Untargeted Metabolomics of the Sea Cucumber, *Bohadschia marmorata* (Jaeger, 1833)

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Abstract

The marine invertebrates such as sea cucumber are regarded as sources of diverse bioactive compounds with promising health and nutritional benefits. Many commercially traded sea cucumber species have been explored, yet none of the local low-value types in the province of La Union, Philippines. This study aimed to establish a baseline metabolomic profile of Bohadschia marmorata (Jaeger, 1833). The identification of compounds in its body wall utilized an Orbitrap MS-based approach. Metabolites were annotated using Compound Discover 3.2, and similarity searches were performed from online databases. Eleven putatively identified compounds, which included saponin (3beta,5xi,9xi)-28-Hydroxy-28-oxoolean-12-en-3-yl-beta-Dgalac topyranosyl-(1-3)-[beta-D-glucopyranosyl-(1-2)]-beta-D-glucopyranosiduronic acid, fatty acids and their byproducts such as eicosanoid (11,12-Epoxy-(5Z,8Z,11Z)-icosatrienoic acid) and prostanoids (13,14-dihydro Prostaglandin F1?; 13,14-Dihydro-15-keto Prostaglandin A2), dicarboxylic acids (tetradecanedioic acid; dodecanedioic acid; suberic acid; NP-001596), and amino acid (4-Oxoproline). The metabolic profile of the B. marmorata body wall showed a diverse group of representative metabolites common among holothurians associated with innate defense mechanisms, structural composition, and products of metabolic pathways. There is, however, a need to further isolate these compounds using other methods and test their biological activities.

Keywords*:* holothurians, metabolites, Philippine sea cucumber, sea cucumber spicules, triterpenoid saponin

1. Introduction

Marine invertebrates, specifically sea cucumbers, are usually consumed as seafood (Olivera-Castillo *et al.*, 2013; Shi *et al.*, 2016; Chieu *et al.*, 2019; Puspitasari *et al.*, 2022; Siddiqui *et al.*, 2022). Traditionally, it is considered a delicacy in China, Japan, Korea, and the Philippines (Wen *et al.*, 2016; Sánchez-Solís *et al.*, 2021). Holothurians generally have high concentrations of cerebrosides, polysaccharides, and saponins, which are the most abundant and significant (Hu *et al.*, 2012). The nutritional effects of sea cucumbers have increased interest among locals since they could facilitate wound healing besides reducing gastric ulcers, inflammation, arthritis, pain, gout, asthma, eczema, hyperglycemia, and hypertension (Ridzwan *et al.*, 2014; Kwan, 2011; Rasyid and Putra, 2023).

Holothurians are also remarkable sources of vitamins, minerals, and amino acids associated with various biological properties, including anti-cancer (Janakiram *et al.*, 2015; Sajwani, 2019), antithrombotic (Glauser *et al.*, 2013; Yan *et al.*, 2021; Hosseini *et al.*, 2022), antimicrobial (Cusimano *et al.*, 2019), antioxidant (Althunibat *et al.*, 2013; Guo *et al.*, 2020; Senadheera, 2020), anti-hyperlipidemic (Wu *et al.*, 2016; Li *et al.*, 2017), anti-hyperglycemic (Wang *et al.*, 2016), anti-inflammatory (Song *et al.*, 2015; Kareh *et al.*, 2018; Zhang *et al.*, 2021), and anti-hypertensive properties (Sadegh Vishkaei *et al.*, 2016). Metabolically active compounds, like triterpene glycosides and cerebrosides, polysaccharides and acid mucopolysaccharides, glycolipids, sulfated glycosaminoglycan, and phosphatidylcholines present in sea cucumbers are also linked to their therapeutic properties and medicinal benefits (Dhinakaran and Lipton, 2014; Silchenko *et al.*, 2016; Mondol *et al.*, 2017; Bahrami *et al.*, 2018; Kalinin *et al.*, 2019; Mansur, 2022; Mohamadzadeasl and Khodabandeh, 2023).

More recently, studies on the chemical profile of sea cucumber species' whole body or body parts were done. Ceesay *et al.* (2019) identified the presence of phenols, terpenoids, carbohydrates, flavonoids, saponins, glycosides, steroids, phlobatannins, and tannins from organs of *Holothuria leucospilota*. High concentrations of 2-Pentanone, 4-hydroxy-4-methyl, phenol-2,4-bis (1,1-dimethyl ethyl)-, and 2-Chlorooctane were also detected (Ceesay *et al.*, 2019; Hossain *et al.*, 2022). Yin *et al.* (2020) detected through Liquid chromatography-mass spectroscopy (LC-MS) low levels of docosahexaenoic acid, eicosapentaenoic acid, and glycerophospholipids in hot water-treated *Apostichopus japonicas* body wall (Keipour *et al.*, 2023).

On the other hand, Popov *et al.* (2017) applied LC-MS coupled with electrospray ionization to various body parts of *Eupentacta fraudatrix*, identifying a total of 54 compounds including sulfated, non-sulfated, and disulfated glycosides. Hossain *et al.* (2022) also revealed phenolic compounds and flavonoids from the internal organs of the *Cucumari frondosa*, an Atlantic sea cucumber, using ultra-high performance liquid chromatography-mass spectrometry (HPLC-MS) with quadrupole time-of-flight.

Torreno *et al.* (2021) established a mass spectrometry saponin profile from *Stichopus horrens* sea cucumbers in the Philippines. They identified 22 saponins, including holothurinosides, impatienoside, and stichloroside (Popov, 2002), which prevent tumor formation and have anticancer properties and antifungal activities.

The province of La Union in Region 1, Philippines is bordered to the west by the shores of the South China Sea; hence, fishing is one of the means of livelihood for local folks. The South China Sea abounds with sea cucumbers, and these are collected and processed by the fishermen (Choo, 2008). Naturally, sea cucumbers are found in shallow intertidal zones such as mangroves, mud or sand flats, sea grass beds, and shallow reefs (Altamirano, 2017). They are easily gathered by hand or with the aid of basic instruments like spears and nets (Mercier and Hamel, 2013). Desirable cucumbers are sold in the market since sea cucumbers are considered a delicacy locally. However, some low-value, sea cucumber species are often discarded.

Bohadschia marmorata (Jaeger, 1833) is a harmless species that has an Indo-Pacific distribution and reef-associated in a depth range of up to 20 m (Schoppe, 2000). *B. marmorata* is said to be rich in calcium, iron, protein, omega-3, selenium, vitamin A, and zinc (SeaLifeBase, 2023). However, there is incomplete knowledge of the metabolomic profile of this species. Since this sea cucumber species is common in La Union, this study on the untargeted metabolomic profile of *B. marmorata* was conducted to add more knowledge on the chemical profile of *B. marmorata* that will not only benefit researchers and aqua-scientists, but also the students, science teachers, and local folks in La Union. It can pave the way for conservation efforts not only for this species in particular but also for other sea cucumbers abundant in the area in general. It may also open possibilities for other research that explores the isolation and testing of the medicinal uses of the identified metabolites.

2. Methodology

2.1 Study Design and Locale of the Study

The descriptive study (Protocol No. SLU-REC 2022-035) utilized laboratory techniques to identify and characterize the detected metabolites. Prior informed consent was secured from the Municipality of Balaoan, La Union, Philippines. Permission (Gratuitous Permit No. 0236-022) was obtained from the Bureau of Fisheries and Aquatic Resources, Quezon City, to use the purchased samples in the study.

2.2 Collection and Taxonomic Identification

Fresh sea cucumber samples were bought from a local market in Barangay Paraoir, Balaoan, and selection were based on observed features before morphometric assessment (Kim *et al.*, 2013; Nishanthan *et al.*, 2018; Purschke, 2020; Keipour *et al.*, 2023). Following the procedure by Keshavarz *et al.* (2021), the sea cucumber samples were transported in seawater to the Ilocos Training and Regional Medical Center (ITRMC), Department of Laboratories at Barangay Parian, City of San Fernando.

Sea cucumbers with a body length ranging from 10-15 cm (Desmelati *et al.*, 2020) were randomly selected. Identification was done through the observation of external morphology (Arriesgado *et al.*, 2022; Jontila, 2023), microscopic examination of dermal ossicles from tissue samples, identification keys (Olavides *et al.*, 2010; Kim *et al.*, 2013), and field guides (Jontilla, 2023).

The tegument's dorsal and ventral sections were sampled and tissues with a diameter of 1.0 mm were prepared and soaked for 30 min in 0.5-mL dilute sodium hypochlorite solution. Then 10-µL of the sediment was placed on a glass slide mounted with a coverslip and observed through a bright field microscope with a blue filter (Olympus CX22, China) (Lakshmi *et al.*, 2008), particularly its ossicles. Documentation was done through a Vivo X80 phone camera (Vivo Communication Technology Co. Ltd., Chine).

2.3 Metabolomic Profiling

The internal organs of the sea cucumber (*B. marmorata*) was removed, body wall was rinsed with distilled water, sliced into pieces that were about 1 cm

by 1 cm, sun-dried for five to seven days, ground into a powder using a blender, sieved through fine mesh, and kept in a cool, dry place. The powder-dried sea cucumber body wall was submitted to the Philippine Genome Center-Protein, Proteomics, and Metabolomics Facility, University of the Philippines, Diliman, Quezon City for the untargeted metabolomic analysis. Based on the protocol of the laboratory, the test raw material was extracted using HPLC-grade methanol (Husni *et al.*, 2011). Subsequent analysis followed the procedures described by Li *et al.* (2019). Reverse-phase liquid chromatography was undertaken using a C18 column (Thermo Scientific™ Hypersil GOLD™ VANQUISH™, Massachusetts, United States) attached to Thermo Scientific Vanquish UHPLC system (Massachusetts, United States) (Mansur, 2022). Compounds were eluted using mobile phase gradients in formic acid (0.1%), ammonium citrate (10 mM), acetonitrile (95%), and acetonitrile (50%) at a flow rate of 0.2 mL/min (Campi *et al.*, 2023). Temperatures were maintained at 40 and 4 °C in the column and auto-sampler, respectively. Pre-programmed protocol in the equipment for linear gradient elution (Li *et al*., 2019) was used as follows: 0-1 min – 2% B; 1 min – 2% B; 17-17.5 min – 50% B, returning to 2% B for 18-20 min. Thermo Scientific's Heated-Electrospray Ionization Probe (HESI II) (Massachusetts, United States) was used with a spray voltage of 3,500 V. The sweep, sheath, and auxiliary gas flow rates were programmed at 2, 2.1, and 2 arbitrary units, respectively (Li *et al.*, 2019). The ion transfer tube temperature was at 275 °C. The mass scan was performed using Thermo Scientific™ Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Massachusetts, United States) at a 40 Hz rate ranging from 120-1,200 m/z (Maritha *et al.*, 2023).

2.4 Data Acquisition and Processing

Compound Discoverer 3.2 (Thermo Fisher Scientific, Massachusetts, United States) performed data pretreatments on the acquired raw mass spectrometry files. The accuracy of the identified metabolites was screened for their retention time, m/z ratio, and peak alignment (Stanstrup *et al.*, 2015; Fernandes *et al.*, 2022). The measurements were performed using 0.2 min retention time deviation and 5 ppm mass deviation as cited by Yin *et al.* (2021) and Tan *et al.* (2021). The sample's mass spectrometry matrix data, which included information on the ions' intensities and m/z-retention time pairings, was generated and exported. mzCloud™ and ChemSpider online databases integrated the target ions and determined the chemical formula. With blank samples, the background ions were eliminated. The potential identity of compounds was subsequently derived from the data (Ganna *et al.*, 2016; Li *et al.*, 2019; Purschke, 2020; Gray *et al.*, 2023).

2.5 Treatment of Data

The mzCloud™ mass spectrum database search was used in the compound identification, considering the matched peaks, collision energy, and other factors and parameters used in the analysis (Vinaixa *et al.*, 2016). Both high match and confidence scores provided high confidence in the annotated feature. ChemSpider database search was used to supplement the library search for unsuccessful hits in the mzCloud™ database (Pence and Williams, 2010; Ayers, 2012). Available data in METLIN™ facilitated the identification of chemical entities from the mass spectrometry experiments and the unknowns (Guijas *et al.*, 2018). The untargeted metabolomic profile analysis was done in the negative mode, for there were little to no peaks detected in the positive mode and no molecular networks established. The annotation of a putatively identified compound was based on an online database library search (Shang *et al.*, 2018; Vincenti *et al.*, 2020).

3. Results and Discussion

3.1 Taxonomic Description and Identification

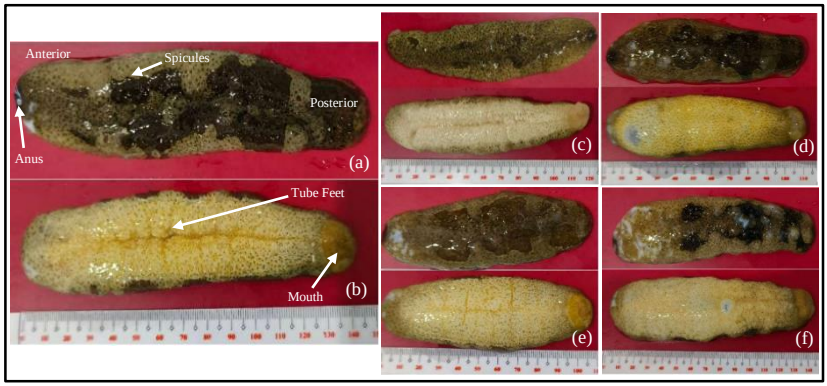
Five representative sea cucumber samples (Figures 1a to 1f) were measured, and the average length of its body was 12.47 cm, the average width was 3.66 cm, and the average weight of the wet samples was 49.97 g (Table 1). *Bohadschia* sp. are generally large; however, the average length and weight of *B. marmorata* in the study were less than those recorded by Eissa *et al.* (2017) and Arriesgado *et al.* (2022). The weight and size of sea cucumbers vary according to the species and stages at which they were collected. The chances of viscera loss and excretion of excessive water prior to measurement may have also contributed to the discrepancy in weight of the bought sea cucumbers (Ngaluafe *et al.*, 2013).

The body was cylindrical with a light-yellow ventral surface area and dorsolaterally located with variable light brown blotches in different shapes and sizes (Figure 1) (Kim *et al.*, 2013; Olivera-Castillo *et al.*, 2013; Masre, 2018). The mouth is at the anterior tapering end with a ring of refractile tentacles, and the anus is located at the posterior terminal end. The body wall is tough and leathery during tissue sampling for spicule analysis (Eissa *et al.*, 2017; Arriesgado *et al.*, 2022). Spicule shape was observed using brightfield

microscopy at 400x magnification. Typical smooth and rosette buttons, tables, spinous rods, and anchors were the observed shapes of the spicules (Figure 2) (Toral-Granda *et al.*, 2005; Eissa *et al.*, 2017; Torres *et al.*, 2019; Puspitasari *et al.*, 2022).

Table 1. Length, width, and weight of the five sea cucumber samples

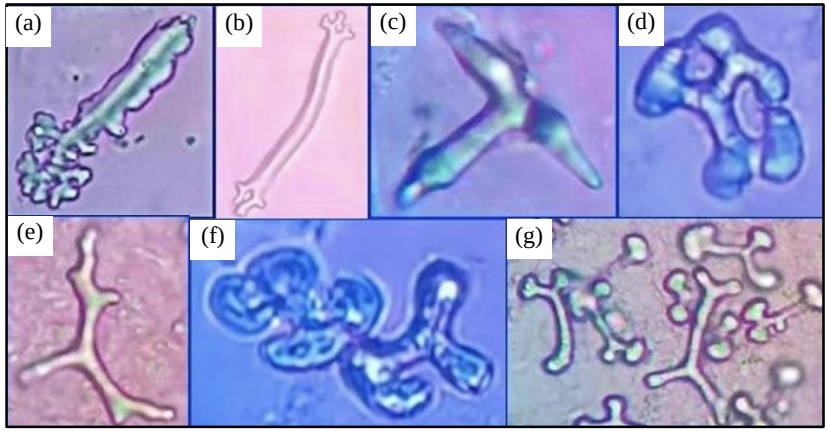
Sample no.	Length (cm)	Width (cm)	Weight (g)
092022-A	14.07	3.5	49.54
092022-B	11.90	3.4	35.07
092022-C	11.20	3.7	41.11
092022-D	10.67	3.8	58.05
092022-E	14.53	3.3	66.06
Average	12.47	3.66	49.97



Dorsal surface of sea cucumber sample 1 (a); ventral surface of sea cucumber sample 1 (b); sea cucumber samples 2 (c), 3 (d), 4 (e), and 5 (f)

Figure 1. External features of representative sea cucumber samples

The College of Fisheries, Don Mariano Marcos Memorial State University identified and confirmed the sea cucumber samples as *B. marmorata* (Jaeger, 1833). Diagnostic characters of *B. marmorata* include the following: length and width ratio less than 6 cm (average: 3.53 cm); lack of protuberances in the body wall; lack of anal teeth; the presence of nebulous spots on its dorsal surface wall and mamillated ventrolateral papillae; and a creamy to light brown back and a white to yellowish belly (Kim *et al.*, 2013; Künili, 2022; Di Simone *et al.*, 2022; Jontilla, 2023).



Note: A, B – rods; C – anchor; D – rosettes; E, F, G – branching rods

Figure 2. Observed shapes of mid-dorsal and mid-ventral body wall spicules of the sea cucumber (*bohadschia marmorata*) under the binocular microscope (400x, although printed enlarged for clearer view)

3.2 Metabolomic Profile of B. marmorata

The untargeted metabolic profile of the powder-dried *B. marmorata* body wall showed around 47 compounds, predominantly saponins, fatty acids (i.e., polyunsaturated fatty acid [PUFA]), and their derivatives, such as eicosanoids, prostanoids, dicarboxylic acids, and amino acids. Of the 47 detected, 11 were putatively identified. Table 2 presents the retention times of the compounds identified in the negative mode, the mzCloud™ match score, the detected mass, and the proposed formula. The match score corresponds to the score for the best hit from the mzCloud™ mass spectrum database. The confidence score considers the matched peaks, collision energy used, and other factors and parameters, such as the FISh (fragment ion search) score used in the analysis. Both high match and confidence scores provided high confidence for the putative identification and annotation of the compounds.

The predominant compound detected was the triterpenoid saponin similar to (3beta,5xi,9xi)-28-Hydroxy-28-oxoolean-12-en-3-yl-beta-D-galactopyranosyl-(1-3)-[beta-D-glucopyranosyl-(1-2)]-beta-D-glucopyranosiduronic acid as cited National Center for Biotechnology Information (2023) and Al Saedi *et al.* (2022). This saponin was not reported in the literature among *B. marmorata* and needed further chemical characterization. Its high proportion in the sample is supported by the study of Sroyraya *et al.* (2018), demonstrating saponins localized in the sea cucumber epidermis.

Table 2. *B. marmorata* body wall putatively-identified compounds

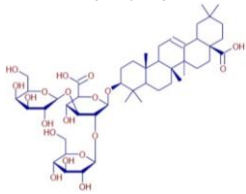
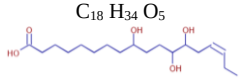
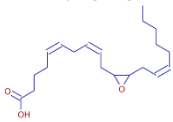
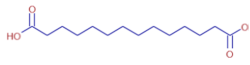
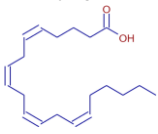
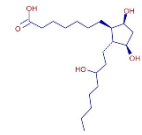
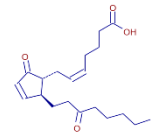
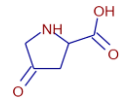
No.	Compound name	Ret. time (min)	mzCloud match score[a]	Detected mass	Proposed formula[b] and chemical structure
1	(3beta,5xi,9xi)-28-Hydroxy-28-oxoolean-12-en-3-yl-beta-D-galactopyranosyl-(1-3)-[beta-D-glucopyranosyl-(1-2)]-beta-D-glucopyranosiduronic acid	14.867	89.7	956.4983	$C_{48}H_{76}O_{19}$ 
2	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	13.102	99.6	330.2401	$C_{18}H_{34}O_5$ 
3	11,12-Epoxy-(5Z,8Z,11Z)-icosatrienoic acid	16.513	80.2	320.2346	$C_{20}H_{32}O_3$ 
4	Tetradecanedioic acid	14.819	98.4	258.1828	$C_{14}H_{26}O_4$ 
5	Arachidonic acid	18.252	99.8	304.2398	$C_{20}H_{32}O_2$ 
6	Dodecanedioic acid	13.693	99	230.1516	$C_{12}H_{22}O_4$ 
7	Suberic acid	9.976	99.6	174.0892	$C_8H_{14}O_4$ 
8	NP-001596	15.816	99.7	286.2142	$C_{16}H_{30}O_4$ 

Table 2 continued.

9	13,14-dihydro Prostaglandin F1?	13.959	99.5	358.2714	$C_{20}H_{38}O_5$ 
10	13,14-Dihydro-15-keto Prostaglandin A2	14.745	98.5	334.2139	$C_{20}H_{30}O_4$ 
11	4-Oxoproline	3.707	100	129.0426	$C_5H_7NO_3$ 

[a]Co-eluting peaks may occur during analysis, which can be observed for hits with relatively close retention times. [b]Proposed formula is provided by software and corresponds to the permutations of the chemical formula with the lowest ppm error.

Saponin is a biologically active molecule with pharmacological significance (Claereboudt *et al.*, 2019). Holothurians secrete saponins in their body wall and viscera as a chemical defense mechanism against predators (Van Dyck *et al.*, 2010; Bahrami, 2015; Feng *et al*., 2021; Yang *et al.*, 2021). Achmad *et al.* (2020) showed that sea cucumber saponins have anti-inflammatory potential by reducing cyclooxygenase-2 (COX-2) stimulation (Huang *et al.*, 2021; Hoang *et al.*, 2022). Lakshmi *et al.* (2008) evaluated the spermicidal activity of n-butanol fractionated lanostane triterpenoid bivittoside-D from *Bohadschia vitiensis* whole-body methanol extract. Results showed that bivittoside D was a potent spermicide, killing human sperm in seconds with a dose of 350-µM in vitro. Lakshmi *et al.* (2012) further showed the activity of bivittoside D against yeasts and fungi.

Three putative dicarboxylic acids were all secondary metabolites, namely Tetradecanedioic acid, Dodecanedioic acid, and Suberic acid, which are alpha, omega-dicarboxylic acids, with their methyl groups oxidized to their corresponding carboxylic acids (National Institute of Health, n.d.). Tetradecanedioic acid is an endogenous metabolite used as a drug-drug interaction biomarker for anion transport (Cambridge Bioscience, 2024). Dodecanedioic acid has a role as an alcohol dehydrogenase inhibitor (National Institute of Health, n.d.). Suberic acid has potential applications in the delivery of anti-cancer drugs and the fluorescent detection of dicarboxylic acids

(National Center for Advancing Translational Sciences, n.d.). NP001596, with the chemical formula $C_{16}H_{30}O_4$, is presumably another fatty acid. Rachmawati *et al.* (2023) classified a similar compound from a mixed vegetable fermentation extract as an endogenous metabolite. Other studies found the metabolite to prevent inflammation (Aparna *et al.*, 2012; Abdel-Motaal *et al.*, 2022; Trifani *et al.*, 2022), cytotoxicity (Ravi and Krishnan, 2017), anti-cancer activity on HT-29 cells (Bharath *et al.*, 2021; Cortes *et al.*, 2022; Kiashi *et al.*, 2022), and decoupling properties (Semenova *et al.*, 2021).

Two eicosanoid derivatives were identified, namely 13,14-dihydro Prostaglandin F1? and 13,14-Dihydro-15-keto Prostaglandin A2 (Walton *et al.*, 2002). Prostaglandins in sea cucumbers significantly promote germ cell proliferation, including the development of gonads and their tube diameter, including the gonadal-somatic index (Nontunha *et al.*, 2022). The amino acid identified, 4-Oxoproline, was also a secondary metabolite. Secondary metabolites may serve as defense or signaling molecules (Human Metabolome Database, 2021). Some studies show other amino acids (Bechtel *et al.*, 2013; Masre, 2018) present in sea cucumbers, like glutamic acid, leucine, and lysine, present in *Cucumaria frondosa* (Zhong *et al.*, 2007; Sales *et al.*, 2021), and glycine (Wen *et al.*, 2010; Feng *et al.*, 2021).

Arachidonic acid (ARA), an omega-6 PUFA, is an essential cell membrane component (Tallima *et al.*, 2015; Li *et al.*, 2018). It gives fluidity and flexibility, essential for its function, especially in nerve and muscle cells (Tallima and El Ridi, 2017). Careaga *et al.* (2013) also found ARA in *Athyonidium chilensis*. Similarly, it was the major PUFA in the eight common sea cucumbers evaluated by Wen *et al.* (2010).

4. Conclusion and Recommendation

This untargeted metabolomics for *B. marmorata* showed the presence of putatively identified essential compounds. However, there were challenges encountered in the identification of the compounds. For one, significant peaks in the chromatogram did not have good resolution. Identification was also a result of similarity searches in the online metabolite databases, considering the detected features of the compounds; hence, identification was only tentative. Nonetheless, this study provided initial results on possible essential

compounds found in low-value local sea cucumbers. Further studies for more definite identification of these compounds should be undertaken not only on *B. marmorata* but on other local low-value sea cucumbers. Isolation and testing of the biological activities of these compounds are also recommended.

5. Acknowledgment

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