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The Latest Research on 3 (Three) Types of Sea Cucumber Extract from Tegal Mas Island, Lampung

(Penelitian Terbaru terhadap 3 (Tiga) Jenis Ekstrak Teripang di Pulau Tegal Mas, Lampung)

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Abstract: Sea cucumbers are deep-sea-dwelling invertebrate animals that are rich in nutrients and have been empirically used to prevent various degenerative diseases, especially due to their ability to dampen free radicals. The purpose of this study was to identify, and determine antioxidant activity, total phenolic content, and toxic properties in extracts of several sea cucumber species originating from Tegal Mas Island, Lampung. The identification results of the three sea cucumber species were *Stichopus vastus* Sluiter (SV), *Stichopus monotuberculatus* Quay & Gaimard (SM), and *Stichopus quadrifasciatus* Massin. (SQ). The extraction was performed by kinetic maceration against all parts of the sea cucumber using a 70% ethanol solvent. Antioxidant activity test using the DPPH (2,2-diphenyl-picrilhydrazyl) free radical suppression method and determination of the total phenolic content using the Folin-Ciocalteu method. The results showed that SV had the highest total phenolics and antioxidants with the lowest toxicity. However, optimisation of extraction between wet and dry sea cucumbers is still required. Extraction optimisation with various solvents can also be done to gain secondary metabolites more effectively.

Keywords : Antioxidant, BSLT, DPPH, sea cucumbers, total phenolic content.

Abstrak: Teripang merupakan hewan invertebrata penghuni laut dalam yang kaya akan nutrien dan sudah secara empirik digunakan untuk mencegah berbagai penyakit degeneratif. Teripang berpotensi untuk dikembangkan dalam bidang kesehatan, terutama dalam kemampuannya untuk meredam radikal bebas. Tujuan dari penelitian ini yaitu untuk mengidentifikasi, menentukan aktivitas antioksidan, kandungan total fenol, dan sifat toksisitas pada ekstrak beberapa spesies teripang yang berasal dari Perairan Tegal Mas, Lampung. Hasil identifikasi dari ketiga spesies teripang adalah *Stichopus vastus* Sluiter (SV), *Stichopus monotuberculatus* Quay & Gaimard (SM), dan *Stichopus quadrifasciatus* Massin (SQ). Ekstraksi dilakukan dengan teknik maserasi kinetik terhadap semua bagian teripang menggunakan pelarut etanol 70%. Uji aktivitas antioksidan menggunakan metode peredaman radikal bebas DPPH (2,2-difenil-pikrilhidrazil). Penentuan total fenolik menggunakan metode Folin-Ciocalteu. Uji toksisitas dilakukan menggunakan metode *Brine Shrimp Lethality Test* (BSLT). Hasil menunjukkan bahwa SV memiliki total fenolik dan antioksidan tertinggi dengan toksisitas yang terendah. Namun demikian, diperlukan optimasi ekstrasi antara teripang basah maupun kering serta variasi pelarut untuk mendapatkan metabolit sekunder lebih efektif.

Kata kunci: Antioksidan, BSLT, DPPH, teripang, total fenolik.

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INTRODUCTION

FREE RADICALS are highly reactive compounds that oxidise other molecules, resulting in damage to lipids, proteins, DNA, and cell membranes. Those damages could trigger degenerative diseases such as cancer, atherosclerosis, diabetes, and hypertension(1). Free radicals in the body emphasise the importance of a substance that can scavenge free radicals, which is an antioxidant. Antioxidants donate one of their electrons to free radicals, resulting in the inactivation and inhibition of free radicals(2).

Based on the sources, there were natural and synthetic antioxidants, yet some of the synthetic antioxidants were carcinogenic, leading to gene mutations and cancer. Natural antioxidants can be obtained from land or marine organisms. Sea cucumber was one of marine product that has antioxidant activity. Some commercial sea cucumbers that have been well known for their benefits were *Holothuria scabra*, *Holothuria edulis*, *Holothuria vacabunda*, *Holothuria vatiensis* and *Holothuria marmorata*(3).

Sea cucumber contents were scientifically proven to scavenge free radicals and then degenerative diseases. Compounds that play a role were triterpene glycosides (saponin), chondroitin sulphate, glycosaminoglycans (GAGs), phenolics, and essential amino acid(4). Previous studies reported that *Stichopus hermanii* was potentially an antioxidant due to its IC_{50} of 65.08 ppm(5). Another sea cucumber, *Holothuria scabra* has antioxidant activity, with an IC_{50} of 33.77±0.24 mg/mL(6).

Research that has been done before proves that sea cucumbers have the potential to be antioxidant agents. Besides that, the antioxidant activity of non-commercial sea cucumber in Tegal Mas Sea Waters, Lampung hasn't been widely researched. Tegal Mas Sea waters, Lampung, Indonesia, were one of the sea cucumber resources(7). An effort to optimise the utilisation of Indonesian marine natural resources was to assess the antioxidant activity of several sea cucumbers found in Lampung. Therefore, this research needs to be done. This research needs to be conducted to identify, determine the antioxidant activity, total phenolic content, and toxicity of selected sea cucumber extracts from Tegal Mas Sea waters, Lampung.

MATERIALS AND METHODS

MATERIALS. *S. vastus* Sluiter (SV), *S. monotuberculatus* Quay & Gaimard (SM), and *S. quadrifasciatus* Massin (SQ) sourced from sea at Tegal Mas, Pesawaran, Lampung, 5-15 meters in depth (104.92º–105.34º East Longitude, and 5.12º–5.84º South Latitude, Figure 1). Sea cucumbers were harvested in June 2021. It was harvested at night when sea cucumbers were active, preferably when the moon rises for easy harvesting. SV, SM, and SQ were determined to ensure its veracity. Determination was conducted at Indonesian Institute of Sciences (LIPI) Oceanography Jakarta.

Figure 1. Tegal Mas Island location.

Chemical and Reagent. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), DMSO 1%, sodium sulphate anhydrate, magnesium powder and H_2SO_4 were purchased from Sigma-Aldrich, Missouri, USA. Methanol for analysis, Folin-Ciocalteu reagent, sodium carbonate, ethyl acetate, acetic acid, chloroform, acetic anhydrate, Mayer reagent, NaOH, KOH solution, amyl alcohol, ammonia, nutrient agar, $FeCl_3$ and potato dextrose agar were purchased from Merck KGaA, Darmstadt, Germany. Ascorbic acid (ASEAN Reference Standard, Jakarta, Indonesia), distilled water, ethanol 96% (Mallinckrodt Chemicals, Dublin, Ireland), gallic acid, HCl, Dragendorff reagent, Stiasny reagent, *Artemia salina* Leach larvae, synthetic sea water and phosphate buffer.

Equipments. Spectrophotometer UV-visible (Shimadzu UV-1800, Shimadzu Access Corp., Japan), analytical balance (KERN: ABS 220-4, KERN & SOHN GmbH, Germany), No. 4 and No. 18 mesh, waterbath, microbalance (Mettler Toledo Balance XPR2U, PT. Mettler-Toledo, Indonesia), UV cabinet, *Artemia salina* Leach incubator, TL lamp 18W, rotary vacuum evaporator (Hei-VAP Core, Heidolph Instruments GmbH & Co. KG, Germany), incubator (Memmert UM300, Memmert USA, LLC., USA), macerator.

METHODS. Sea Cucumber Extraction. Sea cucumbers were washed, gutted out, and then sun-dried. Then dried sea cucumbers were grinded and turned into powder with a fine degree of 4/18 as required on *Materia Medika* Indonesia (MMI). Powder was macerated three times using ethanol 70%, and concentrated using vacuum rotavapor at 40 °C, 175 mmHg, and 60 RPM.

Phytochemical Screening. Sea cucumber chemical metabolites were identified based on Phytochemical Screening Farnsworth, including alkaloid, flavonoid, saponin, tannin, quinone, steroid/triterpenoid, coumarin, and volatile oil qualitative analysis[8].

Antioxidant Activity Assay. The DPPH method was used at extract concentration of 50, 100, 150, 200, and 250 ppm. Ascorbic acid was used as a positive control in concentrations 2, 4, 6, 8, and 10 ppm[9]. The solution was then analysed using a spectrophotometer UV-Vis at 516 nm. IC_{50} (inhibition concentration) was obtained by a linear regression equation. The smaller IC_{50} shows higher antioxidant activity[10]. %Inhibition was calculated with equation:[11]

$$Q = \frac{A_0 - A_1}{A_0} \times 100\%$$

Note : Q = Inhibition (%); A_0= DPPH absorbance; A_1 = DPPH + extract absorbance.

Total Phenolic Content (TPC) Assay. Gallic acid was used as a standard solution with a concentration of 2 to 10 ppm. Each of them was added 5 mL of aquadest and 0.5 mL of Folin-Ciocalteu 50% reagent ($^v/_v$). After 5 minutes, solution was added with 1 mL of analytical Na_2CO_3 7.5% then homogenized and incubated in dark conditions for 60 minutes. Absorbance was measured with spectrophotometer UV-Visible at 775 nm. Subsequently, each sea cucumber ethanol extract (10 mg) was treated the same as the standard. TPC was expressed as Gallic Acid Equivalent (GAE)[12].

$$TPC = \frac{X\,x\,Va\,x\,df}{Wu}$$

Note: X = Concentration (μg/mL); Va = Final volume (mL); Fp = Dilution factor; Wu = Sample weight (g).

Brine Shrimp Lethality (BSLT) Toxicity Assay[13–15]**.** *Artemia salina* eggs were hatched in synthetic sea water (38 g NaCl in 1000 mL water) below TL lamp 18W. 48 hours passed, eggs were hatched into nauplii and ready to be used as animal tests. *Artemia salina* larvae were put into vials containing extract solutions (50, 100, 200, 500, and 1000 ppm) in 3 repetitions. Larvae were then incubated at room temperature for 24 hours below TL lamp 18W.

After 24 hours, died *Artemia salina* larvae were observed on each concentration. The LC_{50} of extract was analysed in μg/mL or ppm using probit analysis with confidence value 95%.

RESULTS AND DISCUSSION

Animal Determination. Determination results at the Indonesian Institute of Oceanography, Jakarta, showed that the sea cucumbers used were *Stichopus vastus* Sluiter (SV)*, Stichopus monotuberculatus* Quay & Gaimard (SM)*,* and *Stichopus quadrifasciatus* Massin (SQ) (*Stichopodidae*). The determination results can be seen in Figure 2. SV were identified with a brownish-black, white-spotted body. SM tends to have less visible spotting with a yellow-black body. SQ were more red-brownish, white-spotted body.

The biodiversity of sea cucumbers on Tegal Mas Island was extensive. The determination was aimed at knowing the specific species of each sea cucumber. It also decides which species can be further explored by considering their bioavailability. SV, SM, and SQ were the three sea cucumber species that have more bioavailability than other species on Tegal Mas Island.

Figure 2. Sea cucumber in Tegal Mas, Lampung Sea.

Dried Fine Degree Assesments. SV dried powder passed 100% No. 4 sieve, and 20.43% No. 18 sieve. SM dried powder passed 100% through the No. 4 sieve and 23.37% through the No. 18 sieve. SQ dried powder passed 100% through No. 4 sieve and 23.28% through No. 18 sieve. From the measurement of the fine degree of 4/18, the results of the powder meet the requirements of powder size according to Materia Medika Indonesia (100% passes No. 4 sieve and does not exceed 40% passes No. 18 sieve). The purpose of making 4/18 powder was so that the powder was easily wetted by the solvent used, making it easier and giving maximum results in the extraction process. The smaller dried sample particles have a larger surface area and could harden extraction and filtering processes[16].

Extraction Yield. SV extraction yielded 15.91% and DER-native 6.28; SM obtained 14.12% and DER-native 7.08; and lastly, 14.46% and 6.92 DER-native were yielded from SQ extract. In a previous study on Seira, ethanol extraction on SV yielded 5.1%, which was less than the results obtained. SM on Luang Island with the same extraction method yielded 1.62%. The extraction yield depends on which sea cucumbers were harvested, since each region would not be in the same condition[17].

Cold kinetic maceration was intended to prevent compound degradation[18]. Nevertheless, some loss of secondary metabolites can occur when drying sea cucumbers, preparation with fresh and wet sea cucumbers prevents the active compounds from being damaged by high temperatures or contamination during drying[19].

Phytochemical Screening. Secondary metabolites from SV, SM, and SQ dried powders and concentrated extracts were shown in Table 1.

Both dried powder and extracts of SV, SM, and SQ have contained saponins, steroids, triterpenoids, and coumarins. In previous studies, methanol extracts of SV contained saponins and terpenoids, which have potency as an antioxidant[20]. 70% ethanol was commonly used as a universal solvent for the extraction of natural materials[8]. Therefore, extraction with ethanol allows to extract secondary metabolites with a broad spectrum of polarity.

Antioxidant Activity of Sea Cucumber Extract. Antioxidant assessments were conducted for DPPH radicals with Vitamin C as a positive control to determine the activity of each species of sea cucumber extract in reducing DPPH radicals. The amount of antioxidant activity was defined by the IC_{50} (inhibition concentration) value, the concentration of the sample that provides 50% suppression of DPPH free radicals. The IC_{50} value was inversely proportional to the antioxidant activity of a compound. The smaller the IC_{50} value, the stronger the antioxidant ability of a compound. The results of the antioxidant activity test on the positive control vitamin C and the 70% ethanol extract of sea cucumber can be seen in Table 2.

Vitamin C as a positive control has very strong antioxidant activity with an IC_{50} value of 14.56±0.08 µg/mL, while the IC_{50} value for sea cucumber extract was smaller than that of vitamin C, namely SV at 164.2±0.67 µg/mL, SM at 377.2±2.20 µg/mL, and SQ at 520.7±8.72 µg/mL.

Table 1. Phytochemical screening results.

Metabolites	*Stichopus vastus* Sluiter		*Stichopus monotuberculatus*		*Stichopus quadrifasciatus* Massin	
	Dried	Crude extract	Dried	Crude extract	Dried	Crude extract
Alkaloid	-	-	-	-	-	-
Flavonoid	-	-	-	-	-	-
Saponin	+	+	+	+	+	+
Tannin	-	-	-	-	-	-
Quinone	-	-	-	-	-	-
Steroid	+	+	+	+	+	+
Triterpenoid	+	+	+	+	+	+
Coumarin	+	+	+	+	+	+
Volatile oil	-	-	-	-	-	-

Table 2. IC_{50} of vitamin C and sea cucumber extract.

Series	Vitamin C (µg/ml)	*Stichopus vastus* (µg/mL)	*Stcichopus monotuberculatus* (µg/mL)	*Stichopus quadrifasciatus* (µg/mL)
1	14.4982	163.3294	376.8427	531.9890
2	14.6825	164.7568	374.6131	519.4227
3	14.5148	164.7433	379.9778	510.7603
Average	14.56±0.08	164.2±0.67	377.2±2.20	520.7±8.72

Among these three sea cucumber species, SV extract has the highest inhibition in suppressing DPPH radicals. However, three sea cucumber extracts were still much lower than the positive control (vitamin C). This may be due to secondary metabolites in sea cucumbers with low concentrations, thus influencing antioxidant activity. Factors that influence the secondary metabolite content in organisms include the condition of the environment from which the sample originates. Organisms that live in more threatening habitats, such as those with large waves, sun exposure, pollution, etc., produce secondary metabolites to survive so that they have greater antioxidant potential. Another factor that influences antioxidant activity was none other than sea cucumber extracts were still in form of crude, resulting in other compounds for example salts, minerals and other nutrients were not have a synergic effect towards antioxidant activity. This contrasts with research conducted in Thailand, where purification of the research compounds showed that *Holothuria sp.* had an EC_{50} value of 14.63 µg/mL[6]. Friedelin, 3-Hydroxybenzaldehyde and 4-Hydroxybenzaldehyde, from sea cucumber were determined using DPPH, Folin-Cioccalteau reagent. The results indicated the total phenolic contents at 30.52.28±0.21 GAE/g dry weight equivalent and the effective concentration (EC50). Other sea cucumber antioxidant activity research has suppressed radicals by 35,35% at concentrations of 1 mg/mL[21]. Other researchers report that depth, temperature, and sunlight intensity will affect the composition of pigments and other active compounds in the sample[22].

The body part of the sea cucumber extracted also affects the antioxidant potential. The antioxidant ability in the viscera was higher than in the body wall because it was associated with the food eaten by the sea cucumber itself, mainly phytoplankton containing many phenolic compounds[23].

TPC of Sea Cucumber Extracts. The determination of TPC was carried out for antioxidant activity testing because phenolic compounds were known to play a key role in preventing oxidation[24]. This measurement was based on the formation of molybdenum-tungsten complexes due to the reduction of phosphomolybdate-phosphotungstate contained in the Folin-Ciocalteu reagent by phenolic compounds present in the sample. Gallic acid was used as a standard solution because it was a simple, pure, and stable phenolic compound.

The test results of the TPC in each extract of SV, SM, and SQ consecutively 12.10±0.07, 10.26±0.02, 8.16±0.03 mg GAE/g sample. This result was different from the results of research conducted previously, where the TPC in the crude extract of *Holothuria scabra* methanol fraction was 69.09 GAE/g sample and the n-hexane fraction was 58.01 GAE/g sample. In other research with the same sea cucumber species, crude extract TPC was 30.52±0.21 GAE/g[6]. The differences in total phenolics in sea cucumber extracts were caused by several factors, such as species type, geographical, physiological, and environmental conditions that vary[25]. The presence of phenolics in sea cucumbers was influenced by their diet. The main source of sea cucumber food, which was phenolic-rich material, namely phytoplankton and particles derived from marine macroalgae, most likely affects the presence of active phenolic compounds in the sea cucumber body wall[23].

Low TPC values in sea cucumbers have become one of the factors contributing to low suppression against DPPH radicals. A synergistic relationship exists between total phenol content and antioxidant activity. *B. vitiensis,* which has the highest TPC, could be more effective at suppressing free radicals compared to other species and other types of sea cucumbers, as evidenced by the lowest IC_{50} value. This was in line with the statement of Fitriansyah et al. (2017), the higher the TPC in a substance, the higher its antioxidant activity, which was indicated by a lower IC_{50} value[24].

The Correlation between TPC and Antioxidant Activity. Determination of total phenolic content by measuring three times in a row on each sea cucumber sample used and obtained an average total phenolic content in the extract of 12.10 ± 0.07 mg GAE/g for SV, 10.26±0.02 mg GAE/g for SM, and 8.16±0.03 mg GAE/g for SQ. These results can be seen in Table 3.

The obtained TPC and IC_{50} results indicate that the higher the total phenolic content, the stronger the ability of antioxidants to donate electrons in terms of suppressing the progression of free radicals.

Sea Cucumber Toxicity Assay. A toxicity assay was done with the BSLT method on each extract of SV, SM, and SQ to determine the toxicity level of the extract to *Artemia salina*. If the extract belongs to a non-toxic group, it was likely that its use can be used for a wide range of purposes. For example, as a food supplement or raw material for cosmetics, if it belongs to a toxic compound group, it was likely that its use can be developed for medicinal raw materials. Extract with an LC_{50} value above 1000 µg/mL was categorised as non-toxic[13]. Toxicity results from three sea cucumber extracts were shown in Table 4.

Table 3. Total phenolic content and antioxidant activity results.

Series	TPC (mg GAE/g)	IC_{50} (µg/ml)
S. vastus	12.10	164.2
S. monotuberculatus	10.26	377.2
S. quadrifasciatus	8.16	520.7

Based on the phytochemical results of the dried powder and extracts of the three sea cucumber species, it was presumed that the active ingredients concerned were terpenoids because many of them from natural materials have properties as toxic compounds.

The toxicity level of *Holothuria scabra* against salina shrimp larvae for ethanol extract has an LC_{50} of 286.031 ppm. When compared with the results of other sites, it turns out that the toxicity level of ethanol extract of sea cucumbers on Seira Island was smaller at 109.64 ppm, while on Luang Island it was greater at 804.21 ppm[17]. The toxicity level of ethanol extract towards salina shrimp larvae depends on the season when sea cucumbers were harvested, processed, and stored. It was also being said that the environment of sea cucumbers strongly influences the safety and quality of raw extracts and its products[26].

CONCLUSION

Table 4. LC_{50} of sea cucumber extract.

Series	LC_{50} (µg/mL)	Intensity
S. vastus	73.72	Toxic
S. monotuberculatus	155.31	Moderately Toxic
S. quadrifasciatus	118.94	Moderately Toxic

The exploration of sea cucumbers on Tegal Mas Island, Lampung, concluded that among the three selected sea cucumbers, Stichopus vastus had the highest total phenolics and antioxidants with the lowest toxicity. However, optimisation of extraction between wet and dried sea cucumbers was needed. Extraction optimisation with various solvents can also be done to obtain secondary metabolites more effectively.

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