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Proximate composition and cytotoxicity of selected sea cucumbers (Holothuroidea) in Goso-on and Vinapor, Carmen, Agusan del Norte, Philippines

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Abstract

Sea cucumbers are seafood that provides potential therapeutic properties, impressive profile of valuable nutrients and unique biological and pharmacological activities (Ozer *et al*, 2004; Samonte, 2016; Shi *et al*.,2016).The proximate composition and cytotoxicity of selected sea cucumbers were determined using the sea cucumbers body wall in dry basis. Oven drying was utilized for moisture, ashing for ash, Soxhlet extraction for crude lipid, Kjeldahl for crude protein and by difference for carbohydrates content. Brine shrimp lethality test (BSLT) was used to test the cytotoxicity of the sea cucumbers extracts. Tests were done in triplicate, and an average of each test was noted and expressed in percentages. Among the three sea cucumbers, *B. graeffei* had the highest moisture (6.00%) and crude lipid content (1.42%), *B. graefffei* showed lowest total ash content (33.49%) while *A. lecanora* had the highest ash content (50.06%) while *H. fucopuntata* had the highest crude protein (40.74%) and carbohydrates content (39.74%). Moisture content did not show any significant difference ($p < 0.05$) among species while significant differences were observed among crude lipid, crude ash, crude protein and carbohydrates ($p<0.05$).*Holuthuria fuscopunctata* extract exhibited the lowest lethal concentration (LC_{50}) in BSLT which was 32.10 ppm. All samples showed high protein and low lipid levels, two sea cucumbers extracts exhibited median lethal concentration (LC_{50}) in BSLT except *A. lecanora* extract (LC_{50}>1000ppm). The study suggests that results could be linked to the presence of a wide array of bioactive compounds found in sea cucumbers.

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Introduction

Sea cucumbers (Holothuroidea) have been popular as a traditional food tonic (Chen *et al.,* 2011), valued and prized as food and folk medicine in Asia (Mamelona *et al.*, 2007). These marine invertebrates are usually processed into dried products in which the body wall are the main market demand, commercial value are graded as to the type of species, size, abundance, appearance, odor, color and thickness of the dried body wall (Wen, Hu, and Fan, 2010). Sea cucumber is one of the most expensive marine commodities in the world (Samonte, 2016). Traditionally, sea cucumbers are eaten raw , boiled or dried among the local folks (Ozer, Mol, &Varlik, 2004).These are exploited for drug development, bioactive compounds are extracted from them and have been used to cure impotence, joint pains, burns, and fatigue (Samonte, 2016). Several biological and pharmacological properties like anticancer, anticoagulant, antimicrobial, antioxidant and many more have been ascribed and isolated from sea cucumbers (Shi *et al.*, 2016).

Chemical compounds of these benthic echinoderms are influenced in seasonal variations and feeding behavior (Wen, Hu and Han, 2010) or to individual or geographical variations (Chang-Lee *et al.*, 1989). Handling procedures can also affect the chemical composition of sea cucumbers (O¨zer *et al., 2004*). Sea cucumbers are seafood with high protein and low fat levels (Wen *et al.,* 2010), however the amino acid contents were similar but fatty acid profiles were different among species. Protein, lipid and carbohydrate each contribute to the total energy content of an organism while water and ash only contribute mass. A wide array of bioactives are found in sea cucumbers extract (Bordbar, Anwar and Saari; 2011). Holothurins or triterpene glycosides (saponins) are abundant in sea cucumbers which exhibited cytotoxic activities to some animals including mammals (Sarhadizadeh *et al.,* 2014).

Sea cucumbers are found abundantly in almost all of the Philippine coastal waters, with more than 200 species wherein 40 species are of high value (Samonte, 2016). Philippines is the second largest producer of sea cucumbers, second to Indonesia (Conand and Byrne, 1993). Unfortunately, in the Philippine setting, a little information on the studies of sea cucumbers bio-chemical characterization as to its bioactive properties and activities (SPC, 2000). Philippines considered as the hotspot of sea cucumbers in Asia due to massive commercial exploitation and exportation to other countries (Choo, 2008) in which species are at risk to extinction.

This study highlight the proximate composition and cytotoxic effect of the sea cucumber extracts with a view of exploring their potential uses as functional food and a natural source of new and novel multifunctional drugs. The study might be of great help as to the utilization, management and preservation of marine resources most specifically on sea cucumbers and will recognize its potential value as to medicinal, nutritional and therapeutic properties.

Materials and methods

Prior to the tests, all materials used were washed and some were sterilized. All tests were done at the Chemistry laboratory of University of Science and Technology of Southern Philippines, Cagayan de Oro City.

Materials

Live specimen of sea cucumbers *Holuthuriafuscopunctata* (Fig.1.A), *Bohahschiagraffei* (Fig.1.B) and *Actinopygalecanora* (Fig.1.C) were collected from the seafloor of Barangay Goso-on and Vinapor, Carmen, Agusan del Norte, Philippines. Methanol and hexane solvents and other chemicals were obtained and purchased from Elmar Marketing, Iligan City.

Sample preparation

The body wall of selected sea cucumbers were washed with tap water, cut into cubes with a dimension of about 2 x 2 x2 cm. Dried in an oven with nitrogen blanketing until 10 % moisture or less was attained. The dried sample was homogenized and ground into

powder using a grinder. The powder was stored in a closed plastic bottle in a freezer which was used all throughout the different tests.

Extraction procedure

Each of the individual sample powder in a desired weight was soaked with 95% methanol for 48 hours. Then the filtrate was concentrated at a maximum temperature of 40 ^{O}C, using rotary evaporator to yield the crude extract. The extract was diluted to a desired concentration for the test of toxicity.

Proximate composition

Moisture content: Moisture content of the sample was determined using the oven drying method. About 5.00 g sample was carefully weighed using analytical balance. The weighed sample was then oven dried at a temperature of 110 ^{O}C for one hour. The sample was cooled in a desiccator for 30 minutes and after which, the sample was then remove from the desiccator and its weight was recorded until constant weight was attained. The moisture content was obtained by drying the samples to a constant weight. Percent moisture was calculated through dividing the difference of the weight of wet sample and weight of dry sample by the weight of the dry sample times 100. Percent moisture average will be calculated by getting the sum of percent (%) moisture in three trials per species.

Ash content: The ash content was determined using dry ashing method. About 2.00 g of the sample was placed in a pre-weighed crucible. The sample was then charred; which was placed in a muffle furnace for about 30 minutes to an hour at a temperature of 300 ^{O}C. As soon as the fumes ceased, the temperature was raised to 600 ^{O}C until all of the carbon has been oxidized. Ash content is calculated through dividing the recovered % ash by the mass of solids times 100.Mass of the solid is calculated through dividing the difference of 100 and percent moisture (dry basis) by 100 and then multiplied by 100.

Crude lipid: The crude lipid content was determined using the semi-continuous solvent extraction method or Soxhlet method. Before the extraction was conducted, all parts of the apparatus were rinsed with hexane solvent. About 15.00 g of the sample was placed in pre-weighed rolled Watt man filter paper which was placed in a beaker. The rolled filter paper with the sample was then placed in an extraction chamber suspended above a round bottom flask containing 400 mL of hexane below a condenser. The flask was heated vaporizing the solvent for six hours. The condensing units were then removed and allowed to cool. Then, all the solvents used in the extraction process were recovered and collected. The residue was then placed in an oven at a temperature of 110 ^{O}C until the remaining solvent was vaporized. After this, the residue was then placed again in the oven at a temperature of 110^{O}C for an hour and cooled in the desiccator for 30 minutes, then, the sample's weight was recorded. Percent (%) lipid was calculated through dividing the % weight of extracted lipids by the difference of weight of the sample and the product of weight of sample and % moisture of the sample then multiplied by 100.

Crude protein: Kjeldahl method (Kjeldahl, 1883 ; Chow *et al.*,1980) was used to get the desired protein. The method utilized 1 g of each sample and place in Kjeldahl flask, which then added by 10 g K_2SO_4, 0.7 g HgO and 20 mL concentrated H_2SO_4. The flask was placed in the digester and tilted at a certain angle. The mixture was brought to boil which was done until the solution is clear and it was continuously heated 30 minutes more until foam disappear, and it was cooled by adding gradually 90 mL distilled water or de-ionized water. When the mixture cooled, a 25 mL Na_2SO_4 solution was added and then stirred. The flask was kept tilted and the mixture was added with one glass bead and 80 mL of 40 % NaOH solution to form two layers. Then, the flask was quickly connected to the distillation unit and heated. A 50 mL of distillate was collected which contained ammonia in 50 mL of indicator solution. As the distillation ended, the receptor flask was removed, the end of the condenser was rinsed, and then the solution was titrated with the standard HCl solution. Percent protein in dried basis was calculated by dividing

percent protein by the percent of solids times 100. Percent of solids was calculated by the difference of 100 minus percent moisture of a sample times 100 then divided by 100.Crude protein percent (%) of the three specimens were calculated as to the quantity of nitrogen present in each sample multiplied by 6.25.Carbohydrates content: Total carbohydrates content of each species of selected sea cucumbers was done by difference. Each trial of percent (%) ash, crude lipid and crude protein was sum up and subtracted from 100%, all in dry basis.

Cytotoxicity

Brine shrimp preparation: Brine shrimp eggs (*Artemia salina* cysts) were obtained from Butuan City, Agusandel Norte. Natural seawater from Aplaya, Jasaan, Misamis Oriental was used for hatching the shrimp eggs. The seawater was put in a hatching chamber, a small plastic container with a partition, the covered and light areas. Shrimp eggs were added into the dark side (covered) of the chamber while the lamp above the other side (light) to attract the hatched shrimp. Two days were allowed for the shrimp to hatch and mature as nauplii larva. After two days, when the shrimp larvae are ready, 4 mL of the natural sea water was added to each test tube and brine shrimps were introduced into each tube. Thus, there were a total of 30 shrimps per dilution. Then the volume was adjusted with natural seawater up to 5mL per test tube. The test tube left uncovered under the lamp. The number of surviving shrimps were counted and recorded after 24 hours.

Test for Toxicity: Toxicity assay of the crude extracts was conducted separately using Brine Shrimp Lethality Test (BSLT). Prior to the test, methanolic extractions of the three dried sea cucumbers were done with the use of rotary evaporator to evaporate the methanol. Then, stock solution (4,000 ppm) was prepared by dissolving 1 g of crude extract in 10 ml methanol and volume was raised to 100 ml with distilled water. From these different dilutions of 125 ppm, 250 ppm, 500 ppm, 1000 ppm, 2000 ppm and 4000 ppm were prepared in 200 mL deionized water in 250 ml beaker and 10 live and active larvae were released in it and mortality was noted after 24 hr. Results were compared to the positive control (concentration with dried methanol) and negative control (natural sea water).The beakers were kept in a temperature controlled room at 28°C ± 2°C. Each treatment was replicated three times. Percent larval mortality was calculated using Probit analysis technique (Finney, 1981), a software used to determine the lethal concentration. Percentage Mortality (%M) was calculated by dividing the number of dead nauplii by the total number nauplii used, and multiplied by 100.

Statistical analysis

Proximate composition: All treatments were replicated three times and results were reported as means. A one way analysis of variance (ANOVA) was performed using the general linear models procedure of MINITAB 13.30 (Minitab Inc., State College, PA., USA). The test was used to compare the average moisture, ash, fat, and protein content between the three species of selected sea cucumber. Tukey Pairwise Comparison was also utilized to compare the percent of the three selected cucumbers in terms of average moisture, ash, lipid, protein and carbohydrates content. Pairwise comparison among means with 0.05 significant level was used.

Toxicity: Using Probit analysis, software developed by Finney (1981) and adopted by Raj (2016), the lethality concentration (LC_{50}) was assessed at 0.05 significance level (Olowa and Nuñeza, 2013). The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the napulii is attributed to the bioactive compounds present in sea cucumber extract. All measurements were carried out in triplicate.

Results and discussion

Proximate composition

The averages value (Table 1), indicated that *B. graeffei* had the highest moisture content (6%) followed by *H. fuscopuntata* (5.51%) and A. *lecanora*

showed the lowest (4.51%). On the other hand, *A. lecanora*ash content (50.06%) was more than *H. fuscopunctata* (36.29%) and *B. graeffe*i (33.49%). Among the three species, *B. graeffei* shows high crude lipid content (1.46%), *A. lecanora* comes next (0.86%), and *H. fuscopunctata* has the very least crude lipid content (0.42%), this may be attributed since *B. graeffei* contains cuevarian tubules among other species; these are clusters of fine tube located at the base of respiratory tree, a white sticky substance, which can be discharged through the anus and serves as their defense mechanism.

Table 1. Proximate composition content (%) of selected sea cucumbers.

Sea Cucumbers	Moisture	Ash	Lipid	Protein	Carbohydrates
H. fuscopunctata	5. 51±1.03	36.29±1.13	0.42±0.07	40.74±0.44	39.74±1.38
B. graeffei	6.00±0.73	33.49±0.92	1.46±0.11	21.92±1.10	21.91±0.79
A. lecanora	4.51±1.25	50.06±1.21	0.86±0.11	18.97±0.71	18.96±1.07

Values are expressed as means±SD. Values are calculated based on the dry weight of sea cucumber body wall.

The crude lipid of *A. lecanora* may be attributed to the juvenility of species, small in size, thin body walls, unlike the other two species of sea cucumbers, which were matured and large in size. Furthermore, *B. graeffei* had the highest crude protein (40.74%), while *A. lecanora* has the very least protein content (18.97%). A least carbohydrates content (18.96%) showed of the species *A. lecanora* while *H. fuscopunctata* gave the highest carbohydrate content (39.74%).

Table 2. One-Way ANOVA of proximate composition of selected sea cucumbers.

Values	Moisture	Ash	Lipid	Protein	Carbohydrates
F-value1.65	189.99	87.54	87.54	459.05	310.92
p-value0.268	0.000*	0.000*	0.000*	0.000*	0.000*

Values with (*) are significantly different at $p<0.05$.

For the purpose of comparison with literature values, results of the moisture content among sea cucumbers were very similar to the study of Chang-Lee and Lampila (1989) where dried sea cucumber contained between 2-6 % moisture and it varies differently to the moisture content in fresh sea cucumber as reported by Chang-Lee *et al.* (1989) which ranged between 89-91% moisture which moisture dropped about 85% in dried basis. Results of this study on moisture content was negated to the result of Chen (2003) on the dried various species of sea cucumber in which moisture profile has a minimum and maximum moisture content of 8.25% and 21.55%, respectively. Ash content in the present study was more than the range reported by Chang-Lee *et al.*(1989) which ranged from 16% to 24%, but it was similar to the result of the study of Wen *et al.* (2010) which ranged from 15.4% to 39.6% and to the study of Reid *et al.*(2006) on the processed *H. scabra* which was determined to be 17.9 – 44.5% ash but differently comparable to the result of Chen (2003) were ash content ranged between 7.56% and 21.09 %

The data in Table 1 on crude lipid content (0.4 -1.4 %) in dried formed were less than the range reported by Chang-Lee *et al.(* 1989) which is 2-3%, but it is closely similar to the study of Reid *et al.(*2006) and Chen (2003) which ranged 1.2 – 2.4% and 0.55 – 3.70%, respectively. Moreover, crude protein content which ranged from 24.15 – 42.42% (Table 1) is comparable to the study of Wen *et al.* (2010) which was determined between 40.7% to 63.3% but lower than to the report Chang-Lee *et al.*(1989) on were content ranged between 61-70% protein, all were in dried form. Similarly, report Chang-Lee *et al.*(1989) was supported by the study of Chen (2003) were the highest protein content in sea cucumbers was reported as 69.72% and the lowest content was 55.51%. Results negated the study of Chang-Lee

&Lampila (1989) were dried sea cucumbers contained 2-3% carbohydrates which were too small compared to 18-40%.

Method used and sample preparation are some factors that contribute to the differences of results (Ozer *et al.,* 2004). Consequently, chemical compounds of these benthic echinoderms are influenced in seasonal variations and feeding behavior (Wen *et al.*, 2010) or to individual or geographical variations (Chang-Lee *et al.*, 1989).

Table 3. Tukey-Pairwise comparison - proximate composition of selected sea cucumbers.

Sea Cucumbers Moisture	Moisture	Ash	Lipid	Protein	Carbohydrates
H. fuscopunctata	5.51/A	36.29/B*	0.42/C*	40.74/A*	39.74/A*
B. graeffei	6.00/A	33.49/C*	1.46/A*	21.92/B*	21.91/B*
A. lecanora	4.51/A	50.06/A*	0.86/B*	18.97/C*	18.96/C*

Values are expressed as means/grouping. *Means of different letter are significantly different.

To compare the significant difference among other species, data were tested using One Way ANOVA at p-values less than 0.05 ($p<0.05$).Table 2 indicated that among other proximate composition only moisture content did not show significant difference while a significant difference was showed between ash, crude lipid, crude protein and carbohydrates at $p<0.05$.

The collected data were further subjected to Tukey Pairwise Comparison as indicated in Table 3, grouping information using the Tukey Method at 95% level of confidence to compare of which among the proximate compositions, moisture content, ash, crude lipid, crude protein and carbohydrates in their result mean in all species confirm significant difference.

Cytotoxicity

All tested concentrations exhibited different levels of lethality against *Artemia sp* nauplii. Table 4 showed the values of LC_{50} which *H. fuscopunctata* and *B. graeffei* exhibited LC_{50} lower than 1000 ppm.*H. fuscopunctata* exhibited highly toxic concentration with the LC_{50} of 35. 10 mg/L, moderately toxic for *B. graeffei* while no value of LC_{50} exhibited for *A. lecanora* which means non-toxic.

As reported, the effectiveness or the concentration-mortality relationship of each concentration does not produce death in half; consequently, LC_{50} is usually expressed as a median lethal concentration (Finney, 1981). The mortality of the nauplii in shrimp lethality test is attributed to the bioactive compounds present in the sample (Rivera and Uy, 2011).

This result is supported by the study of Meyer *et al.* (1982) and others on the index of toxicity, LC_{50} value of less than 1000 µg/mL is toxic while LC_{50} value greater than 1000 µg/mL is non-toxic. Clarkson's toxicity criterion for the toxicity assessment mentioned that extracts with LC_{50} above 1000µg/mL are non-toxic, LC_{50} of 500-1000 µg/mL are low toxic (least toxic), extracts with LC_{50} of 100-500µg/mL are medium toxic (moderately toxic), while extracts with LC_{50} of 0-100 µg/mL are highly toxic (Clarkson *et al.* 2004). Olawa and Nuñeza (2013) emphasized that LC_{50} of less than 1000 ppm will be considered as potent (active). Specimens withLC_{50} less than 1000 ppm indicates the presence of potent cytotoxic and probably antitumor and anticancer components (Rivera and Uy, 2011). The more toxic the sample, the more potent or active it is (Meyer *et al.,* 1982), as it contains biologically-active compounds and as mentioned by the study of Omron (2013) that functional food that contains biologically-active compounds is an important source for prevention, management and treatment of chronic diseases. Since sea cucumbers served as dietary delicacies among Asian countries (FAO, 2008), eaten raw, boiled or dried as food for human consumption in many tropical and subtropical countries (Subasinghe, 1992), it is therefore considered as potential functional food (Bhakuni and Rawat, 2005).

Table 4. Cytotoxic activity of methanolic extracts of the three selected sea cucumbers.

Sea Cucumber	Concentration (ppm)	% Lethality (24 h)	LC_{50} 24 h* Toxicity (ppm)
	4000	90.0	
	2000	100.0	
H.fuscopunctata	1000	93.3	32.10 ±1.37
	500	83.3	(highly toxic)
	250	80.0	
	125	63.3	
	4000	100.0	
	2000	100.0	
B. graeffei	1000	93.3	384.80 ±4.08
	500	83.3	(moderately toxic)
	250	20.0	
	125	13.3	
	4000	40.0	
	2000	26.7	
A. lecanora	1000	23.3	> 1000
	500	36.7	non-toxic
	250	30.0	
	125	3.3	

*mean ± standard deviation, n =3.

Furthermore, result might be attributed of the type of chemical components present in the specimen in which LC_{50} can be attained in a certain concentrations and since *A. lecanora* is eaten raw among the local folks, then therefore the toxicity level might be tolerable and negligible to *Artemia sp.* at a certain concentrations while *H.fuscopunctata* which is less graded in the local market, eaten boiled or dried, exhibited very toxic (Table 4) and this possibly due to the substances release of the species which is itchy, especially when it comes contact to skin.

According to studies that, a high toxicity of methanolic extract probably contributed to the presence of saponins of sea cucumber (Chanley *et al.*, 1955: Kelly 2005), and as reported, saponins or triterpene glycoside or also known as holothurins are poisonous, and this chemical component is also responsible for antifungal activity against tested fungus (Habermehl *et al.*,1990).

Saponins are one of the major isolated products from sea cucumber and these compounds attributed to cytotoxic activities of sea cucumbers (Bhakuni and Rawat, 2005). Holothurins from sea cucumber are considered to have good pharmacological potential as neuromuscular and anticancer agents, since the biological action of holothurins was vested in the steroid moiety of the molecule and it is hoped that one may find some useful chemotherapeutic agents from sea cucumbers species (Erickson *et al.*, 2009).

Furthermore, as mentioned in several studies, that results of bioassay may vary from one place to another. Sea cucumbers of the same species but in different geographical regions provide discriminative functions in medical applications (Zao *et al.*, 2008; Wang *et al.*, 2009).

Variation holothurin content is due to the changes in ecological conditions (Habermehl *et al.*, 1990).

Chemical compounds may be subjected to seasonal variations and feeding behavior of sea cucumbers (Chang-Lee, *et al.*, 1989) or to individual or geographical variations (Ginger *et al.*, 2001).

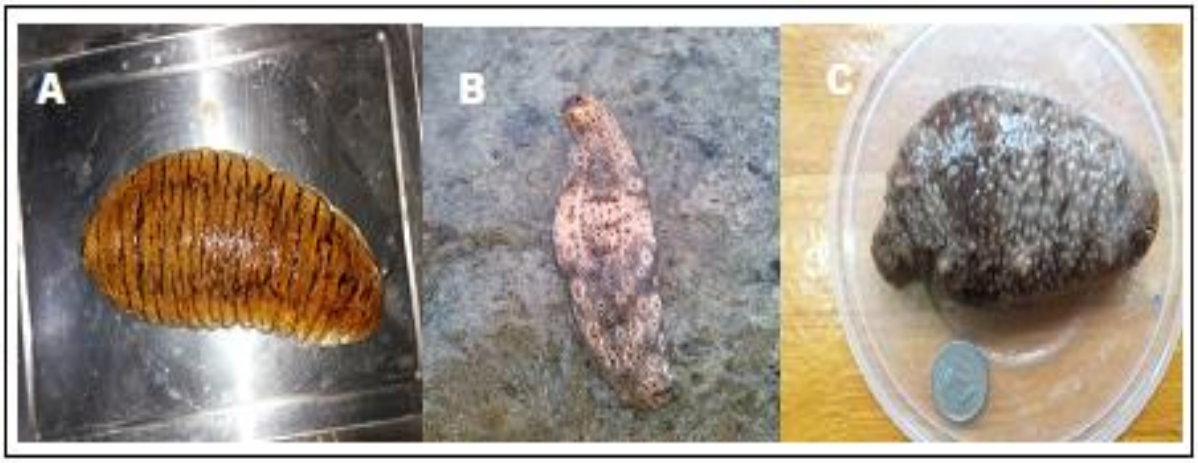

Fig. 1. Selected specimen of sea cucumbers collected from Goso-on and Vinapor, Carmen. A, *Holuthuriafuscopunctata;* B,*Bohadschiagraeffei*; C, *Actinopygalecanora.*

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