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Article

Species identification and the biological properties of several Japanese starfish

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Abstract: Marine organisms are a rich source of natural products with potential secondary metabolites that have great pharmacological activity. Starfish are known as by-catch products in the worldwide fishing industry and most of starfish have been got rid of by fire destruction without any utilization. On the other hand, starfish are considered as extremely rich sources of biological active compounds in terms of having pharmacological activity. In the present study, molecular identification of starfish species, micronutrient content and hemolytic activity from *Luidia quinaria*, *Astropecten scoparius*, and *Patiria pectinifera* were examined. Nucleotide sequence analysis of the 16S rRNA gene fragment of mitochondrial DNA indicated that partial sequences of PCR products of the species was identical with that of *L. quinaria*, *A. scoparius*, and *P. pectinifera*. From the results of micronutrient contents, there were no great differences on the micronutrient among species. However, Cd, Cu, and as contents had species-specificity. The crude extract of three starfish showed hemolytic activity against 2% rabbit erythrocytes with 50% hemolytic concentration of 10-1000 µg/mL. The findings of the present study provided some basic information about identification of starfish species, potentialities of starfish which could be utilized in food and pharmaceutical industry.

Keywords: starfish; identification; micronutrient content; hemolytic activity

1. Introduction

Starfish are star-shaped echinodermata, belonging to the class Asteroidea. The large outbreak of starfish has been observed in worldwide ocean. Starfish have the ability to consume a wide range of food source including mussels, scallops, clams, and some seabed fish, which causes considerable decline in the number of commercial shellfish (Global Invasive Species Database, 2016). Starfish are considered as an extremely rich source of biologically active components, such as glycosylceramide, steroidal glycosides, ceramide, and cerebrosides (Inagaki *et al*., 2006; Ishii *et al*., 2006; Suh *et al*., 2011). Steroidal glycosides are the main metabolites of starfish and possess most toxicity (D'Auria *et al*., 1993). In particular, steroidal glycosides and related compounds are predominant metabolites in starfish and have a broad variety of biological activities such as cytotoxic, hemolytic, ichthyotoxic, repellent, antineoplastic, antimicrobial, antifungal, antiviral and anti-inflammatory (Lee *et al*., 2014; Thao *et al*., 2014; Nina *et al*., 2003).

Starfish has identified as a serious pest species because of its ability to consume a wide range of food sources including mussels, scallops, and clams (Global invasive species database, 2016). The wastes of them results in serious environmental pollution. Furthermore the large outbreak of starfish causes significant loss of the marine ecosystem and fishing gears (Kim, 1969). There is no effective method to control these large outbreaks of starfish. Extensive investigations of starfish, chemically and pharmacologically analysis in now demanded for utilization of starfish resources.

Therefore, it is necessary to identify the starfish species and to know their biological properties for advanced utilization. Firstly starfish species identification was done by PCR amplification method and then micronutrient content of starfish was evaluated. In addition, hemolytic activity was determined and the activity was compared with two other plant saponins, quillaja bark and ted seed.

2. Materials and Methods

2.1. Sample collection

Luidia quinaria, *Astropecten scoparius*, and *Patiria pectinifera* was collected from the coast of Kobe, Hyogo prefecture, Japan in February 2014 and immediately brought to the laboratory in fresh conditions in ice. Freshly collected samples were immediately washed to remove mud and other particles and subsequently stored at -60°C until use.

2.2. Species identification based on DNA analysis

Genomic DNA was extracted from tube feet of starfish sample by using Quick gene-810 (Kurabo, Tokyo, Japan) as recommended by the manufacturer. The DNA concentration (ng/µL) was measured by a Biospec Nano (Shimadzu Corporation, Tokyo, Japan). A partial region of the mitochondrial 16S rRNA gene was amplified by the conventional polymerase chain reaction (PCR) using universal primers (16SarL, 5'-CGCCTGTTTATCAAAAACAT-3' and 16SbrH, 5'-CCGGTCGAAACTCAGATCACGT-3'). Briefly, the reaction mixture for PCR was carried out in the 50 µL volume containing 5µL (50 ng) of genomic DNA, 4 µL of dNTP (2.5 mM each), 5 µL of 10 × Ex Taq buffer, 0.4 µL of Ex Taq DNA polymerase (Takara Shuzo, Japan) (5 U/µL), and 1.5 µL of 20 µM of each primer,16SarL and 16SbrH. PCR amplification was performed with Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 30 cycles consisting of denaturation at 98°C for 10 s, annealing at 53°C for 30 s and extension at 72°C for 60 s. The amplified PCR products were run in 1.2% agarose gel containing SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA). The gel was run at 100 V for 30 min and visualized using LAS-4000 mini documentation system (Fujifilm Cooperation, Tokyo, Japan). The PCR samples were sequenced with BigDye® terminator V3.1 Cycle Sequencing Kit and ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the obtained sequence data were analyzed by SeqEd Version 1.0.3 (Perkin Elmer, Foster City, CA, USA) software. The sequences were subjected to blast search with national center for biotechnology information (NCBI) data base. These sequences were then aligned using the default settings in ClustalW package in the MEGA 6 software (Tamura *et al.*, 2013).

2.3. Analysis of inorganic components

The micronutrient content of starfish was determined. Briefly, 1 g of sample was dissolved in a furnace and diluted to 200 mL with 1N HCl, and the diluted solution was analyzed using combustion thermal conductivity (CTC) and inductively coupled plasma spectrophotometer (ICP) method. Moisture content was determined with minced starfish sample by drying in an oven at a temperature of 105°C for 5 h.

2.4. Preparation of crude extracts

The extraction procedure was followed according to the method described by Yasumoto *et al.* (1966). The sample preparation method with various solvents is shown in Figure 3. Briefly, one kilogram of starfish was cut into small pieces and then minced using a food grinder (Kitchen Aid, St. Joseph, Michigan, USA). The extraction was carried out with 3 L of methanol and repeated twice with 2 L of methanol. The extract was filtered through Whatman filter paper No. 2. The filtrate was concentrated up to 250 mL with a rotary evaporator (EYELA, Tokyo, Japan) under reduced pressure at 45 ºC. The concentrate was stirred with an equal volume of water (250 mL) and defatted with 250 mL benzene. After being freed from benzene, pH of the extract was adjusted to 3 with 1N hydrochloric acid and then neutralized using 1N sodium hydroxide. The extract was dialyzed through an ultra-filtration membrane (MWCO: 1000, Millipore-amicon, Billerica, MA, USA) and then partitioned with *n*-butanol three times. After the *n*-butanol extract was concentrated up to 150 mL, three volume of diethyl ether and a half volume of water were added. Finally, the aqueous layer was freeze-dried.

2.5. Hemolytic activity

Rabbit blood was obtained from the Japanese Biological Center (Tokyo, Japan). Hemolytic activity was determined according to the method described by Charles *et al.* (2009) with slight modifications. Briefly, 2 mL of aliquot of blood were washed three times with phosphate buffer saline (PBS) solution (0.15 M NaCl-0.01M Tris-HCl, pH 7.0) by centrifugation at 1,090 ×*g* for 5 min at 4 ºC. Washed erythrocytes were suspended in the

PBS solution to obtain a concentration of 2%. Then, 0.5 mL of erythrocytes and 1 mL of PBS solution were mixed with 0.5 mL diluents containing 1, 5, 10, 50, 100, 500, and 1,000 µg/mL of individual crude starfish saponin in PBS solution. The mixtures were incubated for 30 min at 37 ºC and centrifuged at 270 ×*g* for 5 min. A volume of 1.5 mL PBS and distilled water were used as minimal and maximal hemolytic controls, respectively. After centrifugation, the presence of a suspension of a uniform red color was considered to indicate hemolysis, and a button formation in the bottom of the wells constituted a lack of hemolysis. A volume of 250 µL of each supernatant was transferred to a 96-well flat bottom micro plate, and the absorbance at 540 nm was measured with a micro plate reader (680 Microplate readers, BIO-RAD, Tokyo, Japan). The experiment was done in triplicate, and each sample was transferred three times into a 96-well micro plate. The concentrations that induced hemolysis of 50% of erythrocytes hemolysis (HC_{50}) in the different crude extract were calculated. Quillaja bark and Tea seed saponins were obtained from Wako Pure Chemical industry (Osaka, Japan) and Sigma Aldrich (St. Louis, MO, USA), respectively, and were used for comparison with starfish extract. All experiments were done in triplicate for the analysis of each HC_{50} and expressed as mean ± standard deviation (SD).

3. Results and Discussion

3.1. Identification of starfish species

Molecular identification of starfish by DNA-based method was carried out by a direct DNA sequencing analysis. Partial nucleotide sequence data of 16S rRNA gene was compared with NCBI gene data base. Figure 1 shows agarose gel electrophoresis results and Figure 2 shows aligned DNA sequences of the amplified partial 16S rRNA region from *L. quinaria*, *A. scoparius*, and *P. pectinifera.* Sequencing analysis confirmed that the PCR products of three species of starfish had 99 % identity with the partial regions. Molecular identification of starfish is enabled by using the nucleotide sequence encoding 16S rRNA gene of mtDNA. From the results of the alignment with the estimated species, it was found that the partial sequences of the PCR products from four samples were almost identical with those of *L. quinaria* (99.20%), *A. scoparius* (99.17%), and *P. pectinifera* (99.06%) (Table 1).Thus, it was confirmed that identification of starfish is enabled by using the nucleotide sequence encoding 16S rRNA gene of mtDNA. Based on mitochondrial 16S rDNA sequences, Wada *et al.* (1996) suggested that the Luidiidae (*L. quinaria*) is a sister group of the rest of asteroids, including the Astropectinidae. It was hypothesized that the universal primer (16SarL and 16SbrH) could be amplified the partial region of *L. quinaria*, *A. scoparius*, and *P. pectinifera*. As a result, partial 16S rRNA region of all the starfish used in this study could be amplified by using the universal primers (Figure 1). PCR products of *L. quinaria*, *A. scoparius*, and *P. pectinifera* had a length of approximately 500 bp (Figure 2).

3.2. Analysis of inorganic components

The result of moisture and micronutrient content of three starfish *L. quinaria*, *A. scoparius*, and *P. pectinfera* are shown in Table 2. The Cu content was found in *P. pectinfera* with the value of 10.0 µg/g and followed by *A. scoparius.* The As and Cd content were found higher in *P. pectinfera* with the value of 2.10 µg/g. Likewise, high level of Ca content was observed in *A. scoparius* with 16.30% of dry weight in the inorganic analysis coupled and relative high level of C content was found in *P. pectinfera* with 9.00% dry weight. Furthermore, the water content was observed higher in *L. quinaria* with 64.20% followed by *P. pectinfera.* The results of Table 2 showed that there are no great differences on the micronutrient content among starfish species. However, Cd, Cu, and As contents had species-specificity. Ca and P are minerals that have an important role to the development and maintenance of the skeleton, together with many other physiologic functions in the body. Ca content was found to be higher in *A. scoparius* with 16.30% followed by *L. quinaria* with 10.60%. On the other hand, moisture content of the starfish *L. quinaria* was detected as 64.2%, which was higher to the starfish of *A. planci* with the value of 67.7 to 69.1% by weight (Luo *et al.*, 2011). When formulating diets for animals (pigs, chicken, and fish) or fertilizer for agriculture sector, it is necessary to consider an appropriate amount of micronutrient content since excess or deficiency in one of the minerals will cause impaired utilization of the other (Gonzalez-Vega *et al.*, 2013). Living starfish contain on average 1.65% N and 0.15% P of wet weight. Therefore, the removal of 10,000 t of starfish, would directly remove approximately 165 t N and 15 t P from the coastal area (Petersen *et al.*, 2014). Nitrogen is considered to be the most important nutrient, and plants absorb more than any other elements. Thus, starfish has a great possibility to use as plant growth enhancer.

3.3. Hemolytic activity

Hemolytic activity of starfish saponin was evaluated using rabbit erythrocytes. Positive control (100 % hemolysis) by distilled water and negative control (0% hemolysis) by PBS were used as standard references.

The activity was expressed as the concentrations inducing 50% of erythrocytes hemolysis (HC_{50}) and HC_{50} was determined at the concentrations of 10-100 µg/mL. The HC_{50} values indicate that starfish crude extract *L. quinaria*, *A. scoparius*, and *P. pectinifera*, induced hemolysis against rabbit erythrocyte with HC_{50} values of 34.1 ± 4.00, 37.8 ± 2.16, and 31.1 ± 6.52 µg/mL, respectively (Figure 4). Furthermore, the HC_{50} values of plant saponins (18.6 ± 2.51µg/mL for quillaja bark and 27.0 ± 5.94 µg/mL for tea seed) were significantly different from those of starfish crude extract such as *L. quinaria*, *A. scoparius*, and *P. pectinifera.* The present results support the findings that Imamichi and Yokoyama (2013) reported that crude extract from pyloric caeca of *Asterias amurensis* showed high hemolytic activity in rabbit erythrocytes. The crude extract of *Ophiocoma erinaceus* showed hemolytic activity at 80% ethanol fraction against rat erythrocytes (Amini *et al.*, 2014). Pant saponin and starfish extract showed different hemolytic activity. Choi *et al.* (2001) reported that all saponins not have hemolytic activity.

Table 1. Homology analysis results of sequence.

Sample no	Species name	Identities	Max identity
A	*Luidia quinaria*	498/502	99.20%
B	*Astropecten scoparius*	453/457	99.17%
C	*Patiria pectinifera*	525/530	99.06%

Table 2. Micronutrient contents of three starfish collected from Japan.

Content	Unit	*L. quinaria*	*A. scoparius*	*P. pectinfera*	Method
Water	%	64.20	50.00	58.90	105ºC
C	%	6.50	7.70	9.00	CTC
N	%	0.87	0.67	1.60	CTC
P	%	0.05	0.04	0.06	ICP
K	%	0.49	0.48	0.93	ICP
Na	%	0.25	0.28	0.34	ICP
Ca	%	10.60	16.30	10.40	ICP
Mg	%	0.78	1.50	1.00	ICP
Cd	µg/g	0.03	0.05	1.70	ICP
Cu	µg/g	2.50	4.90	10.00	ICP
Pb	µg/g	0.64	0.58	0.43	ICP
Hg	µg/g	0.02	0.01	0.06	AAS
As	µg/g	0.77	0.92	2.10	ICP

CTC, comblustion thermal conductivity method; ICP, inductively coupled plasma spectrophotometer method; AAS, atomic absorbtion spectrometer method.

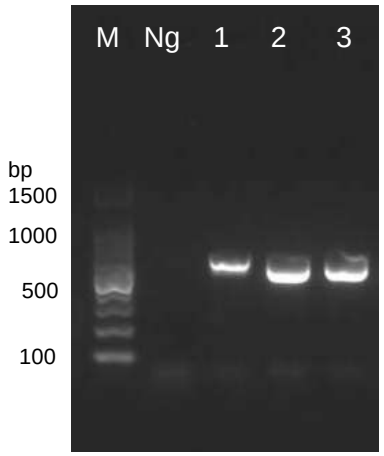

Figure 1. 1.2% agrose gel electrophoresis of PCR amplified product. M, 1500 bp DNA ladder. A, B, and C were samples *L. quinaria, A. scoparius* and *P. pectinifera,* respectively. Universal primers 16SAR-L, 5'-CGCCTGTTTATCAAAAACAT- 3' and 16SBR-H, 5'- CCGGTCTGAACTCAGA TCACGT- 3' for partial 16S rRNA gene were used.

(A)

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Sample A        - - - C T G C C C A G T G A C T T A G T T A A A C G G C C G C G G T A T C T T G A C C G T G C A A A G G T A G C A T A A  57
Luidia quinaria T G C C T G C C C A G T G A C T T A G T T A A A C G G C C G C G G T A T C T T G A C C G T G C A A A G G T A G C A T A A  60
                  * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample A        T C A T T T G C C T C T T A A A T A G A G G C T G G T A T G A A T G G C A A G A C T G G G G T T A A G C T G T C T C T T  117
Luidia quinaria T C A T T T G C C T C T T A A A T A G A G G C T G G T A T G A A T G G C A A G A C T G G G G T T A A G C T G T C T C T T  120
                * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample A        T C T T A T A A C T T G A A T T T T A T A T T T T C G T G A A G A A G C G G A A A T A A A C T C G T A G G A C G A G A A  177
Luidia quinaria T C T T A T A A C T T G A A T T T T A T A T T T T C G T G A A G A A G C G G A A A T A A A C T C G T A G G A C G A G A A  180
                * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample B        G A C C C T G T C G A G C T T T A G T A A A A A T A T T A G T G G T A A T A A A G A A T A A T A A A T C T A A T G T T A  237
Luidia quinaria G A C C C T G T C G A G C T T T A G T A A A A A T A T T A G T G G T A A T A A A G A A T A A T A A A T C T A A T G T T A  240
                * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample A        A A C T T A T A A A A A T T T T A T A A A A T C T A A T T T T T T A T T C T T A G C C T T T T A A T A T A C T A A C T T  297
Luidia quinaria A A C T T A T A A A A A T T T T A T A A A A T C T A A T T T T T T A T T C T T A G C C C T T T A A T A T A C T A A C T T  300
                * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample A        T G A T T G G G G C A A T C G C G G A G T A T A A A A A T C C T C C G C T A A A A A C A T A A A A A A - T A A C C A T T  356
Luidia quinaria T G A T T G G G G C A A T C G C G G A G T A T A A A A A G C C T C C G C T A A A A A C A T A A A A A A A T A A C C A T T  360
                * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample B        T T A A A A T A A A G T G A T C C G C T A C A T A G C G A G C A A A G G A A T A A G T T A C C G C A G G G A T A A C A G  416
Luidia quinaria T T A A A A T A A A G T G A T C C G C T A G A T A G C G A G C A A A G G A A T A A G T T A C C G C A G G G A T A A C A G  420
                * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample A        C G T A A T T T T T T T G G A G A G T T C A T A T T G A T A A A A A A G T T T G C G A C C T C G A T G T T G G A T C G A  476
Luidia quinaria C G T A A T T T T T T T G G A G A G T T C A T A T T G A T A A A A A A G T T T G C G A C C T C G A T G T T G G A T C G A  480
                * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample A        G A T T T C C T A G A G A T G C A G C A G T C T C T  502
Luidia quinaria G A T T T C C T A G A G A T G C A G C A G T C T C T  506
                * * * * * * * * * * * * * * * * * * * * * * * * *
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(B)

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Sample B            A A A G A G T C C T G A C C T G C C C A G T G A C G A A A G T T A A A C G G C C G C G G T A T T T T G A C C G T G C A A   60
Astropecten scoparius - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - C G T - - - -    3
                                                                                                                        * * *

Sample B            A G G T A G C A T A A T C A T T T G C C T T T T A A A T G G A G G C T A G T A T G A A T G G C A A G A C G G G G G T T T  120
Astropecten scoparius - - - - - - - - - - - - - - T T T G C C T T T T A A - T G G A G G C T A G T A T G A A T G G C A A G A C G G G G G T T T   48
                                                  * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample B            A G C T G T C T C T C T T T T A G G A G C T T G A A T T T A T T A T C T T A G T G A A G A A A C T A A G A T A A G G T C  180
Astropecten scoparius A G C T G T C T C T C T T T T A G G A G C T T G A A T T T A T T A T C T T A G T G A A G A A A C T A A G A T A A G G T C  108
                      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample B            G T A G G A C G A G A A G A C C C T A T C G A G C T T T A G C T A T T A G T T A A A G T T A A G G G T T T T T T A T T G  240
Astropecten scoparius G T A G G A C G A G A A G A C C C T A T C G A G C T T T A G C T A T T A G T T A A A G T T A A G G G T T T T T T A T T G  168
                      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample B            T T A A G C T A A T A A A G T T A A A A A T T G A T T T T A C T T A A T A A T A A G A A T A A A C T G T T A A T A A A G  300
Astropecten scoparius T T A A G C T A A T A A A G T T A A A A A T T G A T T T T A C T T A A T A A T A A G A A T A A A C T G T T A A T A A A G  228
                      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample B            T G T T T T G G T T G G G G C A A C C A C G G A G A A A A A T C A A C C T C C G G T T A T A A A A T A G A A A A A A T T  360
Astropecten scoparius T G T T T T G G T T G G G G C A A C C A C G G A G A A A A A T C A A C C T C C G G T T A T A A A A T A G A A A A A A T T  288
                      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

SampleC             A C T A T T T T G T G A A A T G T G A A T T A T T A A G A A G T G A T C C A C T G T G A A A G T G A T C A A A G G A A C  420
Astropecten scoparius A C T A T T T T G T G A A A T G T G A A T T A T T A A G A A G T G A T C C A C T G T G A A A G T G A T C A A A G G A A C  348
                      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample B            A A G T T A C C G T A G G G A T A A C A G C G T A A T T T T T T T G G A G A G T T C A T A T T G A T A A A A A - G T T T  479
Astropecten scoparius A A G T T A C C G T A G G G A T A A C A G C G T A A T T T T T T T G G A G A G T T C A T A T T G A T A A A A A A G T T T  408
                      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Sample B            G C G A C C T C G A T G T T G G A T C G G G A C T T C C A G G A G A T G C A G C A G T - - C C A A G G - - - - - - - - -  528
Astropecten scoparius G C G A C C T C G A T G T T G G A T C G G G A C T T C C A G G A G A T G C A G C A G T T T C C A A G G G T T G G T C T G  468
                      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
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(C)

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Sample C          TGCCTGCCCAGTGACTTAGTTAAACGGCCGCGGTATCTTGACCGTGCAAAGGTAGCATAA   60
Patiria pectinifera TGCCTGCCCAGTGACTTAGTTAAACGGCCGCGGTATCTTGACCGTGCAAAGGTAGCATAA   60
                  **********************************************************

Sample C          TCATTTGCCTCTTAAATGGGGGCTGGTATGAATGGCAAGACGGGAATCAAGCTGTCTCTC   120
Patiria pectinifera TCATTTGCCTCTTAAATGGGGGCTGGTATGAATGGCAAGACGGGAATCAAGCTGTCTCTC   120
                  **********************************************************

Saample D         CCTTATATCTTGAAGTTAATATTTCTGTGAAGAAGCAGAAATGAGATCGCAGGACGAGAA   180
Patiria pectinifera CCTTATATCTTGAAGTTAATATTTCTGTGAAGAAGCAGAAATGAGATCGCAGGACGAGAA   180
                  **********************************************************

Sample C          GACCCTATCGAGCTTTAGTAGAAATAAGATATAA-CTTGTTAATAGTGTTTGTGCTGCTA   239
Patiria pectinifera GACCCTATCGAGCTTTAGTAGAAATAAGATATAAGCTTGTTAATAGTGTTTGTGCTGCTA   240
                  ************************************ ************************

Sample C          ATTCAATTGTTAAAAACAGTTCCTATAGTTTTACTGCTAAGAACATTAGTGTTGTATATA   299
Patiria pectinifera ATTCAATTGTTAAAAACAGTTCCTATAGTTTTACTGCTAAAAACATTAGTGTTGTATATA   300
                  ****************************************** ******************

Sample C          TAATACTTAGGTTGGGGCAACCGCGGAGAATAATTACCCTCCGTTAATTTTATTGAAAAA   359
Patiria pectinifera TAATACTTAGGTTGGGGCAACCGCGGAGAATAATTATCCTCCGTTAATTTTATTGAAAAA   360
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Sample D          ATAATTATTTTTCAATATAAGATTTATTAAAAGAGTGATCCACTGAAATGGTGAGCAAAA   419
Patiria pectinifera ATAATTATTTTTCAATATAAGATTTATTTAAAGAGTGATCCACTGAAATGGTGAGCAAAA   420
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Sample C          GAATAAGTTACCGTAGGGATAACAGCGTAATTTTTTTGGAGAGTTCATATTGATAAAAAA   479
Patiria pectinifera GAATAAGTTACCGTAGGGATAACAGCGTAATTTTTTTGGAGAGTTCATATTGATAAAAAA   480
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Sample C          GTTTGCGACCTCGATGTTGGATCGGGATT-CCTGGGGATGCAGCAGTCCC-  528
Patiria pectinifera GTTTGCGACCTCGATGTTGGATCGGGATTTCCTGGGGATGCAGCAGTTCCC  531
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Figure 2. Aligned nucleotide sequences of partial mitochondrial 16S ribosomal RNA gene from *L. quinaria* (A), *A. scoparius* (B), and *P. pectinifera* (C) starfish species. The identical nucleotides were shown by dots.

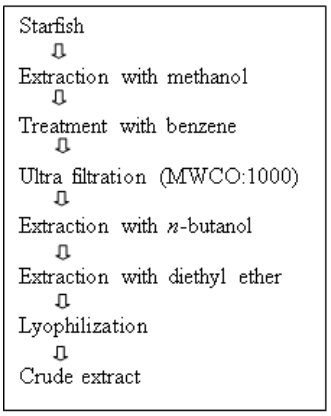

Figure 3. Schematic flow chart of crude extracts extraction.

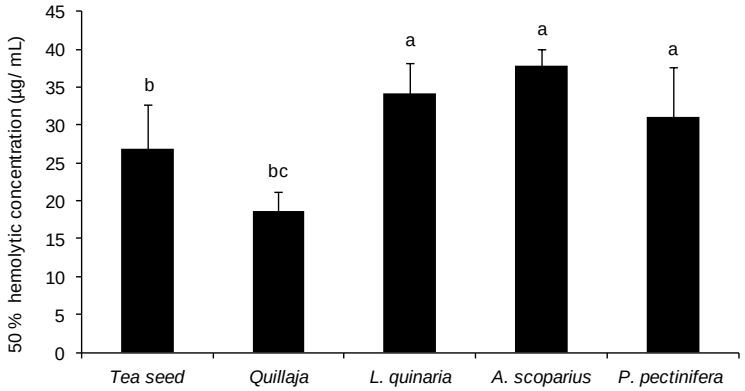

Figure 4. Hemolytic activity of crude extract from starfish against rabbit erythrocytes. Hemolytic activity was expressed as the concentrations of various crude extract that induced hemolysis in 50% of rabbit erythrocytes, and compared using one-way ANOVA followed by Tukey's test. Significant differences ($p < 0.05$) are represented by different letters over the columns.

4. Conclusions

In this study, we analyzed 16S rRNA gene fragment of mitochondrial DNA and confirmed that 16S rRNA markers are useful and applicable to identify *L. quinaria*, *A. scoparius*, and *P. pectinifera* species. The micronutrient results suggested that starfish, one of the marine resources expected to be used as formulation of animal diet and plant growth regulating activity. The hemolytic activity extended by starfish crude extract is very much appreciable for the future development of novel use in pharmaceutical ingredients. Further investigation is required to fully elucidate the hemolytic activity of the secondary metabolites in *L. quinaria*, *A. scoparius*, and *P. pectinifera.*

Conflict of interest

None to declear.

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