academicJournals

Vol. 16(4), pp. 163-170, 25 January 2017 DOI: 10.5897/AJB2016.15776 Article Number: D74472B62537 ISSN 1684-5315 Copyright © 2017 Author(s) retain the copyright of this article http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Molecular identification, micronutrient content, antifungal and hemolytic activity of starfish Asterias amurensis collected from Kobe coast, Japan

Farhana Sharmin*, Shoichiro Ishizaki and Yuji Nagashima

Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato, Tokyo108-8477, Japan.

Received 11 November, 2016; Accepted 6 January, 2017

The starfish Asterias amurensis is one of the common species in Japanese coastal area. The species is considered an extremely rich source of biological active compounds, including saponin. Saponins are secondary metabolites and exhibit a wide spectrum of biological activities. The species of starfish caught in Kobe, Hyogo prefecture, Japan, was analyzed for species identification and micronutrient content. The starfish extract was evaluated for different biological properties and its fractions obtained using thin layer chromatography (TLC). 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 *A. amurensis*. The micronutrient contents results showed that nitrogen (N) content of the starfish was 1.50% of dry weight and the copper content was 2.10 μ g/g. The crude extract of *A. amurensis* exhibited predominant growth inhibitory activity against six human fungal pathogens and also showed hemolytic activity against 2% sheep erythrocyte. The present findings suggest the possible pharmacological applications of *A. amurensis* that can be used as food ingredients and antifungal component.

Key words: Asterias amurensis, crude extract, biological property, saponin, antifungal component.

INTRODUCTION

Nowadays, invasion of exotic species has become a major concern in the marine environment since the number of human-mediated introduction has increased (Ruiz et al., 2000). Many studies researching marine invertebrates to determine the therapeutic potential of their bioactive materials have been showing very promising results (Lee et al., 2014). Starfish are marine

invertebrates and possess many useful pharmacological and biological activities. *Oreaster reticulates, Luidia senegalensis* and *Echinaster* sp. have been used as traditional medicine for thousands of years in China and North-eastern Brazil to treat asthma, bronchitis, diabetes, and heart and stomach diseases (Alves and Alves, 2011). Various biological active compounds and

*Corresponding author. E-mail: ginisaz05@yahoo.co.in.

Author(s) agree that this article remains permanently open access under the terms of the <u>Creative Commons Attribution</u> <u>License 4.0 International License</u> molecules have recently been identified from starfish glycosylceramide, steroidal glycosides, such as ceramide, and cerebrosides (Ishii et al., 2006; Suh et al., 2011). Saponins are a group of natural plant glycosides, characterized by their strong foam-forming properties in aqueous solution that occur in a wide range of plant species (Osbourn, 1996). The presence of saponin has been reported in more than 100 families of plants out of which at least 150 kinds of natural saponins have been found to possess significant anti-cancer properties (Man et al., 2010). Saponins have also been commonly employed in some other sector in medical practice because of their potential health benefits. In addition, many plant saponins have been isolated and they exhibit broad spectrum of biological uses, such as anti-cancer, anti-inflammatory, ion channel blocking, immune stimulating, antifungal, and anti-thrombotic property (Lee et al., 2014; Thao et al., 2014).

The starfish A. amurensis, called the northern Pacific starfish, is a predator of the marine benthic system that is listed in the top 100 invasive species globally (Lowe et al., 2000). The species was 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. Recently, many studies have been conducted to examine the activity of saponin against bacteria, fungi and even tumor cells (Robin et al., 2013). However, little information about the biological properties of starfish saponin has been reported. Extensive investigation of starfish, both chemically and pharmacologically, is now a demandable issue for utilization of starfish resources.

Therefore, it is necessary to clarify the molecular identification and biological properties of starfish for the advanced utilization of starfish resources. In this context, first of all species identification of starfish was done by PCR amplification method from the view point of large species variation. The micronutrient content of *A. amurensis* was evaluated for utilizing these in animal feed and plant growth promotion. Based on these experiments, we evaluated biological properties such as antifungal and hemolytic activity of *A. amurensis*.

MATERIALS AND METHODS

Sample collection

A. amurensis was collected from the coast of Kobe, Hyogo prefecture, Japan (latitude 34°41 24.3° N and a longitude of 135°11 43.84" E) 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.

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'). PCR was performed in 50 µl volume containing 5 µl of genomic DNA, 4 µl of dNTP, 5 µl of 10 × Ex Taq buffer, 0.4 µl of Ex Taq DNA polymerase (Takara Shuzo, Japan), and 1.5 µl of 20 µM of each primer. PCR amplification was performed with Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA). Amplifying conditions were 98°C for 10 s in denaturing, 53 °C for 30 s in annealing, and 72°C for 60s in extension for 30 cycles. 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 Clustal W package in the MEGA 6 software (Tamura et al., 2013).

Analysis of inorganic components

The micronutrient content of starfish was determined. Briefly, starfish was cut into small pieces and 1 g of starfish sample dissolved in a furnace and diluted to 200 mL with 1 N HCI. The diluted solution was then analyzed using combustion thermal conductivity (CTC) and inductively coupled plasma (ICP) spectrophotometer method. Moisture content was determined with minced starfish sample by drying in an oven at a temperature of 105° C for 5 h (Luo et al., 2011).

Preparation of crude extracts

The extraction procedure was followed according to the method described by Yasumoto et al. (1966). 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°C. 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 nbutanol 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 lyophilized and stored in a desiccator until further analysis. The crude extract samples (20 mg/ml) were applied to thin layer chromatography (TLC) plates (silica gel 60 F₂₅₄, 10 × 20 cm, layer thickness 0.25 mm, particle size of 10-20 µm; Merck, Darmstadt, Germany), developed in chloroform:methanol:water (65:35:10, lower phase) as the mobile phase. Plates were then sprayed with 50% sulfuric acid

followed by heating at 110°C for 10 min.

Antifungal activity

The antifungal activity of the starfish extract was performed using standard well diffusion method. Fungal strains were obtained from the Japan Collection of Microorganisms (JCM) and NITE Biological Resource Center (NBRC). Two yeasts Saccharomyces cerevisiae (JCM 2194) and Rhodotorula glutinis (JCM 8173), the three filamentous fungi such as Aspergillus flavus (JCM 12721), Cladosporium sphaerospermum (NBRC 4460), and Fusarium oxysporum (NBRC 5942), and the dermatophyte fungi Trichophyton mentagrophytes (NBRC 32410) was tested. Yeast and filamentous fungi were cultivated on potato dextrose agar, DAIGO (3.9%). Dermatophyte fungi were cultivated on sabouraud agar (4% glucose, 1% bactotrypton and 2% agar). The antifungal activity was evaluated with the disc diffusion method described by Reinheimer et al. (1990). One hundred microliters of cultured fungi was uniformly smeared on an agar plate by a smear loop. Then 8 mm diameter sterilized paper disc (Toyo Roshi Kaisha Ltd, Tokyo, Japan) was loaded with 70 µl of the crude extract sample at the concentrations of 10 mg/ml, and then placed on agar plate. Thereafter, the plates were incubated at 27°C for A. flavus, F. oxysporum, C. sphaerospermum and T. mentagrophytes for 48 h and at 30°C for S. cerevisiae and R. glutinis for 24 h. Tea seed saponin was obtained from Sigma Aldrich (St. Louis, MO, USA) used for positive control and distilled water was used for the negative control. A clear zone with a diameter was taken as antifungal activity. The whole analytical procedure of the experiment was carried out twice and replicated three times. The mean value of each experiment was considered for further data analyses.

Determination of minimum inhibitory concentration (MIC)

The fungal strains were grown on potato dextrose agar and sabouraud agar. After incubation, fungal growths were suspended in normal saline (0.9% NaCl). The minimum inhibitory concentration (MIC) values for each crude extract were determined through microdilution assay following a method described by Kumar et al. (2007) with minor modification. A 20 µl crude extract initially prepared (at the concentration of 10 mg/ml) and added into the first well. From here the solution was transferred into eight consecutive wells and then 10 µl inocula were added and kept in an incubator at 27°C (A. flavus, F. oxysporum and C. sphaerospermum) and 30°C (S. cerevisiae and R. glutinis). The fungal suspensions were adjusted with broth to a concentration of 1.0 to 5.0×10³ spore/ml and stored at 4°C for further analysis. A control was maintained with only culture medium of fungal cells. Values obtained for crude extract was compared with the values from control and the difference is considered as growth inhibit on activity. MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms. The experiments were done in 3 replicates and the mean values of the result were taken.

Hemolytic activity

Sheep 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, aliquot of 2 ml of blood were washed three times with PBS (phosphate buffer saline) solution (0.15 M NaCl-0.01M Tris-HCl, pH 7.0) by centrifugation at 1090 × g for 5 min at 4°C. 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 1000 µg/ml concentrations of individual crude starfish extract in PBS solution. The mixtures were incubated for 30 min at 37°C 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). Each sample was transferred three times into a 96-well micro plate. The experiments was done in triplicate and expressed as mean \pm standard deviation (SD).

Data analysis

Statistical analyses were performed using SPSS software (SPSS 16.0, IBM, USA). Data are expressed as mean \pm SD and compared with one way analysis of variance (ANOVA). Significant differences were determined by Tukey's test at p < 0.05 level.

RESULTS AND DISCUSSION

Identification of starfish species

First of all, species identification of starfish was done based on their phenotypic description including external structural appearance and morphological characteristics. Subsequently, species identification of starfish by DNAbased 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 aligned DNA sequences of the amplified partial 16S rRNA region from the samples. From the results of the alignment with the estimated species, it was found that the partial sequence of the PCR products from the sample was almost identical with those of A. amurensis (98.1%), although. Thus, it was confirmed that identification of starfish is enabled by using the nucleotide sequence encoding 16S rRNA gene of mtDNA. Mitochondrial gene order has been demonstrator to be one of the most useful methods for molecular identification (Matsubara et al., 2005). Previous studies have reported that the rapid diversification and adaptation to modern environments may conceal the primitive status of asteroid groups (Blake, 1987). Therefore, it was hypothesized that the universal primer (16SarL and 16SbrH) could be amplified in the partial region of A. amurensis. As a result, partial 16SrRNA region of the starfish used in this study could be amplified by using the universal primers. PCR products of A. amurensis had a length of approximately 400 bp (Figure 1).

Analysis of inorganic components

The result of moisture and micronutrient contents of A.

Sample	GAACTCTCCAAAAAAATTACGCTGTTATCCCTGCGGTAACTTATTCCTTTGCTCGCTATC 60
Asterias amurensis	GAACTCTCCAAAAAAATTACGNTGTTATCTGCGGTAACTTATTCCTTTGCTCGCTATC 58

Sample	TAGCGGATCACTTTATTTTAAAATGGTTATTTTTTATGTTTTTAGCGGAGGCTTTTTATA 120
Asterias amurensis	T A G C G G A T C A C T T T T T T A A A A T G G T T A T T T T
Sample	CTCCGCGATTGCCCCAATCAAAGTTAGTATATTAAAAGGCTAAGAATAAAAAATTAGATT 180
Asterias amurensis	CTCCGCGATTGCCCCAATCAAAGTTAGTATATTAAAAGGCTAAGAATAAAAAATTAGATT 178
	* * * * * * * * * * * * * * * * * * * *
Sample	TTATAAAATTTTTATAAGTTTAACATTAGATTTATTATTCTTTATTACCACTAATATTTT 240
Asterias amurensis	TTATAAAATTTTTATAAGTTTAACATTAGATTTATTATTCTTTATTACCACTAATATTTT 238
	* * * * * * * * * * * * * * * * * * * *
Sample	TACTAAAGCTCGACAGGGTCTTCTCGTCCTACGAGTTTATTTCCGCTTCTTCACGAAAAT 300
Asterias amurensis	TACTAAAGCTCGACAGGGTCTTCTCGTCCTACGAGTTTATTTCCGCTTCTTCACGAAAAT 298
	* * * * * * * * * * * * * * * * * * * *
Sample	ATAAAATTCAAGTTATAAGAAAGAGACAGCTTAACCCCAGTCTTGCCA- TTCATACCAGC 359
Asterias amurensis	ATAAAATTCAACTTATAAGAAAGAGACAGCTTAACCCCAGTCTTGCCACTTCATACCAGC 358
	******** *** **************************
Sample	CTCTATTTAAGAGGCAAATGATTATGCTACCTTTGCAC 397
Asterias amurensis	CTCTATTTAAGAGGCAA-TGATTATGCTACCTTTGCAC 395
	* * * * * * * * * * * * * * * * * * * *

Figure 1. Aligned nucleotide sequences of partial mitochondrial 16S ribosomal RNA gene from *A. amurensis*. The identical nucleotides were shown by dots.

Content	A. amurensis	Method	Unit
Water	63.90	105⁰C	%
С	8.10	CTC	%
Ν	1.50	CTC	%
Р	0.06	ICP	%
К	1.04	ICP	%
Na	0.32	ICP	%
Ca	9.40	ICP	%
Mg	0.60	ICP	%
Cd	0.13	ICP	µg/g
Cu	2.10	ICP	µg/g
Pb	0.25	ICP	µg/g
Hg	0.03	AAS	µg/g
As	2.10	ICP	ha/a

Table 1. Micronutrient contents of A. amurensis.

CTC, Combustion thermal conductivity method; ICP, inductively coupled plasma spectrophotometer method; AAS, atomic absorption spectrometer method.

amurensis is shown in Table 1. The Cu and As content were found with the similar value of 2.10 μ g/g. Likewise, high level of Ca content was observed with the value of 9.40% of dry weight in the inorganic analysis coupled.

The results in Table 1 showed that there are no great differences on the micronutrient content (Hg, Cd, Pb) in starfish species. However, Cd, Cu and As contents had species-specificity. Ca and P are minerals that have an



Figure 2. Antifungal activity of crude extracts from *A. amurensis* against six fungi, *S. cerevisiae* (JCM 2194), *R. glutinis* (JCM 8173), *A. flavus* (JCM 12721), *C. sphaerospermum* (NBRC 4460), *F. oxysporum* (NBRC 5942), and *T. mentagrophytes* (NBRC 32410) at the concentrations of 10 mg. Values are average of three replicates \pm standard deviation. Values with different letters indicate significant differences for p < 0.05 according to Tukey's test.

important role to the development and maintenance of the skeleton, together with many other physiologic functions in the body (Bonjour, 2011). The Ca content in fresh starfish is reported 12.97% by Sorensen (2015) but in our study we have got 9.40%, which might be due to the variation of species, size, season, source, analytical method etc. Moisture content of the starfish A. amurensis was detected as 63.9%, which was slightly lower than 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. Ishii et al. (2007) reported that the plant growth regulating activities of metabolites from the starfish A. amurensis, suggest that these active natural products could be employed for maintaining ecofriendly agricultural production.

Antifungal activity

The antifungal spectra of the crude extract from starfish against six fungi are presented in Figure 2. *A. amurensis* exhibited predominant growth inhibitory activity against all the human fungal pathogens tested. Growth inhibitory activity of *A. amurensis* was in the range of 14.4 to 25.5 mm. The strongest antifungal activity was observed in *A. amurensis* extract against *T. mentagrophytes*. Next to this, moderate levels of growth inhibitory activity were

found against S. cerevisiae and A. flavus (Figure 2). Choi et al. (1999) noted that the methanol and water extracts of Asterina pectinifera were sensitive to Aspergillus niger and T. mentagropytes. Recently, Suguna et al. (2014) reported that the highest antifungal activity found in nbutanol extract of Luidia maculate against Τ. mentagrophytes was 21.0 ± 1.00 mm. However, in our study, A. amurensis crude extract showed slightly higher zone inhibition activity (25.5 ± 1.15 mm) against T. mentagrophytes than that of L. maculate. In Figure 2, zone of inhibition was found significantly highest (p<0.05) in T. mentagrophytes compared to R. glutinis, C. sphaerospermum and F. oxysporum but not with other two fungal species (S. cerevisiae and A. flavus). The difference in antifungal activity of starfish extract from various species, extracted using different procedures, differ in their biological activities (Sen et al., 1998). Remarkable antifungal activity was exhibited by plant saponins from tea seed against S. cerevisiae and T. mentagrophytes with growth inhibitory activity of 24.1 and 23.1 mm, respectively. There was no growth inhibitory activity observed against A. flavus, R. glutinis, F. oxysporum, and C. sphaerospermum.

Minimum inhibitory concentration (MIC)

The extracts that showed antifungal activity in this assay were subjected to the minimum inhibitory concentrations

Table 2. Minimum inhibitory concentration (MIC) of *A. amurensis* crude extract against six fungal strains.

Fungal strains	MIC (µg/ml)
S. cerevisiae	62.50
R. glutinis	50.00
A. flavus	35.71
F. oxysporum	50.00
C. sphaerospermum	83.30
T. mentagrophytes	35.71

Data are the average of 3 independent replicates. Crude extract was added with amount of 500 μ g/ml.



Figure 3. Hemolytic activity of crude extract from *A. amurensis* against sheep erythrocytes. Hemolytic percents of saline and distilled water were included as minimal and maximal hemolytic controls. The data represent the mean \pm standard deviation; n=3.

(MIC) evaluation and the results are presented in Table 2. The crude extract of *A. amurensis* showed significant antifungal activity against *A. flavus* and *T. mentagrophytes* with the MIC value of 35.71 µg/ml for both. On the other hand, *R. glutinis* and *F. oxysporum* showed similar MIC value of 50.00 µg/ml. Among six fungal strain highest MIC value was obtained in *C. sphaerospermum* (83.30 µg/ml) followed by 62.5 µg/ml MIC in *S. cerevisiae*.

Hemolytic activity

Results of hemolytic activity have shown that, extract of

A. amurensis exhibited the maximum hemolytic activity with 98.78 \pm 7.63% at 1000 µg/ml concentration. There was no hemolytic activity observed at the concentration of 1 µg/ml while slightly activity observed at the concentration of 5 and 10 µg/ml. In general, the increase in concentration of test extracts from 50 to 1000 µg/ml has been found to increase the hemolytic activity (Figure 3). The crude extract of *A. amurensis* exhibited strong activity against sheep erythrocytes. Our results support the findings of Imamichi and Yokoyama (2013) who reported that crude extract from pyloric caeca of *A. amurensis* showed high hemolytic activity in rabbit erythrocytes. Hemolytic assays have also been developed for detecting saponin in drugs or plant extracts



Figure 4. Thin layer chromatography profiles of crude extract from *A. amurensis* solvent chloroform: methanol: water (65:35:10, lower phase). The plate was stained with 50% sulfuric acid followed by heating at 110°C for 10 min.

based on their ability to rupture erythrocytes. Hemolytic activity has been used by researchers to follow the isolation of saponin. It is the simplest and the fastest bioassay employed to detect and quantify some saponins and also for using clinical development (Choi et al., 2001).

Thin layer chromatography (TLC) analysis

TLC analysis of starfish extract revealed 8 fractions in *A. amurensis* (Figure 4). Figure 4 shows the chromatograms of *A. amurensis* crude extract consisted of several components, with R_f values ranging from 0.29 to 0.92. The qualitative analysis using TLC plays an important role in the study of saponin. Based on the analysis of the chromatogram and their retention factor, it was observed that these compounds consist together with the polar (1 to 6) and non polar (7 to 10) compounds. Raphaela et al. (2014) reported that the polarity of saponin varied because of the sugar units linked to its structure. According to Raphaela et al. (2014) the biological activity of saponins may be affected by many factors such as the aglycone, number, position, and chemical structure of sugar side-chains. Many natural products and materials from marine animals have been used to treat various kind of disease and might be good substances for the development drugs in pharmaceutical sector.

Conclusion

In this study, we analyzed 16S rRNA gene fragment of mitochondrial DNA and confirmed that 16S rRNA markers are useful and applicable to identify *A. amurensis* species. The present study revealed the potential antifungal and hemolytic activity of starfish *A. amurensis*. The biological properties of *A. amurensis* extended by starfish crude extract are very much appreciable for the future development of novel functional food and pharmaceutical ingredients. Furthermore, the current results have motivated us to carry further studies on isolation and characterization of the bioactive compounds of this starfish in order to evaluate their mechanism and mode of action.

Conflicts of Interests

The authors have not declared any conflict of interests.

REFERENCES

- Alves RR, Alves HN (2011). The faunal drugstore: animal-based remedies used in traditional medicines in Latin America. J. Ethnobiol. Ethnomed. 10:1746-4269.
- Blake DB (1987). A classification and phylogeny of post-palaeozoic sea stars (Asteridea: 98 Echinodermata). J. Nat. Hist. 21:481-528.
- Bonjour JP (2011). Calcium and phosphate: a duet of ions playing for bone health. J. Am. Coll. Nutr. 30(5):438S-48S.
- Charles G, Jean L, Karl GL, Vakhtang M, André P (2009). Haemolytic activity, cytotoxicity and membrane cell permeabilization of semisynthetic and natural lupane- and oleanane-type saponins. Bioorg. Med. Chem. 17:2002-2008.
- Choi DH, Shin S, Park IK (1999). Characterization of antimicrobial agents extracted from *Asterina pectinifera*. Int. J. Antimicrob. Agents 11:65-68.
- Choi S, Jung SY, Kim CH, Kim HS, Rhim H, Kim SC (2001). Effect of ginsenosides on voltage-dependent Ca²⁺ channel subtypes in bovine chromaffin cells. J. Ethnopharmacol. 74:75-81.
- Global Invasive Species Database (2016). Species profile: Asterias amurensis; http://www.iucngisd.org/gisd/species.php?sc=82, Accessed 17 May 2016.
- Imamichi Y, Yokoyama Y (2013). Purification and characterization of a lectin from the starfish *Asterias amurensis*. Fish. Sci. 79:1007-1013.
- Ishii T, Okino T, Mino Y (2006). A ceramide and cerebroside from the starfish Asterias amurensis Luetken and their plant-growth promotion activities. J. Nat. Prod. 69:1080-1082.

- Ishii T, Okino T, Mino Y, Tamiya H, Matsuda F (2007). Plant-growth regulators from common starfish (*Asterias amurensis* Lutken) waste. Plant Growth Regul. 52:131-139.
- Kim YS (1969). Selective feeding on the several bivalve mollusks by starfish, Asterias amurensis. Bull. Fac. Fish. Hokkaido Univ. 19:244-249.
- Kumar R, Kumar AC, Kumar PS, Lakshmia V (2007). Antifungal activity in triterpene glycosides from the sea cucumber Actinopyga lecanoraq. Bioorg. Med. Chem. Lett. 17:4387-4391.
- Lee CC, Hsieh HJ, Hsieh CH, Hwang DF (2014). Antioxidative and anticancer activities of various ethanolic extract fractions from crownof-thorns starfish (*Acanthaster planci*). Environ. Toxicol. Pharmacol. 38:761-773.
- Lowe S, Browne M, Boudjelas S, De Poorter M (2000). 100 of the world's worst invasive alien species a selection from the global invasive species database. The Invasive Species Specialist Group (ISSG), a specialist group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN), New Zealand.
- Luo P, Hu CQ, Xia JJ, Ren CH, Jiang X (2011). Chemical constituent analysis of the crown-of-thorns starfish Acanthaster planci and potential utilization value of the starfish as feed ingredient for animals. Afr. J. Biotechnol. 10:13610-13616.
- Man S, Gao W, Yanjun Z, Huang L, Liu C (2010). Chemical study and medical application of saponins as anti-cancer agents. Fitoterapia 81:703-714.
- Matsubara M, Komatsu M, Araki T, Asakawa S, Yokobori S, Watanabe K, Wada H (2005). The phylogenetic status of Paxillosida (Asteroidea) based on complete mitochondrial DNA sequences. Mol. Phylogenet. Evol. 36:598-605.
- Osbourn AE (1996). Preformed antimicrobial compounds and plant defense against fungal attack. Plant Cell 8:1821-1831.
- Raphaela GB, Carmen LQ, Ílio MJ, Fernando AC (2014). Fractionated extraction of saponins from Brazilian ginseng by sequential process using supercritical CO2, ethanol and water. J. Supercrit. Fluids 92:272-281.
- Reinheimer JA, Demkow MR, Candioti MC (1990). Inhibition of coliform bacteria by lactic cultures. Aust. J. Dairy Technol. 45:5-9.

- Robin J, Swati S, Poonam D, Madhvi M, Dharmesh K, Shalika B, Harish C, Neeraj K, Shashi B, Arvind G, Ajit KS, Ashu G (2013). *In vitro* cytotoxicity, antimicrobial, and metal-chelating activity of triterpene saponins from tea seed grown in Kangra valley, India. Med. Chem. Res. 22:4030-4038.
- Ruiz GM, Rawlings TK, Dobbs FC, Drake LA, Mullady T, Huq A, Colwell RR (2000). Global spread of microorganisms by ships. Nature 408:49-50.
- Sen S, Makkar HPS, Becker K (1998). Alfalfa saponins and their implication in animal nutrition. J. Agric. Food Chem. 46:131-140.
- Sorensen P (2015). Starfish as feed ingredient for piglets. Master Thesis. Department of Animal Science, Aarhus University, Denmark. P 11.
- Suguna A, Bragadeeswaran S, Natarajan E, Mohanraj M (2014). Antimicrobial properties of partially purified extract of starfish *Luidia* maculata (Muller & Troschel, 1842). Int. J. Nat. Prod. Res. 4:100-103.
- Suh SJ, Ko HK, Song KH, Kim JR, Kwon KM, Chang YC, Lee YC, Kim DS, Park SJ, Yang JH, Son JK, Na MK, Chang HW, Kim CH (2011). Ethylacetate fraction from Korean seaside starfish, Asterias amurensis, has an inhibitory effect on MMP-9 activity and expression and on migration behavior of TNF-induced human aortic smooth muscle cells. Toxicol. In vitro 25:767-773.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725-2729.
- Thao NP, Luyen BT, Kim EJ, Kang HK, Kim S, Cuong NX, Nam NH, Kiem PV, Minh CV, Kim YH (2014). Asterosaponins from the starfish Astropecten monacanthus suppress growth and induce apoptosis in HL-60, PC-3, and SNU-C5 human cancer cell lines. Biol. Pharm. Bull. 37:315-321.
- Yasumoto T, Tanaka M, Hashimoto Y (1966). Distribution of saponins in echinoderms. Bull. Jpn. Soc. Sci. Fish. 32:673-676.