

Isolation and identification of antibacterial compound from common name *Holothuria leucospilota* in the Persian Gulf

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Received: November 2017

Accepted: December 2017

Abstract

Echinodermata are one of the most important branches of invertebrate in marine ecosystems, which today are also known as a rich source of natural products with biological properties. Most of these properties belong to compounds with a terpenoids structure. According to the biological properties of sea cucumbers in the Persian Gulf, this scientific study investigates the antibacterial properties of lanosterol compounds extracted from *Holothuria leucospilota* in Hengam Island in the Persian Gulf. The sea cucumber powder was extracted using acetone, then the extract purified by silica gel column chromatography with n-hexane and ethyl acetate combination to isolate the terpenoid compounds. Isolated compound were run through TLC and sprayed with vanillin-sulphuric acid reagent for detection of terpenoid compounds and profile of isolated compounds obtained by gas chromatography (GC). The lanosterol triterpenoids compound in column fraction was identified by GC. Antimicrobial properties were evaluated by dilution test on bacterial strain *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella nemonya*, *Proteus vulgaris*, *Salmonella typhi*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and *Nocardia brasilinus*. This compound show antibacterial activities against gram-positive bacteria (*B. subtilis*, *B. cereus* and *S. aureus*). Antibacterial results of lanosterol authenticated that could be an efficacious compound in antibiotics production

Keywords: Natural products, Terpenoids, Antibacterial, Hengam Island, Persian Gulf

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Introduction

Echinodermata is one of the most important phyla of marine and contains 7,000 live species. The Sea cucumbers belong to the class Holothuroidea and are found in all seas and oceans in all latitudes, from coastal areas to abyssal plains (Mary Bai, 1994). Sea cucumber *Holothuria leucospilota* is found in the shallow habitats up to 10 m depth, grass, sand, mud and coral reefs. This species is distributed in all warm and temperate waters (Purcell *et al.*, 2012).

Sea cucumbers have been well recognized as a tonic and traditionally remedy in Chinese and Malaysian literature for their effectiveness against hypertension, asthma, rheumatism, cuts and burns, impotence and constipation. Several unique biological and pharmacological activities namely antigenic, anticancer, anticoagulant, anti-hypertension, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, antitumor, and wound healing have been ascribed to chemical compounds extracted from different sea cucumber specie and microbial communities (Bordbar *et al.*, 2011; Aminin *et al.*, 2015; Janakiram *et al.*, 2015; Gao *et al.*, 2017). These medicinal benefits and health functions of sea cucumbers can be attributed to the presence of appreciable amounts of bioactive compounds, especially the triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycan sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, peptides, cerberosides and lectins (Podolak *et al.*,

2010; Bordbar *et al.*, 2011; Li *et al.*, 2013).

Low molecular weight compounds, such as teriterpenoids are one of the most important natural product in poisonous of sea cucumbers to protect against predators and threatening biological agents and today they are used as template to develop therapeutic drugs including; anti-cancer, anticoagulants, antihypertensive, anti-inflammatory, antimicrobial, antioxidant, anti-clotting, and wound healing (Podolak *et al.*, 2010; Kim and Himaya, 2012). Terpenes are relatively small organic molecules, which have a wide variety of structural varieties. Approximately 35,000 terpenes have been identified and the majority of possible functions of these molecules are unknown. Some of them are hydrocarbons and others have oxygen in their structures. Some of them are right-chain molecules and some compounds have one or more rings. Teriterpenoids are fat-soluble chemical compounds, which are typically extracted by petroleum ether (Bhat *et al.*, 2005).

Natural products extracted from sea cucumbers have been proven in several studies as potential antimicrobial agents (Rahman, 2014). Due to the antimicrobial properties of terpenoids compounds extracted from Persian Gulf (Nazemi *et al.*, 2014), this study investigates the isolation and identification of bioactive terpenoid and antibacterial effects of isolated compounds of *H. leucospilota* from the Hengam Island on the Persian Gulf.

Material and methods

Sampling and identification

H. leucospilota have been collected by scuba diving in July 2015; from reef those habitats at depths of 30-35 m around Hengam Island in the Persian Gulf. After identification of species, the internal organism removed and samples have been lyophilized and dried samples used for extraction.

Extraction

The powder of dried cucumber samples (200 g dry weight) extracted with acetone solvent. After 72 hours of soaking in acetone, the solvent filtered and acetone evaporated to dryness, at low pressure at 35-40°C by using Rota vapor (Çitoğlu and Acikara, 2012).

Isolation and identification of lanosterol compound

Silica gel column chromatography with 70 cm height and 2 cm diameter used for purification of sea cucumber acetone extract (6.2 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 fraction collected in 10 ml tube (115 fractions) (Çitoğlu and Acikara, 2012). Obtained fractions from the chromatography columns were performed by thin layer chromatography with a mobile phase including methanol-chloroform- n butanol solvents with ratios of 70:20:10. To identify the beta-amic compound (belong to triterpenoid), 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 triterpenoids were changed to pale purple-full purple colour (Attaway *et al.*, 1965). The spots with purple colour and R_f value of 0.4 were injected into a gas chromatography (Agilent7000 Series Triple Quad GC / MS Main Frame) to identify the lanosterol compound.

Antibacterial assay

Antibacterial activity was determined against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi*, *Proteus vulgaris*, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis* and *Nocardia brasiliensis* by performing the classic broth dilution susceptibility test. Microorganisms 1.5×10^5 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 (1, 2, 4, 10, 20, 30, 50, 100, 200, 300, 400, 500, 1000, 2000 $\mu\text{g ml}^{-1}$) of lanosterol agent and to a tube of the growth control. An uninoculated tube of medium was incubated to serve as a negative 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

(matching the negative growth control), was designated the minimum inhibitory concentration (MIC). Later, the MIC has been determined, a known quantity, 0.1 ml, of inoculums from each tubes of broth that showed on visible turbidity after 22 to 24 hours incubation is subcultured 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 concentrate of antimicrobial agent that allowed less than 0.1% of the original inoculums to survive is said to be the minimum bacterial concentrate (MBC) (Rosenblatt, 1991).

Results

Isolation of fractions containing triterpenoid

Thin layer chromatography used to separate the column chromatography fractions (115 fractions) by vanillin reagent based on colure change of terpenoid content from pink to purple (Fig.1).



Figure 1: Thin layer chromatography of acetone extracts of sea cucumber.

Identification of lanosterol compounds

The lanosterol (lanosta-8,24-dien-3-ol = IUPAC) with the chemical formula C₃₀H₅₀O (Figs. 2, 3), molecular weight of 426/71 g mol⁻¹ belonging to the triterpenoid group identified with 96% purity in fraction 49 (ethyl acetate-hexane 0: 100) at 38-48 min retention time of gas chromatography (Figs. 2, 3).



Figure 2: Gas chromatography chromatogram of fraction no. 49 of column chromatography containing lanosterol from sea cucumber.

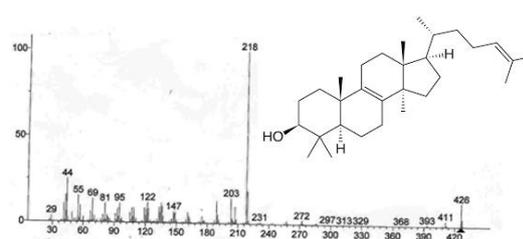


Figure 3: Gas chromatography chromatogram of fraction no. 49 of column chromatography containing lanosterol compound.

Antibacterial activities of fraction containing lanosterol compound

As indicated in Table 1, the minimum inhibitory concentration of bacterial growth (MIC) of lanosterol for *B. subtilis* was 300 µg ml⁻¹ and for *B. cereus*, *E. coli* and *Staphylococcus aureus* equal to 500 µg ml⁻¹. The lanosterol compound has not shown any inhibitory effects on growth of *Nocardia brasiliensis*, *S. typhi*, *K.*

nemonja, *P. vulgaris* and *P. aeruginosa*.

Table 1: Minimum inhibitory concentration of fraction containing lanosterol compound from *H. leucospilata*.

Lanosterol concentration ($\mu\text{g ml}^{-1}$)	Bacteria
300	<i>Bacillus subtilis</i>
1000	<i>Staphylococcus aureus</i>
500	<i>Bacillus cereus</i>
500	<i>Escherichia coli</i>

As per Table 2, the minimum bactericidal concentration (MBC) of the lanosterol compound for *B. subtilis*, *S. aureus* and *Bacillus cereus* is $1000 \mu\text{g ml}^{-1}$ and for *E. coli* is $2000 \mu\text{g ml}^{-1}$.

Table 2: Minimum bactericidal concentration (MBC) of fraction containing lanosterol compound extracted from *H. leucospilata*.

Lanosterol concentration ($\mu\text{g ml}^{-1}$)	Bacteria
1000	<i>Bacillus subtilis</i>
1000	<i>Staphylococcus aureus</i>
1000	<i>Bacillus cereus</i>
2000	<i>Escherichia coli</i>

Discussions

Nowadays, discovery of natural products from marine resources is increasing with the pharmaceuticals application. The studies show that since 2008 more than 1000 natural compounds of marine resources have been identified annually (Hu *et al.*, 2015). So far, more than 20,000 triterpenoid have been extracted and identified from natural resources, such as squalene, lanostrol, lupan, orasan, olanen and hoppan.

This teriterpenoid compound have been identified from *H. scabra* (Stonik and Elyakov, 1988) and *Stichopus*

Californicus with R_f value of 0.4 and 0.6, respectively (Sheikh and Djerassi, 1976). In this study, based on Fig. 2, gas chromatography chromatogram demonstrated that lanosterol compound from *Holoturia locuspilata* isolated and identified in the column fraction No. 49 with R_f value of 0.4. This Study have shown that the lanosterol compound is highly valuable and has antimicrobial, antifungal and anti-cancer properties (Sottorff *et al.*, 2013). Out of 4196 identified natural products from marine resources, 521 compounds (13% of all identified compounds) have antibacterial properties (Hu *et al.*, 2015). Many compounds, such as steroids, glycosides, triterpenoid, peptides, etc. have been identified from sea cucumbers with antimicrobial properties.

The studies have shown that the echinoderms have the most anti-bacterial effects compared to other marine organisms, including porifera, bryozoan, mollusks, coral and worms, etc. (Shakouri *et al.*, 2009).

This research determined that fraction containing lanosterol compound extracted from sea cucumber of *H. lucospilata* in a concentration of $1000 \mu\text{g ml}^{-1}$ has antimicrobial activity on gram-positive bacteria *B. subtilis*, *S. aureus* and *B. cereus* and at $2000 \mu\text{g ml}^{-1}$ concentration has antimicrobial activity on gram-negative bacteria of *E. coli*. This compound in a concentration of $2000 \mu\text{g ml}^{-1}$ also resulted in the death of *Candida albicans*. The results of this study showed that lanostrol and synthesized compounds at concentrations of 4 to $10 \mu\text{g ml}^{-1}$ have

antibacterial effects on gram-positive bacteria (*S. aureus*), gram negative bacteria (*E. coli*) and antifungal effects on different strains of candida (arjami *et al.*, 2014).

Chloroform extracts of gonad and intestine of *H. leucospilota* from Larak Island, the Persian Gulf had more fungicidal activity against *Aspergillus niger* (Farjami *et al.*, 2014). This compound has also resulted in the death of *Candida albicans* in a concentration of 2000 $\mu\text{g ml}^{-1}$. The results of this study were in accordance with the studies on the lanosterol and synthesized lanosterol compounds, at concentrations of 4 to 10 $\mu\text{g ml}^{-1}$ antibacterial effects on strains of *S. aureus*, *E. coli* and antifungal effects on different strains of *candida* (Shingate *et al.*, 2013).

The Studies on *Athyonidium chilensis* cucumber fractions showed that the purified compounds have antimicrobial activities against gram-positive bacteria, *S. aureus* and *B. subtilis*, but does not showed any antibacterial activity on gram-negative bacteria (Sottorff *et al.*, 2013; Nazemi *et al.*, 2014).

The terpenes extracted from sea cucumber *H. locuspilaata* have shown a relatively strong antibacterial effect on gram positive bacteria in this scientific project, although some compounds have also affected on gram-negative bacteria in higher concentrations, but there is no antibacterial activity against gram-positive bacteria.

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