Exploration of Sea Cucumbers *Stichopus hermanii* from Karimunjawa Islands as Production of Marine Biological Resources

Delianis Pringgenies¹, Siti Rudiyanti² and Ervia Yudiati¹

¹ Dept of Marine Sciences, Faculty of Fisheries and Marine Sciences Diponegoro University-Semarang, Indonesia
² Dept of Fisheries, Faculty of Fisheries and Marine Sciences Diponegoro University-Semarang, Indonesia

Email: pringgenies@undip.ac.id; pringgenies@yahoo.com

Abstract. This research aim was to study the potential of *Stichopus hermanii* to determine the amino acid, chondroitin, and glucosamine contents, to discover its antibacterial and anti-cancer agent. The samples were rinsed prior to separation, with only the corpus being used in the study. Sea cucumber extract was then processed using HPLC to trace contents of amino acid, chondroitin, and glucosamine contents. The samples were then put into test against several strains of pathogenic bacteria by means of diffusion for any biological activity. The anti-cancer test was performed by human ovarian cancer cell line (KOC7C) method. The study showed that the extract of *Stichopus hermanii* has the potency to inhibit the growth of active ovarian cancer cells. The qualitative test of the sea cucumber extract showed that it is capable of suppressing the growth of several strains of pathogenic bacteria identified as *Staphylococcus aureus*, *Escherichia coli*, *Vibrio voitivica*, and *Pseudomonas* sp. HPLC results showed that the extract contained amino acid (mg/100g), the highest being Collagen (11200), followed by Glycine (3760), Glutamic Acid (3700), Aspartic Acid (2540), Alanine (2140), Proline (2050), Arginine (2050), Tyrosine (1430), Threonine (1270), Leucine (1170), Valine (1050), Serine (971), Isoleucine (816), Phenylalanine (713), Lysine (639), Methionine (383), Cystine (263) and Histidine (208). The extract also contained Chondroitin Sulfate (4200) and Glucosamine Hydrochloride (<5.00). In conclusion, the study found that the extract of *Stichopus hermanii* has potential as anti-cancer, particularly against ovarian cancer, with the highest content being Collagen within amino acids, as well as chondroitin and glucosamine.

Key words: *Stichopus hermanii*, amino acids, chondroitin, glucosamine, anti bacteria, anti cancer

1. Introduction

*Stichopus hermanii* is a species of sea cucumber classified as marine invertebrate from the Echinodermata phylum. The morphology of this species is of elongated, cylindrical and soft body. Most sea cucumbers are nocturnal in nature/active during the night time and are inactive or in hiding during...
the daytime [1]. This caused fishermen to seek out sea cucumber catch during the night, as hauls during the day have been proven to yield less results. *Stichopus hermanii*, locally known in Indonesia as *teripang emas* (viz: golden sea cucumber), is classified as a deposit feeder species and a sedentary marine life whose natural habitat are hard surfaces or rocks, often feeding off prey on the seabed with its tentacles [1].

*Stichopus hermanii* mainly feed on subsists on small organisms, detritus (decomposed organic materials), diatoms, protozoans, nematodes, filamentous algae, copepods, ostracods and seaweeds. Also included in the diet of this species are radiolarians, foraminifera, sand/coral debris particles, and shells of various marine animals [2].

In several countries in Asia and Russia, sea cucumbers are exploited for traditional medicines to treat wound and a number of diseases. In Malaysia, coastal and fisherman communities use lotion or other salves made of sea cucumber as topical medication for wound treatment, known locally as *gamat* [3].

Information on the medical potency of extracts from various species of sea cucumber shows efficacy in their use in antimicrobial, antifungal, anti-tumor, anti-oxidant, anti-proliferation, wound recovery, immuno-modulator, anticoagulant, and anti-thrombotic purposes. Several researches on the sea cucumber extract also show its potency in controlling excessive cholesterol levels and blood fat contents, in addition to being effective antivirus, anti-malaria and anti-arthritis agents [4-6] [3].

Based on the field survey performed in the waters of Karimunjawa islands on the sea cucumber from the *Stichopus* family, one characteristic of the species could be readily observed, namely its body integrity. During the collection of the samples, the sea cucumbers were observed to be solid, rigid frame. However, when they were taken out of the water, the body of the sea cucumbers would quickly soften and its body walls would drip. When the taken samples were returned into the coast, their bodies would stop disintegrating and they showed rapid body structure regeneration rate. Therefore, sea cucumbers of the *Stichopus* family in the survey shows rapid body cellular regeneration rate, tissue restoration, and possible anti-body or immune system formation as well as enzyme and hormonal balancing. Based on the observation, this study aims to discover the contents of secondary metabolites in sea cucumber of the *Stichopus hermanii* species which acts as potential antimicrobial and anti-cancer as well as to discover the contents of amino acids, chondroitin and glucosamine.

2. Methodology

2.1. Sample Collection

Sea cucumber samples were collected from the waters of Karimunjawa islands, Indonesia. The samples were stored in a cool box to prevent degradation and were transported to Laboratory of Diponegoro University, Semarang. The samples were prepared by intestinal organ removal, ensuring that only the meat remains, which were then diced into 1 cm size. The diced samples were dried under indirect sunlight, by covering the container with black fabric during the day. During the night, sample drying process was carried out by exposure to 5-watt light bulbs. The samples were dried for 5 consecutive days.

2.2 Sample Extraction

Sample extraction was carried out using the methodology in Montano and Glorioso (1994). Each prepared sample was cut into 3-10 cm pieces. The extraction process was accomplished by dissolving 200 grams of prepared sample in 1000 mL of methanol soluent. The filtration result was extracted from the soluent on a rotary evaporator.

2.3 Phytochemical Screening

Phytochemical screening was performed on the sea cucumber extract to determine the contents of secondary metabolites. The Saponin screening is performed by shaking 10 mL of filtrate in reaction tubes for 5 seconds, after which observation for changes in pattern. Formation of bubbles after the
addition of diluted HCl shows positive reaction in saponin screening. Tannin screening was performed by adding 0.5 grams of samples into 5 drops of FeCl₃ 1 %. Dark blue or greenish black coloration signifies positive result in tannin screening. Flavonoid screening is performed by adding 1 mL of extract into 1 gram of sample and 190 mL of concentrated HCl. Red coloration identifies flavonoid content. Alkaloid screening is performed by shaking 1 mL of sample with 10 drops of H₂SO₄, after which the acidic layer is separated into a different reaction tube. The sulfuric acid layer is tested on a test plate with Dragendorf catalyst. Positive result is signified by reddish orange coloration. Terpenoid and Steroid Screening is performed by adding drops of ascetic anhydride or one drop of sulfuric acid H₂SO₄. Red coloration confirms terpenoid content, whereas blue coloration signifies steroid content.

2.4 Analysis of The Type and Contents of Amino Acids in Sea Cucumber Samples Using High Performance Liquid Chromatography

The samples in this amino acid analysis were the extraction of sea cucumber from the *Stichopus hermanii* species. Prior to High Performance Liquid Chromatography (HPLC), the samples were prepared by protein levels analysis by Kjeldahl method (LC 20AD, Shimadzu, Kyoto, Japan). This was followed by acid and base hydrolysis process on the sample. The results of hydrolysis process would then be ready for analysis using standard-procedure HPLC. This analysis essentially involves converting protein into amino acids in order to be detected by the chromatograph. The detector detects the amino acids, after which the results are saved by the recorder. The computer will then display the recorded data in retention time and area graphs with peaks corresponding with the characteristic of each amino acid.

2.5 Antimicrobial Screening

The antimicrobial screening of sea cucumber extract was performed against multi-drug resistant test bacteria from the species *Staphylococcus aureus*, *Escherisia coli*, *Vibrio anguila*, *V. voinivica*, *Bacillus subtilis*, and *Pseudomonas sp* provided from Kariyadi Hospital Semarang, Indonesia. Each paper disc was prepared by adding 10 µL drop of methanol. Observation was then made on the paper disc showing signs of antimicrobial activity, upon which said paper disc would be marked positive[7].

2.6 Anti-cancer Cell Screening

Cancer cells were incultured at 3000 cells per 96-well microplate and pre-incubated. Cancer cell: human ovarian cancer cell line (KOC7C) obtain from Laboratory of Natural Medicinal Chemistry, Kobe Pharmaceutical University Japan. After 24 hours, they were treated without or with test samples and then incubated. After 72 hours, WST-8 reagent was added to each and the cultures were incubated further for 1 hour. The relative viable cell number was determined by measuring the absorbance at 450 nm (Ref. 630 nm). The IC50 value, which reduce the viable cell number by 50 %, was determined.

Cells were incubated with SAB E extracts for two hours before being collected and washed three times with PBS prior to addition of 0.9 ml staining solutions and mixing using vortex. Cells suspension was incubated for 10 minutes in ice prior to addition of 0.1 ml 1.5 M NaCl. Samples were analyzed by flow cytometry. This assay was carried out in duplicate. Staining solution for 10-11 samples consisted of 4.5 ml Propidium iodide stock solution (55.6 mg/500 ml DDW), 1 ml Na-citrate stock solution (0.588/50 ml DDW), 0.45 ml RNAse (10 mg ml⁻¹), 0.1 ml 10% Triton X-100, 3.95 ml DDW.

3. Results and Discussion

3.1 Results

3.1.1 Phytochemical Screening

The screening results showed positive on the saponin, tannin, flavonoid, terpenoid and steroid in the samples. However, no alkaloid content was detected.
3.1.2 Antimicrobial Screening

The sea cucumber extract showed positive activity against pathogenic bacteria from the species *Escherisia coli*, *Pseudomonas* sp., *V. voinivica* and *Staphylococcus aureus* as seen in Table 1 below.

| Sea Cucumber Species | Test Bacteria Species |
|----------------------|----------------------|
|                      | *S. aureus* | *E. coli* | *Pseudomonas* sp | *V. voinivica* | *V. anguila* | *B. substilis* |
| *S. hermanii*        | +          | +         | +              | +             | -           | -             |

3.1.3 Anti-cancer screening

Preparation of Sample for Growth Inhibitory Test against Ovarian Cancer Cells (1)

Anti-cancer test used is the ovarian cancer cell K0c7c. Test results the effect of the concentration of sea cucumber extract, unfiltered and filtered on viability shows that Ic50 between sea cucumber extract, unfiltered (Ic50 : 26.6 µg/mL) and filtered (Ic50 : 24.38 µg/mL) showed that almost the same results as seen in the picture below (Figure. 1)

![Figure 1](image)

**Figure 1.** Test the effect of the concentration of sea cucumber extract, unfiltered and filtered on Viability

Test cell cancer cell (K0c7c line) to extract the results showed that the extract of sea cucumber sea cucumber *Stichopus hermanii* against ovarian cancer cell active as shown in Figure 2.
3.1.4 Amino Acid Contents

HPLC analysis of the sea cucumber extracts revealed, from the highest to the lowest concentration, the following amino acids; Collagen (11200), Glycine (37600), Glutamic Acid (3700), Aspartic Acid (2540), Alanine (2140), Proline (2050), Arginine (2050), Arginine (2050), Tyrosine (Tyrosine), Threonine (1270), Leucine (1170), Valine (1050), Serine (971), Isoleucine (816), Phenylalanine (713), Lysine (639), Methionine (383), Cystine (263) and Histidine (208). The analysis also found Chondroitin Sulfate (4.200) and Glucosamine Hydrochloride (<5.00). Figure 3 showed the findings above in graphic.

Figure 2. Test results with ovarian cancer cell method (K0c7c line) against the sea cucumber extract of *Stichopus hermanii*

Figure 3. Amino acid contents of *Stichopus hermanii* extract

3.2 Discussion
The results of antimicrobial activity test of *Stichopus hermanii* extract showed positive activity in inhibiting the growth of test bacteria *Escherisia coli, Pseudomonas* sp., *V. voinivica* and *Staphylococcus aureus*. The saponin content of the extract was thought to be the main component in this antimicrobial properties. Phytochemical screening of *Stichopus hermanii* extract confirmed that saponin is one of the secondary metabolite contents of the extract, along with tannin, flavonoids, terpenoids and steroids. On the other hand, no alkaloid content was found in the extract. Saponin is a chemical compound with bioactive properties[8]. The first saponin to be found in sea cucumber is categorized as holothurin, signifying a wide spectrum in physiological activity [9]. The saponin was found on the skin, in the flesh and in the gut (tubula cuverian) of Bohadschia sp[10]. Saponin is used by sea cucumbers in high concentration as a self-defense mechanism against predators. Saponin can be used as an antimicrobial compound. The pure sea cucumber extract tend to contain holotoxin, of which effect was found to be similar to that of antymycin in 6,25-25 μg/mL dose[11].

The antimicrobial sensitivity test on the sample of *Bohadschia mamorata* and *B. argus* against *Staphylococcus aureus, Escherichia coli, Vibrio anguila, V. voinivica, Bacillus subtilis, Pseudomonas* sp test bacteria resulted in positive activities[8]. Sample of the Stichopus variegatus sea cucumber was found to inhibit the growth of *Escherichia coli, Pseudomonas* sp., *V. voinivica* pathogenic bacteria [8]. Antimicrobial test of *Stichopus chloronatus* and *Stichopus hermanii* found that both sea cucumber sample were tested positive against *Staphylococcus aureus, Escherichia coli, Vibrio anguila, V. voinivica* pathogenic bacteria.

Cancer cell line test (K0c7c line) of *Stichopus hermanii* extract showed positive activity against the growth of ovarian cancer cells. Saponin is thought to be the key compound in the cancer-inhibiting properties of the sea cucumber extract. Saponin is categorizad as a glycoside, consisting of sapogenin (*steroid; C27*) or triterpenoid (*C30*) group, hexose or pentose group, or uronic acid[12]. These compounds have found application in medical industry as a base in steriodal medicine synthesis. The saponin also has detergent component which causes membrane disintegration, instigating hemolysis of erythrocyt[13]. Saponin has bitter taste, cause bubble in water, toxic for cold-blooded animals and shows anti-exudative and anti-inflammatory properties. Known saponin compounds other than holothurin includes holotoxin, stichoposide and thelothurin, all of which are isolated from sea cucumbers[9]. Another derivative compound with bacteria growth-inhibiting properties is lectin. Mitogenic of nature, this class of protein can develop quickly and be used as an antimicrobial agent. Lectin agglutinates lymphoid cells. Due to its potency in killing would-be predators, sea cucumber toxins are have now been developed as anti-inflammatory and anti-cancer natural antisceptic[14].

Coombs [14] described lectin as a class of proteins which bind glycoproteins on cell surface, inducing agglutination. Lectin showed potency in inhibiting the growth of muscle and lungs cancer cell[11]. This cellular agglutinating properties of lectin in sea cucumbers is thought to explain the fact that sea cucumbers can recover their natural physiology after being taken out from sea water for some time.

Analysis of amino acids contents of sea cucumber extract by HPLC discovered 18 kinds of amino acids. These amino acids, from the highest content to the lowest content (mg/100g), were Collagen (11200), Glycine (37600), Glutamic Acid (3700), Aspartic Acid (2540), Alanine (2140), Proline (2050), Arginine (2050), Arginine (2050), Tyrosine (Tyrosine), Threonine (1270), Leucine (1170), Valine (1050), Serine (971), Isoleucine (816), Phenylalanine (713), Lysine (639), Methionine (383), Cystine (263) and Histidine (208)

Amino acids are the building blocks of proteins. Protein is vital for the body in that it helps in maintaining tissues, in forming other amino acids, in synthesizing various hormones, in neurotransmitter production for the brain and other neural functions, in maintaining body immunity system, in maintaining liquid balance and as a source of energy. A gram of protein can be converted into 4 calories worth of energy[15]. Of all 20 amino acids which form the protein, of them cannot be synthesized by the human body and can only be obtained from nutritional intake. These amino acids are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane dan valine, otherwise known as the essential amino acids. Other than the essential amino acids, there are the conditionally essential amino
acids, which include histidine and arginine. Isoleucine plays a vital role in erythrocyte formation. Leucine prevents muscle degradation. Methionine acts as a precursor of cysteine and creatine, lowers blood cholesterol level, helps liver in detoxification, and regenerates tissue in liver and kidneys. Threonine acts as a detoxifier and helps prevent the accumulation of fat in liver. Tryptophan is an essential material in producing serotonin (a hormone involved in relaxation) and stimulates the release of growth hormone. Valine plays a role in transporting other amino acids, such as tryptophan, phenylalanine, and tyrosine to the brain. Arginine is an important ingredient in creatine formation, and stimulates the production of growth hormones. Histidine is vital in the formation of erythrocyte and leucocyte. Lysine assists in the process of collagen formation as well as in the regeneration of other connective tissues in the body (cartilage and joints). Content analysis of sea cucumber extract showed that chondroitin was the highest (4.200) after collagen (11200) and glycine (3760). As chondroitin is vital for bone formation, it is very useful for those suffering from or vulnerable to osteoporosis, such as mature women, and during formative years of children and teenagers.

4. Conclusion

Based on the findings of this study, it can be concluded that,
1. Sea cucumber has saponin, tannin, flavonoid, terpenoid and steroid content. However, alkaloid content was not found in sea cucumber.
2. Stichopus hermanni sea cucumber extract showed antimicrobial activity in inhibiting the growth of pathogenic bacteria from the species *Escherisia coli*, *Pseudomonas* sp., *V.voinivica* and *Staphylococcus aureus*.
3. The highest content found in sea cucumber (mg/100gr) was collagen (11200), chondroitin (4.200), Glycine (3760), and Glutamic acid (3700).

Acknowledgements

The author would like to thank PT Kalbe Farma tbk for their support in the sample analysis. The author also extends her deepest gratitude to Dr. Nishiyama Yumi and Dr. Shitan Nobukazu of Laboratory of Natural Medicinal Chemistry, Kobe Pharmaceutical University, Japan for their cooperation in the research.

References

[1] Barnes, R. D. 1991. Invertebrata Zooology.ed Sounders College Publishing USA.P : 966 - 975.
[2] Martoyo, J. 1994. Systematic Animals: Invertebrates and Vertebrata For Universities. Second Edition. Publisher Sinar Wijaya . Surabaya: 322 – 333 hlm
[3] Farouk A.E., Ghouse F.A.H., Ridzwan B.H , 2007 *American Journal of Biochemistry and Biotecnology* 3 (2): 60
[4] Abraham TJ, Nagarajan J, Shanmugam SA, 2002 *Indian Journal of Marine Sciences* 31 (2) : 161-164
[5] Althunibat OY, Hashim RB, Taher M, Daud JM, Ikeda MA, Zali BI 2009 *European Journal of Scientific Research* 37 (3) : 376-387
[6] Avilov et al. 2007. Process for isolating sea cucumber saponin frondoside A and immunomodulatory methods of use. United States Patent no. : US 7,163, 702 B1. Available at http://www.freepatentsonline.com/7163702.pdf, accessed November 2010.
[7] Mojica E R, E RJ Layson, MCA Rodil and CC Deocaris 2007 Marine Invertebrates As Source of Potential Antitumor and Antibacterial Agents. http://www.pemrmd.dost.gov.ph/zone2/papers/8th/mojica1.pdf (25 November 2007).
[8] Pringgenies 2013 Antibacterial Activity of Sea Cucumbers Harvested From Karimunjawa. *Squalen, Bulletin of Marine and Fisheries Postharvest and Biotechnology*. 8 (2): 87-94

[9] Hashimoto, Yoshiro 1979 *Marine Toxins and Other Bioactive Marine Metabolites* (Tokyo: Japan Scientific Societies Press) 268-270 pp

[10] Jayasree, V., R. Sen Gupta and P. V. Bhavanarayana. 1991. Bioactive Compound from Marine Organisms, a Toxin from *Holothuria leucospilota*. An Indo-United States Symposium. A.A. Balkema. Rotterdam. 111-120.

[11] Martoyo, Joko, Nugroho Aji dan Tjahjo Winanto. 2006. *Budidaya Teripang*. (Jakarta: Penebar Swadaya) p 75

[12] Winarno, F.G. 1982. *Kimia Pangan dan Gizi*. Gramedia Pustaka Utama. Jakarta. 96 hlm.

[13] Coombs, J. 2007. *Dictionary of Biotechnology*. Oxford. Amsterdam. 330 hlm.

[14] Ayu. 2007. Teripang, Potensi di Dasar Samudra Indonesia. http://www.suaramerdeka/ap_2007 (10 Agustus 2007). 2 hlm.

[15] Schlenker, E.D. & Long Roth, S. (2011). *William’s Essentials Of Nutrition And Diet Therapy*. (St. Louis, Missouri.: Elsevier)