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Research Article

Antibacterial, antifungal, and cytotoxic activity of the fraction contains squalene in the acetone extract of a sea cucumber, *Stichopus hermanni*

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Abstract

It is believed that the marine animals contain many compounds that could be beneficial for the treatment of many diseases. In this study, the acetone extract of *Stichopus hermanni* was fractionated by a liquid chromatography and then fractions were assayed for terpenoids. The fraction C18 that received a positive response for terpenoids was purified further and characterized by the liquid chromatography, mass spectral, and Thin-layer chromatography analysis. A terpenoid compound, squalene, was identified as the constituent of the bioactive extract fraction. Antimicrobial activity of C18-3 was tested against *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Nocardia brasiliensis* pathogens. The lowest MIC and MBC values were observed in the *Bacillus cereus* (50 µg/mL) and *Staphylococcus aureus* (500 µg/mL), MIC and MFC the fungal pathogen Candida albicans (400 and 2000 µg/mL) and was cytotoxic against KB/ C152 and HUT-78/C185 cells (IC50 6.1 µg/mL and 6.29 µg/mL). This research suggests *S. hermanni* can be used as an alternative source for the separation and purification of squalene compound as a medicinal supplement.

Keywords: Antibacterial activity, Anti-fungal activity, Cytotoxic activity, Sea cucumber, Marine natural products

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Introduction

It is believed that the marine animals contain many compounds that could be beneficial for the treatment of many diseases. Several natural chemicals derived from marine resources have been discovered in recent years (Pangestuti and Arifin, 2018). Natural chemicals in marine species can be used to produce compounds for nutritional, pharmacological and medicinal use (Malve, 2016).

In recent years, the discovery of natural compounds with potential medicinal effects from marine resources has been grown. The marine environment holds a great promise for finding new biological compounds with more than 13,000 identified molecules, of which 3,000 have been identified with bioactive capabilities (Vignesh *et al*., 2011; Malve, 2016). So far, more than 20,000 triterpenoids from natural sources such as; Squalene, lanosterone, leopard, urasan, ulnan and hopan have been extracted and identified. Triterpene glycosides with the biological activity such as antifungal, cytotoxic, hemolytic, and immune boosters are the most important natural products in sea cucumbers (Muniain *et al*., 2008; Zhang and Yi, 2011). Marine species' natural chemicals can produce compounds for nutritional, pharmacological, and medicinal use (Bordbar *et al*., 2011; Khotimchenko, 2015).

Sea cucumber study has been restricted for many years to its physiological and biological features. Antibacterial, antifungal, anticoagulant, antiviral, cytotoxic, hemolytic, and even anti-HIV activities are presently being studied in sea cucumbers (Mashjoor and Yousefzadi, 2017). *Stichopus hermanni*, *Thelenota ananas*, *Thelenota anax*, *Holothuria fuccogilva*, and *Actinopyga mauritiana* are among the most interesting sea cucumber species in Asian countries. Triterpene glycosides, carotenoids, bioactive peptides, vitamins, minerals, fatty acids, collagens, gelatins, chondroitin sulfates, and amino acids are all various pharmacological substances found in these species (Masre *et al*., 2012). The health benefits of Sea cucumbers have been proven by scientific research in recent years, and they have shown medical effects such as wound healing (Masre *et al*., 2010), neuroprotection, anticancer, anticoagulant, antibacterial, and antioxidant properties. These functional materials have a potential to be used in a wide range of industries, including food and biomedicine (Pangestuti and Arifin, 2018). Since Echinodermata species have potential medicinal effects were investigated in some studies, so this study aimed to investigate the effect of *Stichopus hermanni* acetone extract on the antibacterial, anti-fungal, and cytotoxic activities. To identify the bioactive compounds, the isolated fraction was subjected to GC-MS analysis.

Materials and methods

Reagent and cell

In this study, SK-BR-3 cells were obtained from the Pasteur Institute of Iran (Tehran, Iran). Moreover, Penicillin–streptomycin antibiotics, and

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XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (FBS) and Roswell Park Memorial Institute Medium (RPMI 1640) were purchased from Gibco (Grand Island, NY, USA). Trypsin/Ethylene diamine tetraacetic acid (Trypsin/EDTA) was also purchased from Bio-Idea. Additionally, all other chemical materials were also obtained from Merck (Darmstadt, Germany).

Source and Preparation of Sea Cucumber Samples

The sea cucumber was manually collected using SCUBA at depths of 15-20 m in the coastal waters (26°50'27.6"N 56°23'17.0"E) of the Larak Island, Persian Gulf. The species was identified in the Persian Gulf and Oman Sea Ecological Research Institute, Bandar Abbas, Iran.

Extraction and isolation method

The sea cucumbers were transferred to the laboratory and then cleaned twice with distilled water. The material was sliced into 1 cm3 pieces and dried with a freeze drier (Spain- TELSTAR). The samples were stored at -24°C until the experiment. Six hundred gram of powder was extracted during 72 hours with 1800 mL of acetone at room temperature (Nazemi *et al*., 2020). Afterward, a rotary vacuum evaporator was used to pool and concentrate the acetone extracts obtained during the whole process at a maximum temperature of 38°C (2 hours) and then to freeze-dry them. Crude extracts were kept at -20°C for later use. By chromatography (Silica gel 0.2-0.6 mm mesh, 500 g, 2 cm 70 cm), the crude acetone extract (30.35 g) was separated into 112 fractions, C1–C112, using a gradient mobile phase of N-hexane-ethyl acetate (100:0–0:100, v/v) and ethyl acetate-methanol (100:0–0:100, v/v).

Thin layer chromatography

The active components in acetone extract were separated and partially purified using thin layer chromatography (TLC). TLC plates containing pre - coated silica gel 60 (Merk aluminum sheet, 20×20 cm, 0.2 mm layer thickness) were utilized. Diluted fractions of acetone extracts were loaded onto the base of the silica gel plates, and allowed to dry. In a solvent saturated environment, samples were chromatographed to 17 cm in Methanol: chloroform: N-butanol (10:70:20 v/v). The fraction C18 (Yield 79 mg) that received positive response for Terpenoids by Vanillin-sulphuric acid reagent was selected and was then separated into five sub fractions, C18-1–C18-5, by Silica gel (0.2-0.6 mm mesh, 500 g, 0.5 cm×50 cm) using eluent of Methanol: chloroform (100:0–0:100, v/v) and chloroform: N-butanol (100:0–0:100, v/v). Then, the sub fraction C18-3 (20 mg) that had a positive response for Terpenoids subjecting it on TLC eluting using Methanol: chloroform: N-butanol (10:70:20 v/v) after eluent the spot that received positive response for Terpenoids by Vanillin-sulphuric acid

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reagent, was selected for determining the use of GC–MS. TLC plates were viewed under white and UV light (254 and 365 nm) to detect separated components, and following eluent, the spot that received a positive reaction for Terpenoids using the Vanillin-sulphuric acid reagent was chosen for GC–MS analysis (Jiang *et al.*, 2016) Cytotoxicity, antifungal, and antibacterial activities were all evaluated on this fraction.

GC-MS analysis

The fraction C18-3 was subjected to GC-MS analysis. To perform this analysis, an Agilent Agilent7000 Series Triple Quad GC/MS (Agilent Technologies, Palo Alto, CA) equipped with an Agilent 19091S-413HP-5MS 5% Phenyl Methyl Silox capillary column (30 m/320 µm/0.25 µm) were used. Thereafter, one µL of the sample was entered into the GC-MS with a split injection mode of 20:1 split ratio. Notably, the program of the oven was as follows: initial temperature was from 80 to 100°C (rate 20°C/min, hold 1.0 min), 100 to 150°C (rate 10°C/min, hold 1.0 min), 150 to 180°C (rate 15°C/min, hold 1.0 min), and 180 to 200°C (rate 10°C/min, hold 1.0 min), followed by an increase up to 280°C (rate 10°C/min, hold 5 min) (Khaledi *et al*., 2020). The injector temperature was at 260°C and Helium gas was then used as a carrier in a steady flow mode at a flow rate of 1 mL/min. Inlet and GC / MS interface temperatures were kept at 250 and 280°C, respectively. EI sources and the quadrupole analyzer was kept at 230 (250°C maximum) and 150°C (200°C maximum), respectively. MS scanning in full scanning mode was ranged from small to high mass (50 M/z- 650 M/z). ChemStation software, version 5.51, was used to control the instruments and collect data. Commercial mass spectrum libraries were used to identify compounds (Wiley 7.1 Mass spectra register).

Cytotoxic activity

The oral epithelial cancer cell line (KB/C152), T-lymphocytic leukemia cell line (HUT-78/C185), and human embryonic kidney (Hek293) cells were purchased from the Pasteur Institute of Iran in Tehran, Iran, and the tests were carried out in the department of hepatitis, HIV, and Bloodborne Viruses' laboratory. The KB/C152, HUT-78/C185, and Hek293 cells were maintained in RPMI-1640 with 10% Fetal Bovine Serum and antibiotics (100 Unit/mL of penicillin and 100 mg/mL of streptomycin). 25,000 cells were also planted in 96-well plates with media containing 2, 10, 50, 100, and 200 g/mL of fraction C18-3. The plates were maintained at 37°C for 24 hours in a humidified environment with 5% CO2 and 95% O2. After 24 hours, 100 liters of XTT were added to each well, and the plates were incubated at 37°C for 4 hours. The median inhibitory concentration (IC50) and the combination index were calculated using the average of triple trials for each dosage. To determine the IC50 values, the concentration of reduced XTT was measured by measuring the absorbance at 490 and 690 nm in an ELISA reader

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(Loizzo *et al*., 2007) . All tests were done in triplicate, and cytotoxicity was computed using GraphPad Prism 5 software, and six separate experiments with three duplicates for each concentration were presented as mean SEM.

Antimicrobial activity

Antimicrobial activity was determined using the micro dilution method (Balouiri *et al*., 2016). Lyophilized six test bacteria were purchased from Persian Type Culture Collection (PTCC). Cultures of *Bacillus subtilis spizizenii* (PTCC No. 1715), *Bacillus cereus* (PTCC No. 11778), *Pseudomonas aeruginosa* (ATCC No. 27853), *Salmonella typhi* (PTCC No. 1609), *Proteus vulgaris* (PTCC No. 1079), *Escherichia coli* (ATCC No. 15224), *Klebsiella pneumonia* (ATCC No. 1053), *Staphylococcus aureus* (TTCC No. 1764) and *Nocardia brasiliensis* (PTCC No. 1422) were grown on BHI Agar medium and used for measuring the antibacterial activity of fraction C18-3. Each bacterial strain was inoculated into Mueller Hinton broth and incubated overnight at 37°C with shaking. The suspension was adjusted to 0.5 McFarland standard turbidity (equivalent to 1.5×108 (CFU/ mL) (Cremers *et al*., 2020) and finally diluted to give approximately 104 CFU/mL for all bacteria. Different dilutions (1, 2, 4, 10, 20, 30, 50, 100, 200, 300, 500, 1000 and 2000 µg/mL) were prepared from the stock solutions of fraction C18-3 (5 mg/mL) and antibiotics (1 mg/mL). 900 µL of each concentration was mixed with 100 µL of sterile BHI broth containing 104 CFU bacteria. One inoculated well was included, to allow control of the adequacy of the broth for organism growth. One non-inoculated well, free of antimicrobial agent and negative controls (DMSO-0.5%) was also included, to ensure medium sterility. Ampicillin and Tetracycline commercial standards were used as positive controls. Tubes were incubated overnight at 37°C. The highest dilution at which 99.9% of the bacteria inoculum was killed was considered as the MBC and the lowest inhibitory dilution at which there was no visible growth was considered as MIC. In order to determine the MBC, 0.1 mL of the tube in which no turbidity was observed was injected into sterile plates and nutrient agar medium was added to it. The plates were then transferred to an incubator and stored at 37°C for 24 h. After 24 hours, the number of CFUs was assessed. In the plates where the bacterium had grown, it was indicated that the extract in question could only inhibit the growth and proliferation of the bacterium, but in the plates where no colony was observed, it indicated that the substance in question had caused the bacterial death. This value is equal to MBC. The assays were replicated and the mean value of 3 experiments were recorded (n=3) with SEM.

Antifungal activity

Yeast and fungal strains *Candida albicans* (ATCC No. 10231) and *Aspergillus fumigatus* (PTCC No. 5009) were purchased from Persian Type

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Culture Collection (PTCC), then each strain was given for initial culture. Briefly, the antifungal potency of the fraction C18-3 was evaluated using *Candida albicans* and *Aspergillus fumigatus*. The antifungal activities were analyzed by macrodilution method (Seniya *et al*., 2012). The suspension of Candida albicans and Aspergillus fumigatus were cultured for 48 hours in 1 mL of sterile Sabouraud dextrose broth at 37°C, then adjusted to a turbidity of 0.5 on the McFarland scale (1.5×108 cells/mL). This culture was used throughout the experiments. Sabouraud dextrose agar (10 mL) was poured into Petri dishes, which were then inoculated with strains of *Candida albicans* and *Aspergillus fumigatus* by the addition of 0.1 mL of cell culture media.

Statistical analysis

For statistical analysis, GraphPad Prism 5 software was used. All values were expressed as mean values. To compare the two groups, One-way ANOVA test was used followed by post hoc Tukey's test for sub-two group comparison. $P<0.05$ was considered as statistically significant.

Results

Isolation and verification of squalene

The acetone extract was packed and eluted with a different solvent system in column chromatography. More than 112 fractions were collected via column chromatography based on the TLC profile. The Fraction C18 (Yield 79 mg) that showed a positive reaction to the Vanillin-sulphuric acid reagent for Terpenoids was chosen and divided into ten subfractions, C18-1–C18-5, using a Silica gel. TLC was used to isolate the organic compound from the C18-3 fractions. After TLC, the band that produced pink to purple color with the Vanillin-sulphuric acid reagent test was wad punched (Fig. 1S), dissolved in acetone, and lastly filtered, yielding crystals of compound (4.5 mg), which were then compared to the Wiley 7.0 library (Figs. 1 and 2) to confirm the compound's identification. squalene (IUPAC=(6E, 10E, 14E, 18E) - 2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22-hexaene) with chemical formula C30H50. The molecular weight of 410.73 g.mol-1, which belongs to the triterpene group, was detected at 92% at a fraction of C18-3 (anhexane-ethyl acetate 40: 60) at 42.87 minutes (Fig. 2).

Antimicrobial activity

The bacteriostatic and bactericidal activities of an extract are denoted by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), respectively. As shown in Table 1, not all the extracts possess both activities. he results of antibacterial activity of fraction C18-3 against the test bacteria are presented in Table 1.

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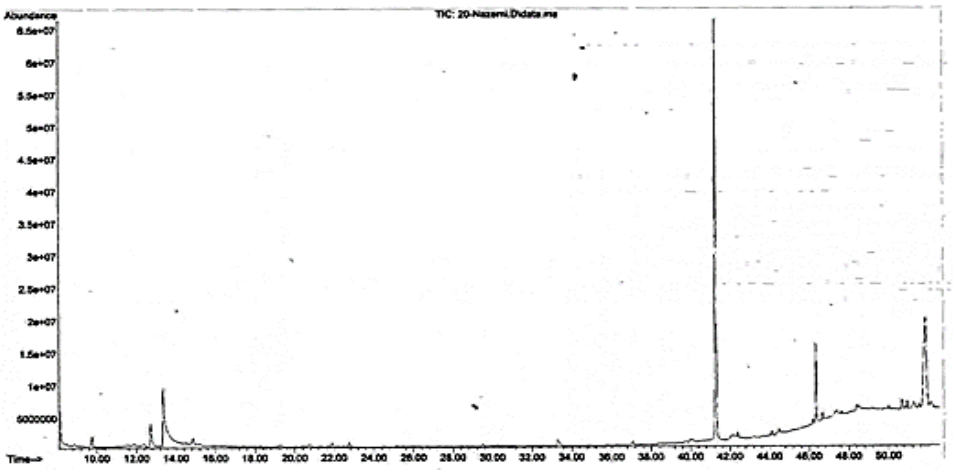

Figure 1: Chromatogram obtained from gas chromatography of fraction C18-3 contains squalene compound from sea cucumber muscle.

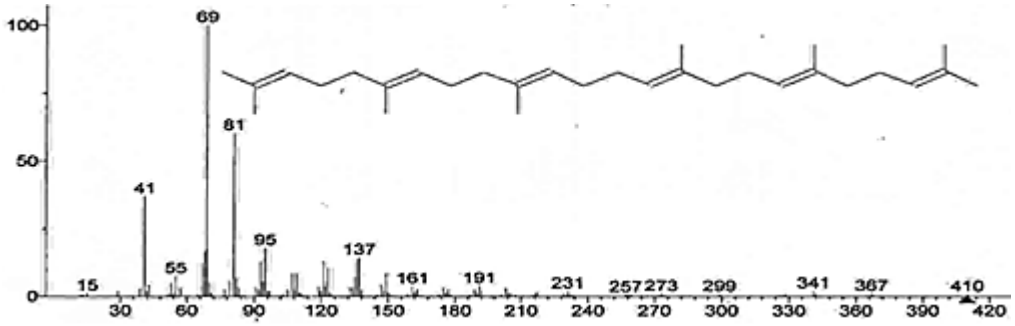

Figure 2: Mass spectrum of the squalene, corresponding to the major peak in the GC.

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of fraction C18-3 contains squalene compound against medically-important bacteria.

Species	MIC[a]	MBC[a]
Gram-positive bacteria		
Bacillus subtilis	500	2000
Bacillus cereus	50	500
Staphylococcus aureus	100	500
Nocardia brasiliensis,	500	1500
Gram-negative bacteria		
Pseudomonas aeruginosa	1500	NA
Klebsiella pneumoniae	NA	NA
Salmonella typhi	NA	NA
Proteus vulgaris	NA	NA
Escherichia coli	2000	NA

[a] The MIC and MBC values are expressed as mean of three consistent independent replicates in µg/mL. abbreviation: NA denotes no activity.

Antimicrobial activity of C18-3 was tested against certain bacteria including Bacillus subtilis, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Nocardia brasiliensis*, and two fungi including, *Candida albicans* and *Aspergillus fumigatus*. The C18-3 fraction revealed antimicrobial activities on test microorganisms with different spectral range and potency. In addition, the strongest bacteriostatic and bactericidal activities, as evident in the lowest MIC and MBC values, were observed in the *Bacillus cereus* (50 µg/mL) and *Staphylococcus aureus* (500 µg /mL), respectively. Minimum inhibitory concentration (MIC) and minimum

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fungicidal concentration (MFC) are used to illustrate the potency of an extract with regard to its fungistatic and fungicidal activities, respectively. *Aspergillus fumigatus* was the most resistant fungus tested; the extract was not effective (Table 2).

Table 2: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of fraction C18-3 contains squalene compound against medically-important fungi.

Species	MIC[a]	MFC[a]
Candida albicans	400	2000
Fungi filamentous		
Aspergillus fumigatus	NA	NA

[a] The MIC and MFC values are expressed as mean of three consistent independent replicates in µg/ml. abbreviation: NA denotes no activity

IC50 values of the bioactive compound

Cytotoxicity of the extracts against KB/C152, HUT-78/C185, and Hek293 cell lines is shown in Table 3. C18-3 fraction was found to be more effective against KB/ C152 cells than the HUT-78/C185 cell line. The IC50 for cell line KB/C152 and HUT-78/C185 were 6.1 µg/mL and 6.29 µg/mL, respectively. The fraction containing squalene compound in the studied concentrations did not show any toxic effect on the human embryonic cell line (Hek 293). These values were within the cut-off point of the National Cancer Institute's criteria for cytotoxicity (IC50 of <20 µg/mL) in the screening of crude plant extracts (Table 3).

Table 3: Cytotoxic Activity (IC50 value in µg/mL) of the fraction containing squalene compound from *S.hermanni.*

Cell line	IC50 (µg/mL)
KB/C152	6.21
HUT-78/C185	6.29

Discussion

Studies show that marine invertebrates have remarkable cytotoxic and anti-cancer effects. The anti-cancer effects of extracts from marine invertebrates have been so far tested on 60 cancer cell lines such as blood, breast, ovary, kidney, prostate, and colon (Datta *et al*., 2015). Studies conducted by the US National Cancer Institute (U.S.NCI) on the biological properties of secondary metabolites of aquatic organisms indicate that 4% of marine species (mostly animal species) have anti-cancer properties, including echinoderms and sea cucumbers (Janakiram *et al*., 2015).

In this study, squalene was identified and extracted from *S. hermanni*, a sea cucumber, at the fraction No. 18. Squalene, a member of triterpenoids, is usually found in fish species, especially sharks, and many oilseeds (Seçmeler and Galanakis, 2019), and commonly used for pharmaceutical and cosmetic purposes (Newmark, 1997). The 6.1 and 6.26 µg/mL concentrations of the fraction C18-3 contains squalene extracted from S.hermanni resulted in the death of 50% of cancer cells of oral epithelial and lymphocytes, whereas it caused the growth of human fetal kidney cells. Kelly *et al*. (1999) showed that the use of squalene as a dietary supplement not only can be effective in the treatment

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of cancers but also reduces the risk of cancers (Kelly *et al*., 1999). Studies have shown that the addition of 6-7% squalene to the diet of F344 mice inhibited the growth of intestinal cancer cells and, finally, resulted in the death of cancer cells over time. This compound also increased the body resistance of subjects (Rao *et al.*, 1998). Similar study have revealed that the squalene extracted from aquatic organisms has anti-cancer effects on soft tissue cells such as breast, uterus, and ovary (Kim, 2015). In another study on the squalene extracted from *Anemopaegma mirandum*, it was shown that this compound inhibited the growth of cancer cells in hamsters with lung cancer (Uchino *et al*., 2004). Another study also showed that the squalene extracted from algae caused the death of cancer cells of Jurkat, sarcoma (a malignant tumor of connective tissue), and cervix (Pacheco *et al*., 2011). Considering the mechanism of action of squalene, it has been shown that this compound is a good choice as an anti-cancer drug because it causes the apoptosis of cancer cells of blood, melanoma, colon, prostate, ovary, liver, lung, and breast (Reddy and Couvreur, 2009). Of the 4196 natural compounds extracted from marine resources, 521 compounds (13%) have antibacterial properties (Hu *et al*., 2015). Many compounds extracted from sea cucumbers, such as steroidal glycosides, terpenoids, and peptides, have exhibited antimicrobial properties. It has been also shown the compounds extracted from echinoderms have greater antibacterial effects than the compounds extracted from other aquatic organisms such as Porifera, Bryozoa, mollusks, corals, and annelids (Shakouri *et al*., 2009). Although there was significant antibacterial and cytotoxic activity for fraction containing squalene of the Body Wall of *Stichopus hermanni*, weak antifungal activity of this extract was observed. As shown in Table 2, fraction containing squalene has Minimum inhibitory concentration and minimum fungicidal concentration were 400 and 2000, respectively, against *Candida albicans*, and also no antifungal activity against *Aspergillus fumigatus*.

The results of this study showed that the squalene extracted from S.hermanni can kill gram-positive bacteria (i.e. *Staphylococcus aureus* and *Bacillus cereus*) at a concentration of 500 µg/mL and *Bacillus subtilis* at a concentration of 2000 µg/mL. In a study on using fractions containing steroid saponins and glycoside-steroid saponins extracted from the muscle wall of the body, *Stichopus hermanni* collected from Lark Island, Persian Gulf, at a concentration of 100 µg/mL leads to the death of *Bacillus subtilis* and only at a concentration of 500 µg/mL prevents the growth of gram-negative bacteria *Pseudomonas aeruginosa* (Salari *et al*., 2018) which compared with the results of this study. The effective concentration of the extract containing saponin compounds of *Sticopus germanium* sea cucumber with a fraction containing squalene extracted shows a stronger antibacterial effect. It can be hence concluded that fraction contains squalene had antibacterial effects on

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gram-positive bacteria but did not exhibit lethal effects on gram-negative bacteria. The study findings are consistent with the results of Liu *et al*. (2018) who conducted a study on natural and synthesized squalene and reported that the 100 nM concentration of this compound killed *Staphylococcus aureus* (Liu *et al*., 2018).

This is the first time, to the best of our knowledge, that the squalene extract from Sea cucumber. It has several advantages, As can be seen from the results of this research project, the amount of squalene in sea cucumber is significant and it seems that it can be introduced to one of the sources containing squalene. Also, according to the cytotoxic assay, squalene has led to the death of cancer cells but has not shown a lethal effect on normal cells. which indicates the potential of this compound for future anti-cancer *in-vivo* investigations. Ultimately, further study is needed to determine the mechanism of action for the anti-cancer activity of squalene. Therefore, it is possible to take an effective step in the treatment of cancer patients by propagating and cultivating sea cucumber and isolating and purifying the squalene compound as a medicinal supplement.

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