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Bioactive compounds proximate composition and cytotoxicity of Echinoidea (Sea Urchin): Basis for future policy preservation and conservation

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Abstract

The presence of Echinoidea in marine ecosystem at the inter-tidal zone of the Philippines is abundant. The proximate composition and cytotoxicity of the combined spines, tests and gonads of the selected three species of sea urchin namely: *Diadema setusom, Tripnuestes gratilla and Astropyga radiata* greatly vary in their proximate composition. Specifically, the three-sample species are potential source of nutrients and minerals. The cytotoxic activity using brine shrimp lethality test differ in selected sea urchins. The test for cytotoxicity showed that LC_{50} is >1000 ppm which is non-toxic. The results confirmed samples non-toxicity since the species are edible and local folks eat and sell them as source of livelihood. Preservation and conservation of sea urchin species are necessary for it is one of the most significant marine invertebrates used as bioindicator and as source for livelihood and commercialization.

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Introduction

The marine environment, which contains a vast array of organisms with unique biological properties, is one of the most underutilized biological resources (Ibañez, Herrero, Mendiola, & Castro-puyana, 2012). The Philippines is the center of sea urchin diversity in an in-depth study from intertidal to abyss according to the Society for Integrative and Comparative Biology of the University of California. As a biodiversity hotspot, the Philippines currently holds the gold medal in species richness (Mooi & Munguia, 2014).

Due to the rich biodiversity of this country, Philippines, though there is general diminishing of some species, this study is timely before sea urchins become extinct. Specifically, this study aims to determine the proximate composition and cytotoxicity of selected species of sea urchins namely the *Diadema setosum*, *Astryopyga radiata* and *Tripnuestes gratilla* using the combined spines, tests and gonads. Furthermore, the study serves as the basis for drafting future policies on preservation and conservation of the sea urchin species.

The UN General Assembly last May 2017 made a resolution for transforming our world through the "2030 Agenda for Sustainable Development". This agenda consists of 17 goals. The present study is aligned to the following goals: "Goal 2 is to … improve nutrition", "Goal 3 is to ensure healthy lives and promote well-being for all ages", "Goal 13 is to take urgent action to combat climate change and its impacts", and lastly, "Goal 14 is to conserve and sustainably use the oceans, seas and marine resources for sustainable development" (UNESCO, 2017).

In the Philippines, there are 70 species of sea urchin located in the different parts of the archipelago (Mooi & Munguia, 2014). A higher density of *T. gratilla* species has been found in Northern Mindanao and in Southern Mindanao (Pates, Responte, Mag-aso, & Dagoc, 2015). In addition, *T. gratilla* is the most known sea urchin that became a major source of livelihood. The populace of the Asian Pacific Region has been using sea urchin for improving quality life and cure for many diseases (Rahman, Arshad, & Yusoff, 2014).

Sea urchins are not only important for their ecological roles in the environment but are found to be very beneficial to humans as well. Gonads of sea urchin also named as roe are highly in demand and economically important food because of its high nutritional value (Jinadasa, Zoysa, Jayasinghe, & Edirisinghe, 2016). The roe of the sea urchin is considered to be a prized delicacy due to its tasty quality in Asian and Mediterranean countries. Also, in Western Hemisphere countries such as Barbados and Chile (Rahman *et al.*, 2014).

Gross chemical composition information of marine organisms contributes largely to the importance of the species. Chemical analyses of the different compositions like proteins, lipids, carbohydrates, nitrogen and phosphorus are significant as main components of living matter (Barbarino & Lourenço, 2009; Diniz *et al.*, 2014). The total chemical composition of an organism can be influenced by many factors such as physical characteristics, habitat and life cycle, in addition to the environmental characteristics (Diniz, Barbarino, Oiano-neto, Pacheco, & Lourenço, 2014).

The biological compounds of the sea urchin gonads, tests, spines and amniotic liquid have been studied in different species. The big quantity of polysaccharides, lipids and proteins are found in the ovary and testis (Tenuzzo, Carata, Mariano, & Dini, 2017). The study on biological composition of edible urchin *Stomopneustes variolaris* determined the proximate composition through moisture and ash, protein, lipid, carbohydrate, fatty acid profile, carotenoid and vitamin analysis, and essential trace metals (Jinadasa *et al.*, 2016).

The use of the brine shrimp *A. salina* has ecological importance in marine environments, as these organisms are a symbol of the zooplankton community and essential on the ecology of seashores. The nuplii is very reactive to toxicants than the adults and microscopial measures are easy to perform using the brine shrimp *A. salina* (Lopes, Fernández, Martins, & Vasconcelos, 2010).

In the Philippine context based on the published literature and studies cited, it dealt only on physical growth, taxonomy and management. It shows that there were no published studies on proximate composition and toxicity of the sea urchins in the Philippines which is the concern of the present study. The present study explored on this topic since sea urchin is also one of the determinants of sea water pollution. It is therefore timely to undergo this study for it might vanish in the future due to rapid human population growth that results to water pollution. This urgency calls for this study since we might lose the species without knowing the extent of its contribution to humanity.

It is in these reasons that this study aims to determine the proximate composition and cytotoxicity of the three species of sea urchin namely the *Diadema setosum, Astropyga radiata* and *Tripnuestes gratilla* using the combined spines, tests and gonads from Vinapor and Goso-on, Carmen Agusan del Norte, Philippines.

Materials and methods

Sampling Area

The study was conducted in Goso-on and Vinapor Carmen, Agusan del Norte, Philippines. The samples were collected from the intertidal zone up to shallow waters of about 0-5m. The map of the collection site is shown in Fig. 1. The Municipality of Carmen is located in the province of Agusan del Norte of CARAGA Region.

Fig. 1. Collection site in Barangay Gosoon and Vinapor, Carmen, Agusan del Norte.

Sampling Scheme

Purposive sampling scheme was adopted to where the sea urchins' species were abundant based on the preliminary survey or reconnaissance of the area. The three sea urchins species were selected from the rest of the species based on the abundance and maturity. The total body weight of each specimen and the average horizontal test diameter were measured perpendicularly without spines using a vernier calliper (Jinadasa *et al.*, 2016).

Preliminary identification was done based on the field guide developed by Schoppe (2000) and the World Registry of Marine Species (WoRMS) using morphological characteristics. Final identification was done at the Marine Center, Xavier University. For further validation, photographs of the specimens were sent to the Institute of Environmental and Marine Science, Siliman University.

The collected mature and fresh sea urchins were thoroughly washed with seawater to remove sediments. Selected species of sea urchins were *Diadema setosum, Tripnuestes gratilla* and *Astropyga radiata* as presented in Fig. 2, 3 and 4 respectively. The amniotic liquid was removed by making small hole in the shell, it was kept in a Ziplock and placed in a styropor box with ice for preservation during transport. The selected, clean and fresh samples were transported to USTP Chemistry laboratory within 24 hours and immediately stored in a freezer to avoid decomposition.

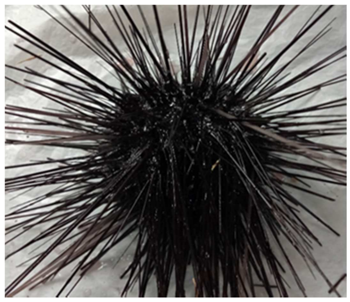

Fig. 2. The *Diademata setosum.*

Fig. 3. The *Tripnuestes gratilla.*

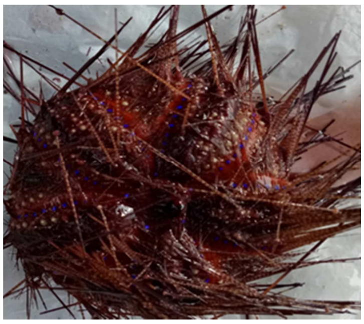

Fig. 4. The *Astropyga radiata.*

Data Analysis

Descriptive statistics was used in the presentation of the different parameters involved. Data gathered such as total ash, crude lipid, crude protein, and total carbohydrates for proximate composition was analysed using one-way analysis of variance (ANOVA) with Tukey Honest Significant Difference Mean Test (Tukey HSD Mean Test) for pairwise comparison. The differences with p-values of less than 0.05 ($p<0.05$) level were statistically significant and data were expressed in mean ± SD.

Procedure and Biochemical Composition Analyses

Methanolic Extraction

Fifty (50) grams of the sample was soaked in 95% methanol. Minimum volume of 200 mL was used to soak the sample. The soaking took about 48 hours. After 48 hours, the sample was filtered using Wattmann filter paper. Another 200 ml of methanol was used for the second soaking and filtered after 1 hour of soaking. For the last soaking, additional 200 ml of methanol was used and the filtrate was then placed in the refrigerator for proper storage.

Proximate composition

The moisture content was obtained by drying the samples to a constant weight.

a. Ash Content

The ash content was determined using dry ashing method according to AOAC (2000). Five grams of the sample was placed in a pre-weighed crucible. The sample was then charred; it was placed in a muffle furnace for about 30 minutes to an hour at a temperature of 300°C. As soon as the fumes ceased, the temperature was raised to 600°C until all the carbon had been oxidized. The following formula was used to calculate the ash content of the samples and expressed as the percentage by weight of the dry sample.

$$\%A_D = \frac{M_{ash}}{M_{dry-}M_{ml}} \text{ x } 100 \qquad (1)$$

Where:

$\%A_D$ = percentage of ash in the sample (dry basis)

M_{ash} = weight of ash sample

M_{dry} = weight of the original dry sample

M_{ml}= mass of the moisture lost (mass of the dry sample minus the mass of sample after moisture determination)

b. Crude Protein

Analysis of crude protein was done using the Kjeldahl's method, which evaluate the total nitrogen content of the sample after it has been digested in sulphuric acid with a mercury as a catalyst. The simple method used was proposed by Chow *et al.* (1980). The following formulas were used to calculate the ash content of the samples:

$$\%N_{sample}\left(\frac{AxB}{C}\ 0.014\right)\ x\ 100 \qquad (2)$$

$$\%\ P_{\text{crude}} = \%N \text{ x } 6.25 \qquad (3)$$

$$\%P_{\text{dry}} = \frac{\%P_{crude}\ x\ 100g}{100\ g - M_{ml}} \qquad (4)$$

Where:

$\%N_{sample}$ = percentage of nitrogen in the sample

$\%P_{crude}$ = percentage of crude protein in the sample

A = chlorhydric acid used in titration (mL)

B = normality (N) of standard acid

C = weight of sample in grams

$\%P_{dry}$ = percentage of crude protein dry basis

M_{ml}= mass of the moisture lost (mass of the dry sample minus the mass of sample after moisture determination)

c. Crude Lipid

The crude lipid was determined using the semi-continuous solvent extraction method or the Soxhlet method. Before the extraction was started, all parts of the apparatus were rinsed using hexane. Then about 25g of the sample was placed in a pre-weighed rolled Wattman filter paper 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 and below a condenser. The flask was then heated vaporizing the solvent for six hours. The condensing unit was then removed and allowed to cool. After which, all the solvent used in the extraction process was recovered and collected. The sample was then placed in the oven at a temperature of 110ºC until the remaining solvent was vaporized and cooled in a dessicator for 30 minutes, then the sample's weight was recorded. The percentage of crude lipid was calculated using the formula below:

$$\% L_{crude} = \frac{M_l}{M_{ds} - M_{ml}} \, x \, 100 \qquad (5)$$

Where:

$\%L_{crude}$ = percentage of crude lipid in the sample (dry basis)

$\%M_l$ = mass of the lipid that remained in the setup

M_{ds} = mass of the original dry sample

M_{ml} = mass of the moisture lost (mass of the dry sample minus the mass of sample after moisture determination)

d. Carbohydrate

The total carbohydrates content for each sample was determined by difference. The total carbohydrate concentration was determined using the following formula

$$\%Carbohydrates = 100\% - (\%Ash + \%Protein + \%Lipid) \qquad (6)$$

Cytotoxicity assay

The Meyer *et al.*, (1982) method was used for cytotoxicity assay. Hatching of brine shrimp eggs were done in a beaker filled with artificial sea water under continuous aerator. After 48 hours, the phototropic nauplii were collected using a pipette. The nauplii were counted macroscopically against a lighted background. The study used eight aqueous extract concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0mgmL-1. After 24 hours of incubation, alive nuplii were counted and percentages of deaths were computed. The control (artificial sea water) was maintained with test organisms (Bragadeeswaran, Kumaran, Sankar, & Prabahar, 2013). The LC_{50} was using the Abott's Formula (7) while the LC_{50} was calculated using Probit method using a statistical program (Finney, 1952).

$$\% P_l = \frac{P_o - P_c}{10 - P_c} \, x \, 100 \qquad (7)$$

Where:

$\%P_l$ = Percentage of brine shrimp mortality

P_O = Observed mortality

P_C = Control mortality

Results and discussion

Proximate Composition of the sea urchin species

Ash content

Ash content was determined using dry ash method charred in the muffle furnace. The selected sea urchin ash content is revealed in Table 1. The *Diadema setusom* has the highest ash content which also means that it has the highest mineral content among the selected species. Standard deviation among the means shows that there is homogeneity of the results.

Table 1. Proximate composition of selected sea urchins combined spines, tests and gonads with Tukey test.

Species	% Ash	% Protein	% Lipid	% Carbo-hydrate
D. setosum	65.86±0.467	9.88±0	1.53±0.405	3.12±0.011
T. gratilla	65.45±0.829	10.65±0.25	1.93±0.082	2.28±0.230
A. radiata	58.44±0.966	17.21±0.13	2.26±0.063	2.72±0.120
*Tukey HSD Mean Comparison**	*DT, DA <TA*	*DT< TA<DA*	*DA, DT, >TA*	*TA, DA>DT*

Legend: *D – D. setosum; T – T. gratilla; and A – A. radiata; *α=0.05 level*

Crude protein

The analysis of hydro-soluble protein in animal tissues revealed high values. This indicated that protein is the most abundant organic compounds in selected sea urchin species. In combined sea urchins' spines, tests and gonad. Table 1 shows values of means and its corresponding standard deviation. The result further shows that *Astropyga radiata* has the highest protein content which means that it could be a good source of energy. The results of this study showed a much higher percentage of protein compared to the amount of protein from studies that make used of gonads only (Mol *et al.*, 2008; Arafa *et al.*, 2012). Protein is the main constituents of gonad of urchins (Verachia *et al.*, 2012) and higher amount of protein is present in gonads (Jinadasa *et al.*, 2016).

Crude lipid

The amount of lipid concentrations differ more than the carbohydrate and protein in marine organisms as reactions to ecological conditions, biological traits and feeding (Diniz *et al.*, 2014). *Astropyga radiata* in this study contains the highest percentage of crude lipid as also shown in Table 1. Standard deviation shows homogeneity among the means. The result of lipid contents of sea urchins below were similar to the values recorded of sea urchin species *Echinometra mathaei* sampled in French Polynesia (Mills *et al.*, 2000).

Carbohydrate

The total carbohydrate in marine invertebrates is primarily low in concentration as evident in the results of this study. The presence of carbohydrates play an important role in body coating and fertilization of sea urchins but generally they are poor in this component (Ghazarian, Idoni, & Oppenheimer, 2012). Table 1 shows the three samples have low concentration of carbohydrate as expected since these are marine organisms. Deep water organisms have lower levels of carbohydrates compared to those in the shallow water since these animals grow more slowly and reduces their reproductive output in a poorer environment (Thompson & Macdonald, 1990).

Table 2. Cytotoxicity effect from aqueous extracts of the selected sea urchin species.

Species	Dosage (ppm)	Percent Mortality			Average Mortality
		Trial 1	Trial 2	Trial 3	
Diadema setosum	125	0	0	0	0
	250	0	0	0	0
	500	0	0	0	0
	1000	20	0	0	6.68
	2000	0	20	20	13.33
	4000	30	20	30	26.68
Tripnuestes gratilla	125	0	0	0	0
	250	0	0	0	0
	500	0	0	0	0
	1000	0	0	0	0
	2000	10	10	20	13.33
	4000	30	10	30	23.33
Astropyga radiata	125	0	0	0	0
	250	0	0	0	0
	500	0	0	0	0
	1000	0	0	0	0
	2000	10	10	20	13.33
	4000	20	20	30	23.33

Cytotoxic activities of the selected sea urchin species

The toxicity of selected sea urchin species methanolic extracts was evaluated using brine shrimp lethality test and summarized in Table 2. For toxicity index, extracts with LC_{50} < 1000μg/mml are considered as toxic, while extract with LC_{50} > 1000μg/ml are considered non-toxic (Meyer *et al.*, 1982). Toxicity criterion for the toxicity assessment of plant extracts classifies in the succeeding order: extracts with LC_{50} above 1000μg/ml are non-toxic, LC_{50} of 500-1000μg/ml are low toxic, extracts with LC_{50} of 100-500μg/ml are medium toxic, while extracts with LC_{50} of 0 – 100μg/ml are highly toxic (Clarkson *et al.* 2004).

Basis for Future Policy on Preservation and Conservation

All governments of countries with coastlines should have a strong policy on sustainable management of coastal resources. Coastal areas are under extreme environmental change pressure with extensive feedback effects between the natural systems and the human systems (Turner, 2000). In the worldwide, marine environments are in serious deterioration as a result of over-harvesting and pollution as impacts of climate change. Climatic changes and anthropogenic stresses have caused detrimental shifts in species composition. This phenomena are often long-lasting and difficult to reverse (Hughes, Bellwood, Folke, Steneck, & Wilson, 2005).

The increase of the human population and the growth in amount of resources used are changing Earth in unprecedented ways. The resulting changes are relatively well documented but not generally valued in their totality, magnitude, or implications (Lubchenco & Nin, 1998). Maintaining social–ecological resilience and effectively managing the ecosystem goods and services requires the ability to detect and to act the ecological feedbacks. Nonetheless, in the world-wide context, the biggest challenge for sustainability of marine ecosystems is to provide an institutional framework for improved linkages between dynamic ecological and social systems (Hughes *et al.*, 2005).

In marine ecosystem, bioindicators play an important role. The term bioindicator is used for organism associations that respond to pollutant load with changes in vital functions, or which accumulate pollutants.

Embryos and larvae of sea urchins are suitable and sensitive bioindicators of marine pollution for the basins affected by the household and industrial effluents. Regular use of bioindicators in impact areas allow to assess the situation with the increasing anthropogenic influence and predict the environmental impacts on biological resources and the whole ecosystem (Lukyanova, Zhuravel, Chulchekov, & Mazur, 2017).

Table 3. Lethality Concentration of Different Sea Star Crude Extracts.

Species	LC_{50} (ppm)			Average LC_{50} (ppm)	Toxicity*
	Trail 1	Trial 2	Trial 3		
Diadema setosum	34193.58	90137.02	33857509324	11285911218.0	Non-toxic
Tripnuestes gratilla	6686.56	1000.01	1200.05	2962.21	Non-toxic
Astropyga radiata	33857509324.38	3987461.10	4892941.28	11288796575.46	Non-toxic

* Meyer *et al.* (1982)

The developmental biology used sea urchin as an important model organism. Similarly, in the study of heavy metals pollution one of the most important marine invertebrates used as bioindicator is also the sea urchin species. Studies designed to determine the effects of chemical pollutants both in the field and in the research laboratory recommended sea urchin as an appropriate model for eco-toxicological and environmental researches. The sea urchin has been recently presented in the list of alternative methods recommended by the European Union Reference Laboratory (EURL) for animal testing, for validation of methods with the use of animals, for safety testing and potency testing of substances and vaccines (Chiarelli & Roccheri, 2014).

Sea urchin organism is sensitive to several aquatic contaminants and adopts several defence mechanisms against any environmental, chemical, physical and mechanical stresses. The sea urchin embryo exemplifies an appropriate model system to probe the adaptive response of cells subjected to stress during developmental stages. Pollutants of anthropogenic origin, specifically heavy metals, are of significant interest for their capability to induce the initiation of defence systems or interrupt the developmental program (Chiarelli & Roccheri, 2014).

Stock enhancement in the Philippine context, is a tool for recovery, increase and sustainability of fisheries stocks. Stock enhancement hastens the recovery of depleted resource, increase the production of the resource and provide potential livelihood for coastal communities. In the hierarchical list of marine species according to Department of Agriculture - Bureau of Fisheries and Aquatic Resources - Fisheries Resource Management Project (DA-BFAR-FRMP) sea urchin is rank 5th in terms of practical selection of appropriate species for stock enhancement in terms of the following criteria: biodiversity conservation status -sea urchin is threatened, tendency to dominate is low, export is high and socioeconomic for livelihood potential is high (Gonzales, 2005). Thus, sea urchin conservation and protection is necessary.

Two echinoderm groups have significant fisheries in the Philippines: sea cucumbers and sea urchins. The herbivorous sea urchins promote higher ecosystem primary productivity in coral reefs and sea grass beds, and facilitate energy flow to higher trophic levels. Their presence and biological activity have been demonstrated to improve sediment quality by decreasing organic matter deposition and inhibiting harmful algal bloom (Michio *et al.*, 2003). The various life stages also form important food web components both in the plankton and benthos of the sea.

The Philippine Constitution and fishery laws safeguard the overexploitation of marine resources, including these invertebrate groups. However, a basic awareness of the biology and life history of fishery resources and important ecological background is very limited at the grassroots (Primavera, 2005). Moreover, there are practically no fishery management regulations for invertebrates in the country.

Harmonization of conservation needs can be undertaken by addressing gaps in existing knowledge. This includes conducting basic research and linking formal sciences and indigenous knowledge systems. Information on Philippine biodiversity is limited, incomplete, and scattered among institutions and individuals. Another major gap in conserving the country's biodiversity is lack of baseline data (Department of Environment and Natural Resources, 2002).

Thus, the results of the different laboratory tests for proximate composition and cytotoxicity showed the used of higher scientific inquiry skills for basic research necessary to answer the gap and for the attainment of the 2030 agenda specifically Goal no. 14 on "conserve and sustainably use the oceans, seas and marine resources for sustainable development". Specifically, for future policy making on preservation and conservation of sea urchins as threatened species.

Conclusion

The species of *Tripnuestes gratilla*, *Diadema setusom* and *Astropyga radiata* results of proximate composition showed great variations and in this case, it is species-specific. The three-sample species are potential source of nutrients and minerals. The cytotoxic activity of the brine shrimp lethality test differs in selected sea urchins. The combined spines, tests and gonads of the samples showed non-toxic. Local folks eat the edible gonads and sell as source of livelihood. The result serves as an excellent base-line data for Philippine sea urchins and as input for future policy making on marine preservation and conservation within the archipelago. Furthermore, zoochemical analysis of the three species is desirable for they contain higher percentage of proximate composition.

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