

RESEARCH ARTICLE

Anticancer Potential of Three Sea Cucumber Species Extracts on Human Breast Cancer Cell Line

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OPEN ACCESS

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Received: 23 June 2022

Accepted: 5 August 2022

Published: 30 August 2022

Academic Editor: Dr. Ariyanti Suhita Dewi

©Squalen Bulletin of Marine and Fisheries Postharvest and Biotechnology, 2021. Accreditation Number: 148/M/KPT/2020. ISSN: 2089-5690, e-ISSN: 2406-9272. <https://doi.org/10.15578/squalen.669>

Abstract

Sea cucumber has long been utilized as a treatment for a variety of ailments, including antibacterial, antifungal, immunomodulatory, and wound healing. As for the first time, the extracts of three sea cucumber species' i.e., *Actinopyga lecanora*, *Holothuria atra*, and *Stichopus vastus*, were compared and tested on the cytotoxicity of cancer cells using MTT and Annexin V/Propidium Iodide assays. This study investigates the protection of sea cucumber extracts against the breast cancer cell line (T-47D). All three extracts were found to be actively declining the cancer cell progression, with the IC₅₀ values of 6.25±0.50 µg/ml (*A. lecanora*), 7.5 ± 1.39 µg/ml (*H. atra*) and 3.25 ± 0.53 µg/ml (*S. vastus*). LC-MS/MS analysis was used to identify chemical compounds in the extracts. The 1,1-diphenyl-2-picrylhydrazyl (DPPH), total phenolic and flavonoid contents, and anti-collagenase activity were also assessed in all the three extracts. The results demonstrated the absence of antioxidant and flavonoid chemicals in *S. vastus*, *A. lecanora* and *H. atra* extracts. However, *H. atra* contained phenolic compounds (0.4794 mgGAE/g DW). Furthermore, all tested extracts showed significant anti-collagenase activity, which supported the reduction of cancer cell proliferation. However, more research into the mechanism of action of the extract is needed before sea cucumbers therapeutic characteristics may be used to combat breast cancer.

Keywords: sea cucumber, cytotoxic, anti-collagenase, phenolic, antioxidant

Introduction

Breast cancer is the second largest cause of mortality and the second most prevalent cancer identified in women, according to the National Cancer Institute in developed and developing countries (Francies et al., 2020). There are about 2.26 million new cases and almost 685,000 deaths from breast cancer in 2020 (IARC Globocan, 2020). Some breast cancers are not inherited, but others are attributable to genetic propensity primarily due to mutations in the tumor suppressor genes of breast cancer 1 (*BRCA1*) and breast cancer 2 (*BRCA2*) genes (American Cancer Society, 2021). Treatments such as surgery, radiotherapy, hormone therapy, chemotherapy, other

targeted treatment or even a mixed treatment are currently available to treat breast cancer (Mustafa et al., 2016). The treatments used for those who have breast cancer somehow depend on the type and stages of cancer. These treatments are categorized into two, local and systemic treatments. Local treatments are meant to treat tumors without affecting other body parts. The treatments are surgery and radiation therapy. As systemic treatments, the patients are treated using drugs, administered orally or intravenously.

The currently available treatments seem to be less effective as it causes side effects to the patients such as nausea, hair loss, loss of appetite and vomiting (Panno, 2010). Since the current modern treatment

has so many side effects on patients, finding a new alternative method that can kill or incapacitate cancer cells without causing harm or excessive damage to the normal cell has become the main priority. Natural products play a main role in developing an anticancer cure, and marine organisms' metabolites content is starting to gain popularity in the field of antitumor drug discovery (Nagle et al., 2004). A marine organism is an excellent natural product source since 70 % of the earth comprises oceans. In recent years, more than 3000 new compounds from marine sources have been discovered to exhibit potential anticancer and antitumor properties and a number of them have gone into clinical trials (Khalifa et al., 2019). Although the marine resources are rich with potential chemicals, most of them are still unexplored and their function remains unknown.

Sea cucumbers are one of the most important marine organisms found almost in every marine environment that belongs to the class Holothuroidea. It is a long worm-like organism with a gelatinous body that usually is soft-bodied echinoderm (Janakiram et al., 2015). In the last two decades, sea cucumber extracts have been widely studied in the medical and pharmaceutical fields as wound healing promoters, exhibiting anticancer, antimicrobial and immunomodulatory properties (Fredalina et al., 1999). Sea cucumber may possess potential compounds for fighting cancer as they consist of protein, vitamin A, thiamine, riboflavin, niacin, calcium, iron, magnesium, zinc, and other unique molecules (Janakiram et al., 2015). There are various kinds of compounds from sea cucumber such as monosulfated triterpenoid glycoside Frondoside A, the disulfated glycoside Frondoside B, the trisulfated glycoside Frondoside C, 12-methyltetradecanoic acid, Griseaside A, Echinaside, Cucumarioside A2-2 and fucosylated chondroitin sulfate. These compounds have been explored and reported as anticancer compounds

for various types of cancer such as colon cancer, liver cancer, lung cancer and cervical cancer (Wargasetia & Widodo, 2022; Wargasetia & Widodo, 2017; Sajwani, 2019). In this study, for the first time, three different sea cucumbers, namely *Actinopyga lecanora*, *Holothuria atra* and *Stichopus vastus* were studied in comparison to their potential cytotoxic effect against T-47D breast cancer cell line and their mode of cell death investigated. This study aimed to provide preliminary data to compare the effectiveness of the three species of sea cucumber extracts as an alternative option to treat breast cancer (Figure 1). This preliminary data will pave the way to study further the molecular mechanisms of bioactive metabolites derived from sea cucumbers and translate them from bench to bedside in the future.

Materials and Methods

Chemicals and Reagents

Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), myricetin, epigallocatechin gallate, coomassie brilliant blue were purchased from Sigma-Aldrich, UK while methanol, aluminium chloride, calcium chloride, sodium chloride and porcine gelatin were purchased from R&M, UK. Other than that, gallic acid, Follin Ciocalteu's phenol reagent, sodium carbonate, and tris hydrochloride were obtained from MERCK, Germany, MTS Assay from Promega, AnnexinV-FITC from Sigma-Aldrich.

Sample Preparation

The fresh samples of *A. lecanora*, *H. atra* and *S. vastus* were obtained from the Repository of Institute Marine Biotechnology (IMB), University Malaysia Terengganu. The taxonomic identity of the sea

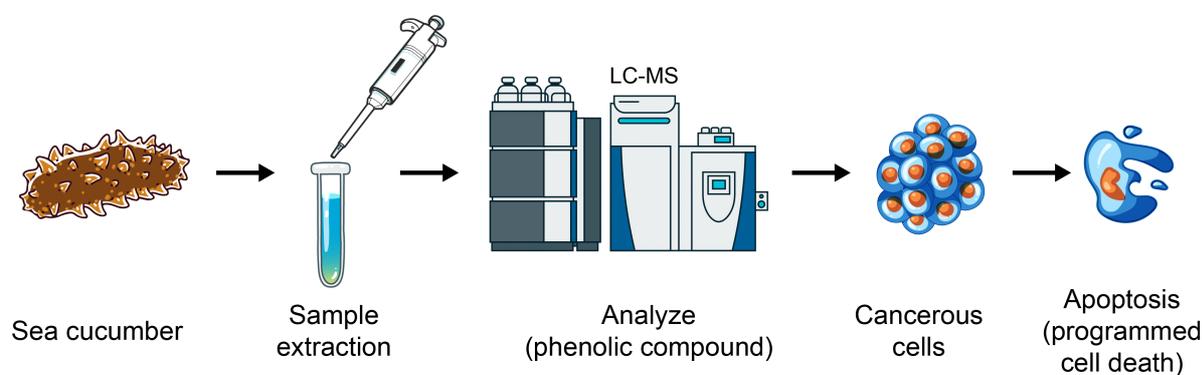


Figure 1. Schematic summarizes the study which involves cytotoxic effect against breast cancer cell line using the extract from different species of sea cucumber.

cucumber is identified based on its morphology and mitochondrial DNA evidence. Specimen ID for the deposited samples in IMB as *A. lecanora* (ECH1015002), *H. atra* (ECH1015003) and *S. vastus* (ECH1015001). Briefly, the fresh samples were washed and rinsed several times with fresh water to remove the sand and salt in the sample. The sample was cut into small pieces and then dried using a freeze dryer to remove the moist and liquid. The yield of each aqueous extract was calculated as the weight of dried extract to the weight of the frozen sample in the range of 1.2–1.5 %.

Sample Extraction

About 200 g of dried powder of sea cucumber samples were soaked in 750 mL of hexane for 24 h and were shaken at 200 rpm on an orbital shaker. Then the hexane was filtered from the sample through Whatman filter paper no. 2 (110Ø) under gravity. The exact process was repeated three times and the hexane extract was discarded. Next, the samples were soaked in absolute methanol for 24 h and kept in an orbital shaker at 200 rpm for 24 h. Dissolved samples were filtered through Whatman filter paper no. 2 (110 Ø) and the process was repeated three times. The filtered solution was dried using a rotary evaporator (IKA, Germany) under vacuum pressure below 40 °C and the methanol crude extract was obtained and stored in –20 °C.

Cell Culture and Maintenance

The breast cancer cell lines (T-47D) were obtained from the Institute of Pharmaceuticals and Nutraceuticals (IPharm), Malaysia and stored in a liquid nitrogen tank. The medium preparation was carried out in biosafety cabinet (BSC) Class II. The medium was prepared using RPMI 1640, which was supplemented with 10% of fetal bovine serum (FBS), 1% (v/v) of non-essential amino acids, 1% sodium pyruvate, 1% antibiotic solution (100 U/ml penicillin and 100 µg/mL streptomycin) to prevent the growth of bacteria. Insulin was added to the media to activate the cell lines and the media was stored at 4 °C. Initially, the cells' vials were thawed in a 37 °C water bath and the vials were transferred to BSC Class II and wiped with 70% of ethanol. Then the cells were transferred into a centrifuge tube containing a 5 mL medium and were centrifuged at 200 g for 5 mins. After that, the supernatant was removed and the pellet form was suspended in a 5 mL medium and cultured in a T-25 flask. Then it was incubated at 37 °C in a 5% carbon dioxide incubator. The medium of the cell culture was then replaced every three days and the cells were

monitored every day to observe their growth and contamination.

Subculture of Cell Lines

The cells were observed daily under an inverted phase-contrast microscope to determine the confluence of the cells in the T-25 flask. Once the cells reached 80% confluence, the old medium was discarded from the flask and the cells were washed with phosphate buffer saline (PBS) to remove any remaining waste. After that, PBS was removed from the flask, and subsequently, Trypsin-EDTA (1X) was added to the T-25 flask and incubated at 27 °C for 10 mins to ensure the full detachment of the cells from the growth surface. After several mins, the complete medium was added into the flask and mixed gently for homogenization to deactivate trypsin. Next, the cells were transferred into a tube containing 10 mL of complete medium. The tube containing the medium was centrifuged at 1000 rpm for 5 mins. The cells were transferred into a completely fresh medium after removing the supernatant. The number of cells was calculated using a hemocytometer. Subsequently, as many as 6000 cells/well of breast cancer cells line T-47D were cultured in a 96-well plate in 100 µL of complete medium for each well. The plate was incubated in 5% CO₂ for 24 h before the treatment.

Cytotoxicity Assay

The cells were treated with various concentrations of *H. atra* extracts (0 µg/ml, 1.56 µg/ml, 3.12 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml and 50 µg/ml). Positive and negative controls were also used in this treatment. One percent (v/v) DMSO served as negative control and vincristine sulfate (10 µg/ml), a vinca alkaloid derived from *Catharanthus roseus*, served as the positive control. The cells were then incubated at 37 °C for 72 h in a 5% CO₂ incubator. Then 20 µl of MTS solution was pipetted into each well and incubated at 37 °C, 5% CO₂ for 3 to 4 h and six replicates were carried out for each concentration. The viability of cells was measured using a microplate reader (Glomax-Multi Detection System, Promega) at 490 nm. Then the percentage of cell viability was calculated using the following formula

$$\text{Percentage inhibition} = \frac{\text{Absorbance of cells treated with samples}}{\text{Absorbance of cells without sample treatment}} \times 100\%$$

Annexin V and Propidium Iodide assay

Annexin V and Propidium Iodide assays were used to detect the early stages of apoptosis. The cells were

seeded in a 96-well plate and incubated with various extract concentrations, *A. lecanora* (6.25 µg/mL), *H. atra* (7.5 µg/mL) and *S. vastus* (3.25 µg/mL) extracts and compared with vincristine sulfate (positive control, 0.25 µg/mL) for an approximate of 12-hour time gap starting from 0 h to 36 h. The medium in each well was removed and 20 µL of Annexin V and Propidium Iodide reagents were added. Cells were then incubated at 25 °C for 15 mins. Next, the cells were analyzed using fluorescence microscopy techniques with an excitation wavelength of 488 nm and a detection range of 540 nm.

LC-MS/MS Analysis of the Sea Cucumber Extracts

An Agilent 1200 series chromatograph (Agilent Technologies, Santa Clara, CA, USA) was used to perform the analysis, which was coupled to a Bruker Impact II QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). For chromatographic separation, a Zorbax Eclipse XDBC18 column (1.0x150 mm, 3.5 m, Agilent Technologies, Santa Clara, CA, USA) was employed with a Zorbax SBC8 guard column (2.1x12.5 mm, 5 m, Agilent Technologies, Santa Clara, CA, USA). One percent formic acid in H₂O (eluent A) and 1% formic acid in MeOH were used as mobile phases (eluent B). The gradient program was as follows: isocratic at 60% of eluent B from start to 3 min, from 60% to 90% eluent B from 3 to 29 min, from 90% to 100% eluent B from 29 to 30 min, isocratic at 100% of eluent B to 35 min, from 100% to 60% eluent B from 35 to 38 min. After returning to the initial conditions, the equilibration was achieved after 15 min. Chromatographic separation was performed at a 0.1 mL/min flow rate at 40 °C. The injection volume was 1 µL. Mass spectrometry detection has been performed using an ESI ionization source. The mass spectra were recorded within the m/z mass range of 100–1500 and 70–1500 for MS/MS spectra (scan time 1 s) (Popov et al., 2017).

DPPH Free Radical Scavenging Assay

The free radical scavenging activity of the extracts on DPPH radical was determined using the method. Test samples (*A. lecanora*, *H. atra*, *S. vastus*) and standard (ascorbic acid) were prepared in various concentrations (0.008, 0.016, 0.031, 0.063, 0.125, 0.250, 0.500 mg/mL) with serial dilution. Then, 0.1 mM DPPH (1.9715 mg in 50 ml methanol) was freshly prepared. Methanol was served as blank or control. In each well of the 96-well plate, 100 µL of sample and 100 µL of DPPH reagent were loaded and left in the dark at room temperature. After 30 min incubation,

the absorbance of the mixture was measured using a spectrophotometer (Perkin-Elmer, New Jersey) at 517 nm and measurements were made in triplicates.

The DPPH radical scavenging activity was calculated using the formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{[(\text{Abs control} - \text{Abs sample}) / (\text{Abs control})] \times 100}$$

Where:

- Abs control is the absorbance of DPPH radical + methanol
- Abs sample is the absorbance of DPPH radical + sample extract/standard

Total Flavonoid Content (TFC)

The total flavonoid content for all the samples was determined using the spectrophotometric method (Santhanam et al., 2013). Test samples (*A. lecanora*, *H. atra*, *S. vastus*) and standard (quercetin) were prepared in methanol with various concentrations (0.016, 0.031, 0.063, 0.125, 0.250, 0.500, 1.000 mg/mL) using serial dilution. Next, 2% of AlCl₃ solution (2 g of AlCl₃ in 100 mL methanol) was prepared. In each well of the 96-well plate, 100 µL of sample and reaction solution AlCl₃ were loaded and samples were made triplicate. Then, the reaction mixtures were incubated for an hour at room temperature. The absorbance was measured using a SpectraMax Plus microplate reader at λ_{max} 415 nm. Total flavonoid contents were determined using the quercetin standard curve and expressed as mg quercetin Equivalent (QE) per gram of dry plant extract (mg QE/g).

Total Phenolic Content

The total phenolic content for all the samples was determined using the Folin-Ciocalteu method (Santhanam et al., 2013). Briefly, 50 µl of extracts prepared in methanol with various concentration (0.016, 0.031, 0.063, 0.125, 0.250, 0.500, 1.000 mg/mL) was mixed with 50 µl distilled water, 50 µl of 10% Folin Ciocalteu's phenol reagent and 50 µl of 1 M sodium carbonate solution in a 96-well plate. Methanol was used as blank and gallic acid was used as standard. Reaction mixtures were incubated for an hour in the dark at room temperature. The absorbance of the reaction mixture was measured using a SpectraMax Plus microplate reader at λ_{max} 750 nm. Then, the total phenolic content was determined using the gallic acid, and the results are expressed as milligram Gallic Acid Equivalents (GAE) per gram of dry extracts. All tests were made in triplicates.

Gelatin Digestion Assay

The gelatin digestion assay was done according to previously described (Santhanam et al., 2018) with slight modifications. Agarose (2%) was prepared in collagenase buffer (50 mM Tris-HCl, 10 mM CaCl₂, 0.15 M NaCl, pH 7.8) and 0.15% porcine gelatin. Next, the mixture is poured into the Petri dish and allowed to solidify (about one h) at room temperature. After solidification, wells were made using a sterile 200 µl microtip. Then, an aliquot of 25 µL of the samples (0.25, 0.50 and 1.00 mg/ mL prepared in collagenase buffer) were incubated with 25 µL of bacterial collagenase-1 (0.1 mg/ mL) for 1 h. After incubation, the reaction mixture (50 µL) was loaded into the well and further incubated overnight. Epigallocatechin gallate (EGCG) was used as the positive control. The degree of gelatin digestion in agarose gel was visualized by Coomassie Brilliant Blue staining. The gelatinase inhibition activity was determined by measuring the area of the light translucent zone over a blue background formed after destaining. All tests were made in triplicates.

Statistical Analysis

Data was provided as mean ± SD. GraphPad Prism version 5 was used for statistical analysis, with a 95% confidence level * (t-test). The significant value was set at $p < 0.05$.

Results and Discussion

Cytotoxicity Effects of Sea Cucumber Extracts on Human Breast (T-47D) Cancer Cells

The present study evaluated the cytotoxic effects of the sea cucumber extracts such as *Actinopyga lecanora*, *Holothuria atra* and *Stichopus vastus* against Human Breast (T-47D) Cancer Cells. Cells were treated with various concentrations such as 0 µg/mL, 0.78 µg/mL, 1.56 µg/mL, 3.13 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL and 50 µg/mL of sea cucumber extracts. Six replicates were prepared for each concentration of the extracts. Table 1 shows the IC₅₀

Table 1. The IC₅₀ value of the sea cucumber extracts towards human breast (T-47D) cancer cells

Samples	IC ₅₀ (µg/mL)
<i>A. lecanora</i>	6.25 ± 0.50
<i>H. atra</i>	7.94 ± 1.39
<i>S. vastus</i>	3.16 ± 0.53

value of the samples against the Human Breast (T-47D) Cancer Cells.

A previous study reported that the ethanolic extract of *H. atra* was found to be significantly cytotoxic to the T-47D breast cancer cell line with an IC₅₀ value of 9.6 µg/mL. Triterpene glycosides such as cucumechinol and philinogenin B were the chemicals responsible for the bioactivity (Nursid et al., 2019). A similar pattern was observed in this study where the methanolic extract of *H. atra* induced cytotoxicity towards T-47D breast cancer cell line with an IC₅₀ value of 7.94 ± 1.39 µg/mL. The saponins, namely holothurin A5, holothurin A, echinoside A and 24-dehydroechinoside A, isolated from the *H. atra* - EtOAc/MeOH (1:1) extract were also reported to possess significant cytotoxicity towards human cervix carcinoma HeLa cell line with the IC₅₀ values ranging from 1.2 to 2.5 µg/mL (Grauso et al., 2019).

Studies on *S. vastus* also revealed that it has an appreciable cytotoxic effect towards MCF-7 cancer cell line with the IC₅₀ value of 65.14±5.59 µg/mL. Triterpene tetraglycosides isolated from the *Stichopus* was reported to possess significant cytotoxicity against MCF-7 breast cancer cell line with the IC₅₀ value ranges from 1.56 µM to 11.45 µM. In this research, the methanolic extract of *S. vastus* possessed appreciable cytotoxic towards T47D breast cancer cells with the IC₅₀ value of 3.16 ± 0.53 µg/mL. Furthermore, studies on the extracts of *Actinopyga* sp. reported that it has moderate cytotoxicity towards the T-47D cell lines with the LD₅₀ value of 87.55 µg/mL (Nurshid et al., 2016). Our study revealed that the methanolic extract of *A. lecanora* showed significant cytotoxicity towards T-47D cancer cell line with the IC₅₀ value of 6.25 µg/mL. Generally, it is clear that the methanolic extract of all three species, namely *A. lecanora*, *H. atra* and *S. vastus*, potentially inhibited the growth of T-47D breast cancer cell lines. As per the literatures, it could be suggested that the cytotoxic effect induced in the T-47D cancer cell line might be due to the presence of triterpene glycosides and saponins present in it (Vien et al., 2018). However, further research is needed to identify the chemical constituents and demonstrate their mode of action towards cancerous cell lines. This research highlighted the cytotoxic effect of three different sea cucumber extracts obtained from the same extraction techniques against T-47D breast cancer cell lines.

Determination of the mode of cell death

T-47D breast cancer cells were treated with the *A. lecanora* (6.25 µg/mL), *H. atra* (7.5µg/mL) and *S. vastus* (3.25 µg/mL) extracts and compared with vincristine sulfate (positive control, 0.25 µg/mL) and

DMSO (negative control) at different time intervals 0, 3, 6 and 24 h to determine the mode of action of cell death using Annexin V/Propidium iodide (PI) assay. The results are shown in Figure 2. Only dark images were seen at zero h since the dye was freshly added to the cell lines. After 3 h of treatment, green staining of Annexin V at the cells' cellular membrane was observed, indicating the inducement of apoptosis. At 6- and 24-h, a gradual increase of distinguishable membrane boundary was detected, indicating the early stage of apoptosis. Annexin is one of the family of calcium- dependent phospholipid binding proteins, which bind to phosphatidylserine (PS) to identify apoptotic cells (Lizarbe et al., 2013). PS is predominantly located along the cytosolic side of the plasma membrane. During the early initiation of apoptosis, PS loses its asymmetric distribution in the phospholipid bilayer and is exposed and translocated to the extracellular membrane, which is stainable by Annexin V and detectable with a fluorescent microscope with the green stained (Ahmed et al., 2015). During the late-stage apoptosis, loss of membrane integrity allows Annexin V to bind to the cytosolic PS and the cell to uptake the propidium iodide (PI) (Ahmed et al., 2015).

From Figure 2, it is clearly understood the cell treated with the extracts and positive control undergo early apoptosis with the green staining of Annexin V at the cellular membrane of the cells. All the treated extracts undergo early apoptosis rapidly within 24 h,

which is higher than the cells treated with the positive control. The results obtained are in accordance with the cytotoxic results. Nurshid et al. (2019) confirmed the apoptosis induction via caspase-3 activation in T-47D cells.

LC-MS/MS Analysis of the Extracts

The LC-MS/MS spectra and the Mass fragmentation obtained from the sea cucumber extracts were provided in the supplementary file. Chemical constituents such as 7-N, N-Dimethylamino-1,2,3,4,5-Pentathiocyclooctane, iophendylate, epibatidine and other unknown compounds were identified in the sea cucumber extracts (Popov et al., 2017). The data were interpreted using the MassHunter Qualitative Analysis-Metlin Database and compared with literature values

DPPH Free Radical Scavenging Activity

The antioxidant is a common medicinal property in plants yet rare in animals. Antioxidants help prevent or reduce cell damage caused by free radicals, which are unstable molecules produced by the body in response to environmental and other stresses (Kumar et al., 2017). They are also known as “free-radical scavengers”. Antioxidants can come from both natural and synthetic sources. DPPH or 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay is usually used to detect the antioxidant effect of an extract. The dark purplish color of the DPPH free radical would be

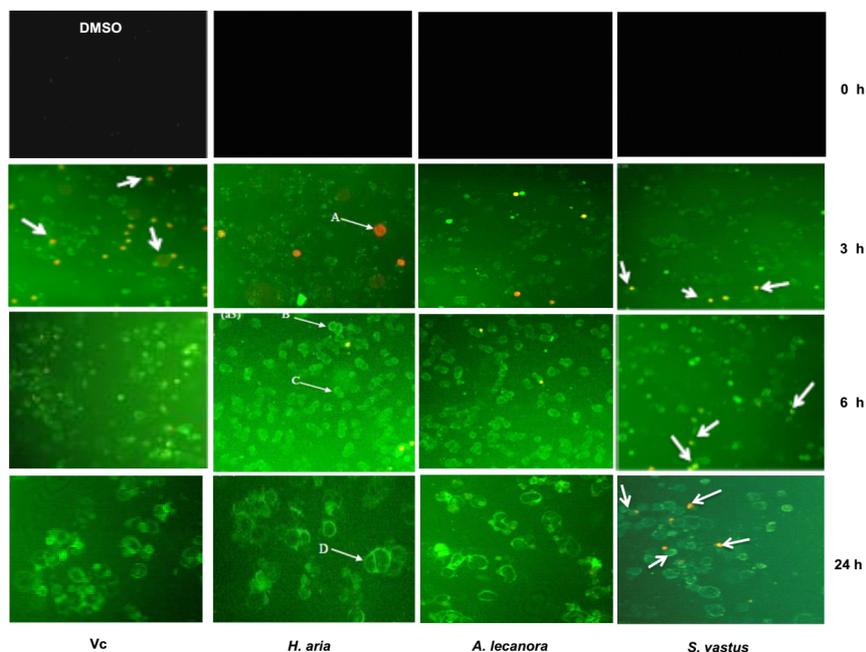


Figure 2. Annexin V and PI stainings of T-47D cells treated with sea cucumber extracts and positive control (Vc, vincristine sulfate). Images were taken within the time interval of 3, 6 and 24 h. This was done to detect the early apoptosis mode of cell death. The arrow pointed (A) indicated necrosis, while (B), (C) and (D) indicated that early apoptosis was induced.

reduced to yellow when it is scavenged by the antioxidants present in the extract (Dos Reis et al., 2018). Unfortunately, our extracts did not manage to reduce the purple color of DPPH, indicating no antioxidant activity of the extracts (*H. atra*, *S. vastus* and *A. lecanora*). Despite that, our result showed a negative value for the percentage of DPPH inhibition (Figure 3).

The negative value also suggested that our extracts may exhibit a pro-oxidative effect. Pro-oxidative indicates the poor effect of the extract by inducing more oxidative stress in the cellular environment. However, a previous study found that pro-oxidative natural products possess the ability as cancer chemopreventive, carcinogenic and chemotherapeutic agents (Martin-Cordero et al., 2012). It is well understood that pro-oxidant substances can generate carcinogenic consequences because they raise cellular levels of reactive oxygen species (ROS). Nevertheless, when pro-oxidant agents raise cellular levels of ROS to lethal levels, they may cause cancer cells to be selectively killed and so be therapeutically beneficial (Martin-Cordero et al., 2012). All these effects can be obtained by substances with both antioxidant and pro-oxidant capabilities, such as curcumin (Martin-Cordero et al., 2012). Substances such as curcumin can obtain all these effects with both antioxidant and pro-oxidant capabilities (Martin-Cordero et al., 2012). Even though the present data showed a negative value (up to 0.5 mg/mL), increasing the extract concentration was found to have some antioxidant activity with a low percentage of DPPH inhibition. A study from Esmat et al. (2013) coincided with our finding where they found small DPPH inhibition activity at a concentration of 0.6 mg/mL. The study found that *H. atra* sea cucumber mixed extract had a hepato-protective effect against liver fibrosis in rats. This outcome also suggests

that *H. atra* strongly possesses anticancer properties as it can reduce hepatic damage through the discarding of the ROS.

Total Flavonoid and Total Phenolic Content

Both flavonoid and phenolic compounds are known for their potent antioxidant properties (Sulaiman & Balachandran, 2012). Flavonoids are chemicals commonly found in plants responsible for their vibrant hues of colors together with carotenoids. With six different groups of flavonoids (anthocyanidin, flavanols, flavonols, flavones, flavonones, isoflavones), plant extract aid in cellular activity regulation and the battle against free radicals that cause oxidative stress in the body (Mustafa et al., 2010). This contributes to plants' antioxidant, anti-carcinogenic, anti-inflammatory, and anti-mutagenic properties (Panche et al., 2016). Due to this reason, flavonoids have become an indispensable ingredient applied in a variety of medicinal, nutraceutical, pharmaceutical, and also cosmetics (Panche et al., 2016). Kartikaningsih et al. (2018) stated that a flavonoid test was not done since it is a phytochemical presence in plants, whereas *H. atra* is an animal species. Little did we know, flavonoid phytochemicals can also be found in certain animal species (Agustina et al., 2021; Rapi et al., 2020). Other than that, phenolic compounds also found ubiquitously in plants exhibit potent antioxidant activity regulated through redox properties acting as reducing agents, singlet oxygen quenchers, hydrogen donors, and metal ion chelating agents (Gulcin, 2020; Kasote et al., 2015). All the available functions allow plants to survive in different extreme environments.

Interestingly, *H. atra* was found to possess phenolic compound in just a small amount while flavonoid compound is absent (Table 2). In *S. vastus* and *A. lecanora* extracts, both the phenolic and flavonoid compounds are absent. The standard calibration curve plotted to determine the total phenolic and flavonoid contents was shown in Figures 4 & 5, respectively.

As stated previously, both flavonoid and phenolic compounds are agents for discarding free radicals and avoiding plant stress. Just like plants, some animals have their antioxidants for species independence in a harsh or extreme environment. Sukmiwati et al. (2020) supported our study where their *H. atra* extract from Cerocok Beach, West Sumatera, Indonesia also presented a phenolic compound without flavonoid compound. However, another part of Indonesia was found absent with phenolic compound, but flavonoid was present in their *H. atra* extract from a different location at Benteng Inong, Aceh Besar, and Panjang Island, Jepara (Agustina et al., 2021; Sibero et al., 2019). Esmat et al. (2013) found both compounds

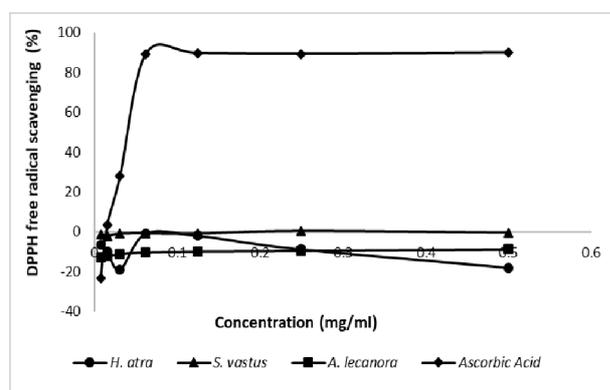


Figure 3. Percentage of DPPH inhibition of *H. atra*, *S. vastus* and *A. lecanora* extracts and ascorbic acid as standard. Ascorbic acid showed high DPPH scavenging activity, whereas *H. atra*, *S. vastus* and *A. lecanora* showed no antioxidant property. The data are expressed as mean \pm standard deviation ($n = 3$).

Table 2. Concentration of flavonoid and phenolic in *Sea cucumber* extracts

Samples	TPC (mg GAE/g)	TFC (mg QE/g)
<i>H. atra</i>	0.4794 ± 0.076 mg GAE/g	-
<i>S. vastus</i>	-	-
<i>A. lecanora</i>	-	-

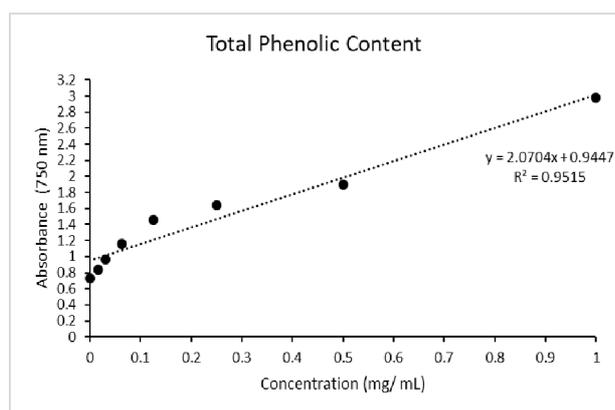


Figure 4. Standard calibration curve to determine the total phenolic content in samples (n=3).

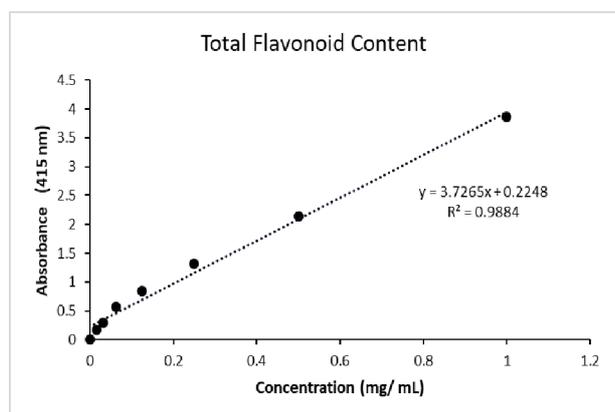


Figure 5. Standard calibration curve to determine the total flavonoid content in samples (n=3).

present in their *H. atra* extract originate from the Red Sea (Hurghada). The phytochemical content of *H. atra* extract was different due to its different origins where their environment and food access available were different, resulting in different medicinal properties of the same species from different places (Kartikaningsih et al., 2018). However, the presence of phenolic compound indicates that our extract might possess an anti-carcinogenic effect and can help inhibit cancer development. A substance with a phenolic compound is also known to have antioxidant properties. As stated before, pro-oxidant and antioxidant substances act as chemo-preventive and therapeutically beneficial to treat

cancer (Martin-Cordero et al., 2012). Since the other two extracts (*S. vastus* and *A. lecanora*) demonstrated the absence of phenolic and flavonoid content, it is evident that other classes of chemical constituents are present in the extracts, which could cause early apoptosis in the breast cancer (T-47D) cell lines.

Gelatin Digestion Assay

The gelatin digestion assay determines the ability of an organism to generate extracellular proteolytic enzymes (gelatinase) used to liquefy gelatin, a type of connective tissue found in vertebrates. The proteolytic enzyme hydrolyzes proteins into amino acids, which are necessary for homeostatic regulation in prokaryotes and eukaryotes (Kasana, 2010). The extracts were examined for gelatinase, inhibition. wherein nature matrix metalloproteinases (MMP-2 and -9) were some examples. MMPs in low concentration has a great function in proteolyze extracellular matrix component, membrane shedding, chemokine processing, and also regulate angiogenesis in wound healing (Castleberry et al., 2016; Löffek et al., 2011). However, high concentrations of MMP-2 and MMP-9 promote intravasation of tumor and neoangiogenesis that form leaky blood vessel that can lead to metastasis of cancer (Adhikari et al., 2020). This condition stipulated that the high value of MMP-2 and MMP-9 is a precursor for cancer and a contributor to a malignant tumor (Adhikari et al., 2020). Captivatingly, our extracts showed inhibition against bacterial collagenase, indicating the protective function against extracellular proteolytic enzyme (Figure 6).

Interestingly, all data showed significant differences against the control showing that our extracts had inhibition activity for gelatin digestion. This in part displayed the anticancer effect of *H. atra*, *S. vastus* and *A. lecanora*. A higher extract concentration showed a lower diameter of the digestion zone, indicating higher inhibition of bacterial collagenase activity. Based on the similar previous study, our study where a higher extract concentration had higher inhibition activity against the enzyme (Prabhu, 2021).

Conclusion

All of the three sea cucumber extracts (*A. lecanora*, *H. atra*, and *S. vastus*) actively inhibit (T-47D) cancer cell growth progression by inducing apoptosis in the cell. The anticancer effect of the extract is might be due to the active chemical constituents such as saponins, triterpene glycosides other than the phenolic/flavonoid compounds. Anti-collagenase activity results supported the protective effect of the extracts against

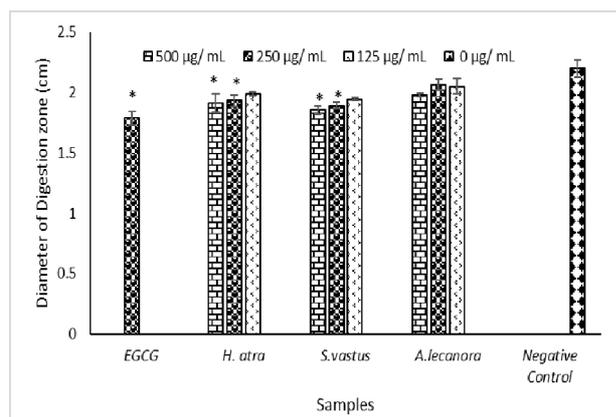


Figure 6: Diameter of digestion showed positive control (250 µg/mL of epigallocatechin gallate) had the lowest digested gelatin, followed by the highest concentration of *H. atra*, *S. vastus* and *A. lecanora* extracts (500, 250, and 125 µg/mL) and negative control showed the highest diameter of digestion zone. Significant different, * (t-test) compared to negative control $p < 0.05$. The data are expressed as mean \pm standard deviation (n = 3).

the cancer cell. Although the results seem promising, but specific mechanism of action of the extracts still unknown and need to be explored further.

Acknowledgement

We would like to thank the researchers from IMB Dr Jasnizat Saidin, Nur Asniza Aziz and Kamaria.h Bakar for helping us with the sample collection.

Supplementary Material

Supplementary material is not available for this article.

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