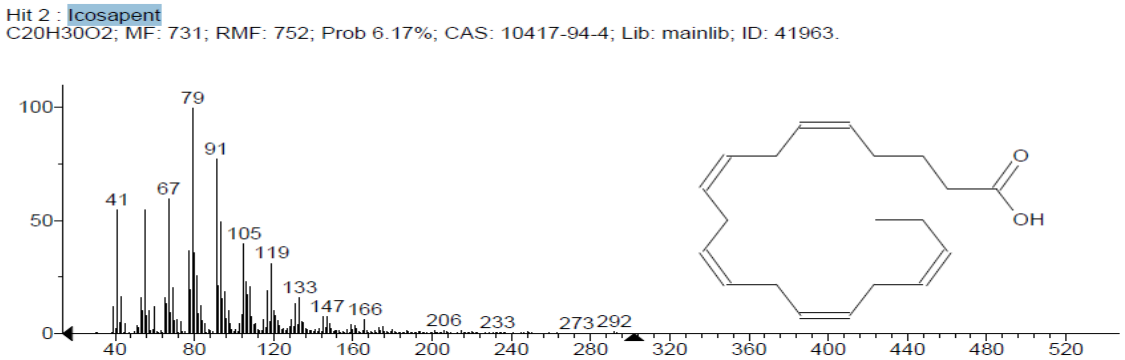


Figure 2: Structure of Icosapent ($C_{20}H_{30}O_2$) present in *Colpomenia sinuosa* extract using GC-MS analysis. This peak represents approximate molecule structure but not exactly the same molecule.

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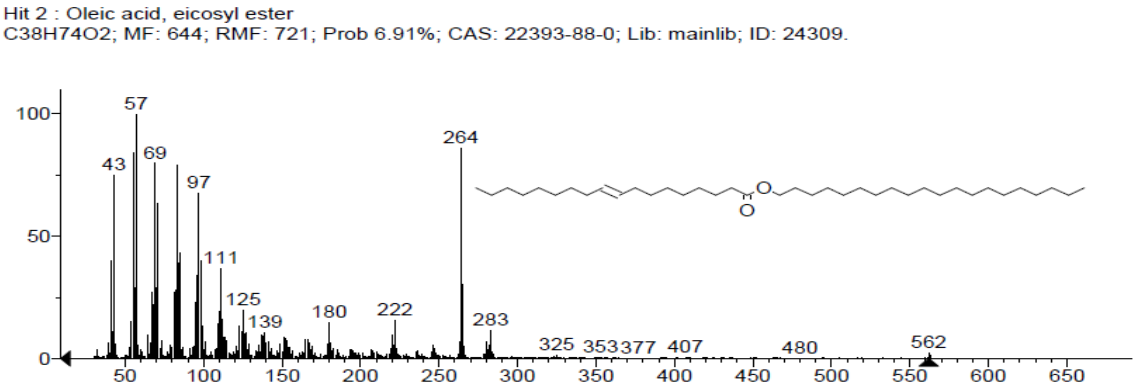


Figure 3: Structure of Oleic acid, eicosyl ester ($C_{38}H_{74}O_2$) present in *Colpomenia sinuosa* extract using GC-MS analysis. This peak represents approximate molecule structure but not exactly the same molecule.

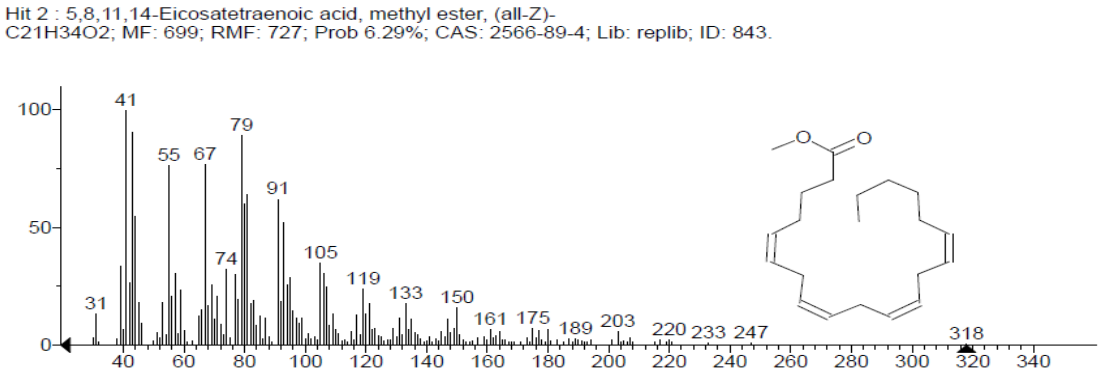


Figure 4: Structure of Eicosatetraenoic acid, methyl ester ($C_{21}H_{34}O_2$) present in *Colpomenia sinuosa* extract using GC-MS analysis. This peak represents approximate molecule structure but not exactly the same molecule.

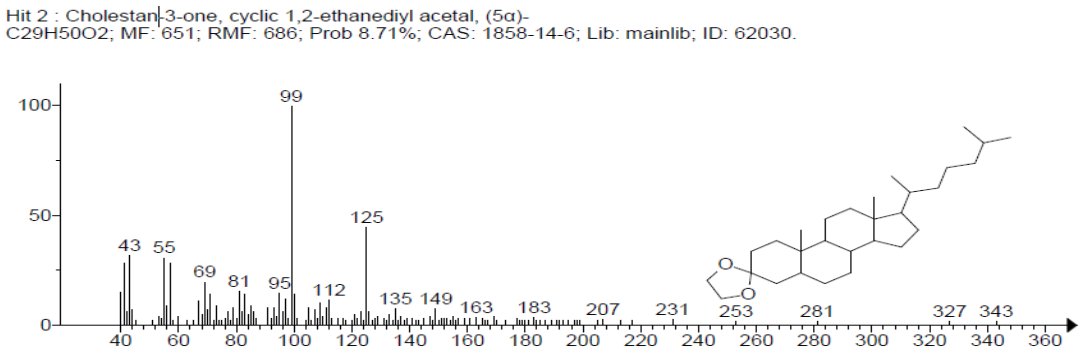


Figure 5: Structure of cholestane-3-one- cyclic-1,2 ethanediyl acetal ($C_{23}H_{50}O_2$) present in *Colpomenia sinuosa* extract using GC-MS analysis. This peak represents approximate molecule structure but not exactly the same molecule.

After further quantification of semi purified steroidal compounds, oleic acid, eicosyl ester, and cholestane-3-one-cyclic-1, 2-ethanediyl acetal have been selected for *in vitro* antimicrobial effects evaluation. The results of the antimicrobial susceptibility tests were expressed in terms of minimum inhibitory concentration (MIC), minimum bactericidal concentration

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(MBC) of the test organisms with respect to the acetone extracts of *C. sinuosa.* The antibacterial potential of the selected steroidal fractions has been investigated by the minimum bactericidal concentration (MBC) and the Minimum Inhibitory Concentration (MIC) against Gram positive bacterial strain of *S. aureus* and Gram negative bacterial strains of *E. coli* and *P. aeruginosa*. The MIC obtained from Oleic acid fraction extracted from *C. sinuosa* against *S. aureus*, *E. coli* and *P. aeruginosa* are shown in Table 1. The MIC has calculated to be 200 µg ml^{-1} against Gram positive bacterial strain of *S.aureus* and 1000 µg ml^{-1} against negative strains of *E. coli*. However, the inhibitory growth effect has not shown for *P. aeruginosa* strains up to 2000 µg ml^{-1} concentration (Table 1). The MBC value has been calculated based on the concentration of isolated steroidal fractions that completely inhibited any visible bacterial colony growth which was 1000 µg ml^{-1} for *S.aureus* strain alone and the other bacterial strains have shown no fatal effect (Table 1). The Minimum Inhibitory concentration (MIC) of fraction containing a mixture of cholestane-3-one-cyclic-1,2-ethanediyl acetal reported to be 100 µg ml^{-1}, 500 µg ml^{-1} and 2000 µg ml^{-1} for *S. aureus, E. coli and P. aeruginosa* respectively (Table 2). In addition, this compound has shown growth inhibitory effect on other pathogens bacteria at present investigation. Based on Table 2 results, The MBC of cholestane-3-one-cyclic-1, 2-ethanediyl acetal fraction for *S. aureus* strain as well as *E. coli* were 500 µg ml^{-1} and 2000 µg ml^{-1}. This steroidal fraction has shown no fatal effect against gram positive bacterial strain of *P. aeruginosa* (Table 2).

Table 1: MIC and MBC growth of the fraction containing a mixture of Oleic acid extracted from *Colpomenia sinuosa* algae.

	Concentration of Oleic acid (µg ml^{-1})														Anti-bacterial effects	
Bacterial strain	2	4	10	20	30	40	50	100	200	300	400	500	1000	2000	MIC µg ml^{-1}	MBC µg ml^{-1}
S.aureus	+	+	+	+	+	+	+	+	-	-	-	-	-	-	200	1000
E.coli	+	+	+	+	+	+	+	+	+	+	+	+	-	-	1000	-
P.aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	>2000	-

KEY: - =No Growth, += turbid with growth

Table 2: MIC and MBC growth of the fraction containing Cholestane compound extracted from *Colpomenia Sinuosa* algae.

	Concentration of Cholestane (µg ml^{-1})														Anti-bacterial effects	
Bacterial strain	2	4	10	20	30	40	50	100	200	300	400	500	1000	2000	MIC µg ml^{-1}	MBC µg ml^{-1}
S.aureus	+	+	+	+	+	+	+	-	-	-	-	-	-	-	100	500
E.coli	+	+	+	+	+	+	+	+	+	+	+	-	-	-	500	2000
P.aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+	-	2000	-

KEY: - =No Growth, += turbid with growth

Discussion

The Persian Gulf is one of the most important environmental areas because of its various living marine organisms. Algae are considered as one of major organisms which are able to supply natural products; however, sufficient information about their biological activities is not available. In the present study, isolated steroid fractions from

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acetone extract of *C. sinuosa* (Phaeophyceae alga) could be effective to all type of the pathogenic bacteria.

The results of the MIC and MBC indicated that the steroidal compound isolated from acetone extracts of *C. sinuosa* have bactericidal properties against *E. coli, P.auroginosa and S.aureus*.

Karkhaneh Yousefi *et al.* (2020) reported the antibacterial activity of algal extract was determined by disc diffusion assay, minimum inhibitory and minimum bacterial concentration tests; and the antioxidant activity through ferric reducing antioxidant power method which *C. sinuosa* algal extract showed a strong ferric reduction power.

Dashtiannasab *et al.* (2012) evaluated the antibacterial activity of ethanolic and chloroformic crude extracts of the brown algae, *Sargassum latifolium* derived from Persian Gulf. The results revealed that both crude extracts showed antimicrobial. activity against shrimp selective pathogen bacteria including *Vibrio alginolyticus*, *V. parahaemolyticus* and *V. harveyi.*

Afzal Rizvi (2010) carried out the investigation of six species green algae, ten brown algae species and ten species of red algae. All samples were collected from different areas of the Karachy beach in Pakistan and the methanolic extract has been selected for evaluation of antimicrobial properties against 11 strains of gram-positive and gram-negative pathogens.

Shameel (2008) reported that acetone extract of *C. sinuosa* and *Iyengaria stellata* which belong to brown algae had the highest antimicrobial effect against gram positive and negative bacteria while green algae had the least antimicrobials effect and red algae showed average level.

Kumar *et al*. (2010) extracted two novel steroidal compounds 3, 6, 17-trihydroxy-stigmasta-4, 7, 24 (28) - triene and 14, 15, 18, 20-diepoxyturbinarin as well as the well-known compound fucosterol from *Turbinaria conoides*. The antimicrobial effects of these compounds have been investigated. The minimum inhibitory concentration (MIC) has done in broth dilution method. All of isolated compounds showed an average antibacterial activity against tested bacteria. Fucosterol compound showed greater potential value against the fungus *Aspergillus niger* and with greater potential value of the MIC of 0.2 microgram per ml. Anti-fungal compounds isolated from *Turbinaria conoides* has been reported for the first time. These results indicated that 14, 15, 18, 20-diepoxyturbinarin can be develope as a new antifungal agent (Kumar *et al*., 2010). However, at the present study, isolated steroidal compounds had 5 fractions in which we have evaluated the anti-bacterial effects of two fractions, oleic acid and cholestane.

Perme (2010) have studied on red algae, *Gracilariopsis persic*, and brown algae, *Sargassum oligocystum*, which have been collected from the coasts of Hormozgan province (Persian Gulf coast and Oman Sea). The red algae were used ethyl acetate solvents and methanol with ratio (1:1) for extraction.

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The bioactive compound of brown algae *S. oligocystum* in comparison with *Colpomenia sinuosa* in the current study, were extracted by chloroform-methanol (3:1) and water. The effects of total extracts investigated on *Artemia salina* larvae at concentrations of 10, 100, 500 and 1000 µg ml^{-1}. Perme (2010) reported that ethyl acetatic and methanolic extracts of the *G. persica* and chloroform-methanolic extract of S. *oligocystum* showed cytotoxic effects with LD50 less than 1000 µg ml^{-1}. Methanolic extracts of *G. persica* and aqueous extracts of *S. oligocystum* demonstrated less cytotoxicity activity with LD50 higher than 1000 µg ml^{-1}. Ethyl acetate extract of *G. persica* with LD50 of 4 µg ml^{-1} was identified as the most effective fraction.

Moreover, by using thin layer chromatography (TLC) methods and HPLC, these steroid compounds have been isolated: Cholesterol and 22-dihydrocholesterol from the methanolic extract of *G. persica* and Cholesterol, 22-dehydrocholesterol, phocostrol, osterstrol, saringestrol, stygmasterol, and two isomeric steroid esters from the ethyl acetate extract of *S. oligocystum* have been isolated and identified and antimicrobial properties have been investigated because of these compounds (Perme, 2010).

Nazemi *et al*. (2014 a) have reported the examination of antifungal and antibacterial activities (*in vitro*) of diethyl ether, methanol and aqueous extracts of *Haliclona* sp. cucumbers from the Persian Gulf and Oman Sea. Similarly, they have been investigated the effects of pathogenic bacteria such as *E. coli*, *P. aeruginosa* and *S. aureus*, *Bacillus subtilisspizizenii* and antifungal effects of *Candida albicans* and *Aspergillus fumigatus*. Based on the findings of the present study, it can be concluded that diethyl ether extract of *Haliclona* sp. was effective against gram-positive bacteria. Furthermore, methanol extract compared to diethyl ether extract has showed better activity against *C. albicans* (MIC: 0.75 mg ml^{-1}, MFC: 1.5 mg ml^{-1}) and *A. fumigatus* (MIC: 2 mg ml^{-1}, MFC: 3 mg ml^{-1}). Aqueous extract showed neither antifungal nor antibacterial activities.

Isolation and characterization of steroid compounds from marine organisms are relatively new with different approaches. Currently, Seaweeds have been used as antibiotics, laxatives, anticoagulants, anti-ulcer products as well as suspending agents in radiological preparations. Because of an increasing demand for screening new therapeutic drugs from natural products, there is a greater interest towards marine organisms. Steroid compounds have been isolated and semi purified using acetonic extracts of *C. sinousa* through GC-MS method. The selected steroid compound demonstrated antibacterial potential against human pathogenic Gram positive bacteria *B. subtilis* and Gram negative bacteria *E. coli* and *P. aeruginosa*. It has been observed that cholestane compound showed better antimicrobial activity compared to oleic acid compound of the same algae in terms of yield and activity. The present study showed that brown algae were more effective compared to other

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groups of algae tested. Thus, with more standardization and procedures these steroid compounds can be further used as therapeutics for targeted drug delivery with minimal side effects and might be used as appropriate candidates for other biomedical applications.

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