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ANTIOXIDANT AND IMMUNOSTIMULATING ACTIVITY OF POLYSACCHARIDES EXTRACTED FROM CUBED-SNAILFISH (*Liparis tessellatus* Gilbert & Burke, 1912) EGGS

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ABSTRACT

An optimized enzymatic extraction using protamex™ was established for polysaccharides from *Liparis tesellatus* eggs (PLE). Total sugars, sulfated glycosaminoglycans, uronic acids, and hexosamines content of PLE were 43.2%, 9.0%, 23.9%, and 1.73%, respectively. Superoxide radical (SOx*) scavenging activity and 2,2-diphenyl-1-picrylhydrazyl (DPPH*) scavenging activity of PLE was tested to proof its antioxidant properties. Results on SOx* scavenging activity revealed that PLE has antioxidant activity although it's not surpass the activity of Ascorbic acid as standard. DPPH* scavenging activity showed that PLE has activity increasing at a concentration-dependent manner. However, at 1.25 mg/ml, the anti-DPPH activities of PLE lowered, possibly due to the pro-oxidant action of the sample. PLE also has effects on NO production of RAW 264.7 cells, suggestive of their possible immunostimulating potential in the human body. On human keratinocyte (HaCaT cell line), HT29 and AGS cells lines, PLE was proofed has no toxicity and showed proliferative effects. This effect showed that PLE could have potency as wound healing promoter. An inference can be made that, PLE has indispensable antioxidant potencies as well as cell proliferating activities on specific cell lines which are deemed necessary in the field of medicine and their utilization as functional food supplements.

Key words: Antioxidant, Fish eggs, Immunostimulator, *Liparis tessellatus*, Polysaccharides.

INTRODUCTION

Polysaccharides are among the most diverse biological macromolecules, consisting of cyclic monomers of varying size and structure. Its extraction is an important process for its application for future research and development. Some methods of polysaccharides extraction include; hot water technology (Chen *et al.*, 2013; Yang *et al.*, 2013), microwave (Chen *et al.,* 2005; Xie *et al.,* 2010) and enzyme-assisted technology (Lee *et al.*, 2011; Yin *et al.*, 2011). Biologically active polysaccharides have been interesting subjects for research for many years now, may it be from animal or plant source. Their therapeutic potentials, especially glycosaminoglycans, is now well documented, and combined with natural biodiversity this will pave the way for the development of a new generation of therapeutics (Senni*et al.*, 2011).

Liparis tesellatus Gilbert & Burke (1912) or 'cubed snail fish', belonging to family Liparidae is a cold water fish usually found in the waters of Korea, Japan, China and Russia (Sokolovskii & Sokolovskaya, 2005). The eggs of this fish are commonly used in soup or traditional side dishes. One of the main components of egg membranes of fishes are mucopolysaccharides. The highest mucopolysaccharides content was found in the early develop-

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ment stage and that in an advanced stage, the membrane is probably free from polysaccharides (Yamamoto, 1956). Fish eggs are commonly called roe or caviar. However, there is a difference between roe and caviar; caviar is unfertilized egg, while roe is fertilized. Fish eggs supply protein, providing 3-4 g/tablespoon in every serving. Most adults consume 46-56 g of protein every day, both to supply energy for promotion of muscle and tissue growth and repair. Fish eggs also provide approximately 1 g of carbohydrates/tablespoon serving, while adults need almost 130 g of carbohydrates to ingest daily (Wolverton, 2012). To date, there is not much information available regarding the extraction and bioactivities of polysaccharides from fish eggs. Therefore, in this paper, we aimed to study bioactivity of Polysaccharides Extracted from Cubed-Snailfish (*Liparis tessellatus* Gilbert & Burke, 1912) eggs.

MATERIALS AND METHODS

Extraction of Polysaccharide from *Liparistesellatus* Eggs

Eggs of *Liparis tesellatus* were purchased from a local fresh market in Tongyeong, Korea. The samples were sorted and washed twice with tap water followed with distilled water and stored at -25°C until use. All reagents/chemicals were purchased from Sigma-Aldrich Chemicals, USA. Protamex was obtained from Novozymes, Denmark.

The frozen *Liparis tesellatus* egg samples were freeze-dried until brittle. After lyophilization, the eggs were ground to powder form using a Waring™ homogenizer for 20 minutes. The comminuted samples were delipidated with acetone changing to fresh solvent everyday for three days. After delipidation, the samples were rinsed with distilled water to remove acetone residues, and lyophilized.

Lyophilized eggs were soaked in 0.5M sodium phosphate buffer pH 6.5 in a ratio of 1: 10 (w/v). One point thirty four percent (w/w) Protamex was added and the solutions incubated at 45˚C for 26.5 hours. After incubation, the enzymes were deactivated at 90˚C for 20 minutes. The solutions were centrifuged and the supernatants were added with sodium sulfate at 60% saturation and centrifuged at 4000 rpm to remove the proteins. The supernatant was dialyzed against deionized water by continuous diafiltration using a Pellicon™ UF membrane with a molecular weight cut-off of 10kDa in a Millipore™ LabscaleTFF (Millipore, France). The dialyzed solutions were concentrated under vacuum at 40˚C, until Brix˚ 9. Four volumes of ethanol were added to precipitate the crude polysaccharides. Precipitates were freeze dried. The freeze dried crude polysaccharides (PLE) were stored at -25^{0}C until use for subsequent assays.

General Methods for Polysaccharides Component Analysis

To determine the sugars contain in the sample was using the method from Dubois *et al.* (1956). The amount of sugar was determined for PLE by reference to the standard curve generated from the data obtained for D-galactose by external standard method. Determination of sulfated glycans in PLE was detected according to Oliveira *et al.* (2007) method. Chondroitin-6-sulfate was used as standard prepared at different concentrations for the generation of a standard curve in the estimation of the presence of sulfates in PLE.The uronic acid content was measured using a modified carbazole reaction assay withglucuronic acidas standard (Cesaretti *et. al*., 2003) and hexosamines were quantified using the MBTH assay

for hexosamines and acetylhexosamines with N-acetyl-D-glucosamineas standard (Anthon & Barret, 2002).

Antioxidant Activity Assay of PLE

The DPPH scavenging activity was measured according to the method from Liu *et al.* (2009) with some modifications. Solutions of PLE Fractions and ascorbic acid (standard) were prepared (0.01, 0.05, 0.25, and 1.25 mg/ml). One milliliter of sample and ascorbic acid of different concentrations was added to 4 ml of 0.004% DPPH (in ethyl alcohol). The prepared solutions were thoroughly mixed in a vortex machine and incubated for 30 min in the dark at room temperature. The absorbances (A) of the solutions were measured at 517 nm using a spectrophotometer (Spectramax M2, Molecular Devices USA).

The reducing power of each fraction was determined according to the method described by Oyaizu (1986). One milliliter of sample solution of each concentration (0.01, 0.05, 0.25, and 1.25 mg/ml) was mixed in a screw-capped test tube containing 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$. Then those mixtures ware incubated at 50˚C for 20 min. After incubation, 2.5 ml of 10% TCA was added to the solution and centrifuged at 3000 rpm for 10 minutes. The supernatant (2.5 ml) was mixed with 2.5 ml DIW and $FeCl_3$ (0.5 ml, 1%). The absorbance of the solutions and the standard were measured at 700 nm. Phosphate buffer (pH 6.6) was used as blank solution. All measurements were carried out in triplicate and expressed as mean values ± standard deviation.

The superoxide scavenging activity of fractions were quantified based on their ability to inhibit nitrobluetetrazolium (NBT) reduction in the presence of a superoxide-generating system that involved phenazinemethosulphate (PMS) and nicotinamide adenine dinucleotide (NADH) (Hazra et al., 2008). Briefly, an assay buffer (phosphate buffer, 100 mM, and pH 7.4) was prepared. Reagent stock solutions in assay buffer: 12.72 mM NADH, 3.18 mM NBT, and 1.28 mM PMS were also prepared. PLE fractions were prepared in different concentrations (0.01, 0.05, 0.25, and 1.25 mg/ml). Using the 96-well microplate, 1 ml of assay buffer was placed onto the wells, added with 10 µl of the sample, and 25 µl of 12.72 mM NADH, 3.18 mM NBT, and 1.28 mM PMS. BHA was used as standard. The solutions were incubated for 5 minutes at room temperature and the absorbances were measured at 560 nm.

Cell Assays for PLE

The effect of PLE to cells viability and proliferation were tested by using HaCat, HT29 and AGS cell lines. The PLE treatment was modified from the anti-cancer drug screening method of natural product branch in U.S. National Cancer Institute (Alley, 1988). Briefly, cells were seeded in 96-well plates at a density of 6,000 cells/well. The cells were grown to about 70-80% confluence, and then synchronized by incubation in DMEM containing 0.5% FBS for 8 h. PLE extracts were added at concentrations of 0.1, 1.0, 10.0, 100.0, and 200.0 µg/mL, respectively. After twenty-four hours, After twenty-four hours, cell proliferation was measured by means of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2(4-sulfophenyl)-2-H-tetrazolium) (MTS) assay.

PLE was tested for NO production using the method from Ohta *et al*. (2007) with slight modification. Briefly, RAW 264.7 cells were incubated upto 80% confluency in a 96-well

plate. Controls were prepared in the absence of samples in the same conditions. The culture supernatants were reacted with Griess reagent at room temperature for 10 min, and then nitrite concentration was determined by measuring the absorbance at 550 nm. The standard curve was obtained using the known concentrations of sodium nitrite.

RESULTS AND DISCUSSION

Liparis tesellatus has low lipid content (0-0.10%) (Lee et al., 2012) and its eggs were high in carbohydrate content (19.50 ± 1.1%), along with 72.1 ± 0.2% moisture, 0.6 ± 0.3% ash, 0.3 ±1.5% lipid, and 7.6 ± 0.9% protein. The notable amount of carbohydrate of *Liparis tesellatus* eggs (LTE) led the researchers to further study the properties of its polysaccharide content. Another consideration was its protein composition (7.6 ± 0.9%), since natural polysaccharides are always attached to proteins to form glycoprotein (Zhang *et al*., 2012). The amino acid composition data of LTE showed 248.880 nmol/40µl total amino acid content. After enzyme-assisted extraction using protamex, the amino acid composition of PLE was lowered to 169.380 nmol/40µl. From these data obtained, it shows that the enzyme-assisted extraction using protease accomplished a high protein removal rate (68.0%), wherein, a protease is often employed in protein removal among natural polysaccharides (Zhu et al., 2011) and that, PLE was a glycoprotein. PLE was tested for its total sugar (43.2%), sulfates (12.0%), uronic acids (23.9%) and hexosamines (3.7%) contents.

PLE was assessed for its DPPH scavenging activity, reducing power, and superoxide radical scavenging activity as reflected in Figures 1A, 1B, and 1C, respectively. The DPPH radical scavenging activities of PLE (43.0%, 48.2%, 49.5%, and 52.0%) and ascorbic acid (36.2%, 41.5, 60.1, and 75.0%) as standard were evaluated at 5.0, 10.0, 15.0, and 20.0 µg/ml concentrations. Results showed that the percentage DPPH radical scavenging activities of PLE as well as the standard were increasing dependently with concentration with 11.30 and 22.80 µg/ml as the IC_{50} of ascorbic acid and PLE, respectively. The presence of sulfates on PLE based on its HPLC analysis (Data not shown) can account for its scavenging activity against DPPH radical. Similarly, sulfated polysaccharide fractions of *Corallina officinalis* have higher DPPH scavenging activity compared with its de-sulfated fractions (Yang *et al*., 2011).

The reducing power (abs. at 700 nm) of PLE (0.0095, 0.0108, 0.0128, and 0.0164) was compared with ascorbic acid (0.0471, 0.0607, 0.1412, and 0.2364) as standard. The absorbance of a certain coumpound is directly proportional to its reducing power. The higher the absorbance, the greater is the reducing power. As can be seen on the behavior of the reducing power of PLE at 5.0- 20.0 µg/ml concentrations, the absorbances were relatively low compared with that of ascorbic acid, a known antioxidant. That is, the reducing power of most natural polysaccharides are lower compared with that of synthetic antioxidants (Zhang *et al.,* 2012).

The superoxide radical scavenging activity of PLE were 46.2%, 53.2%, 54.4%, and 61.0%; those of BHA (used as standard) were 48.0%, 53.2%, 53.7%, and 61.0% measured at 5.0, 10.0, 15.0, and 20.0 µg/ml concentrations, respectively. The values of the superoxide radical scavenging activities of PLE and BHA increased with increasing concentrations and have no significant difference with each other ($p < 0.05$). The superoxide radical scavenging

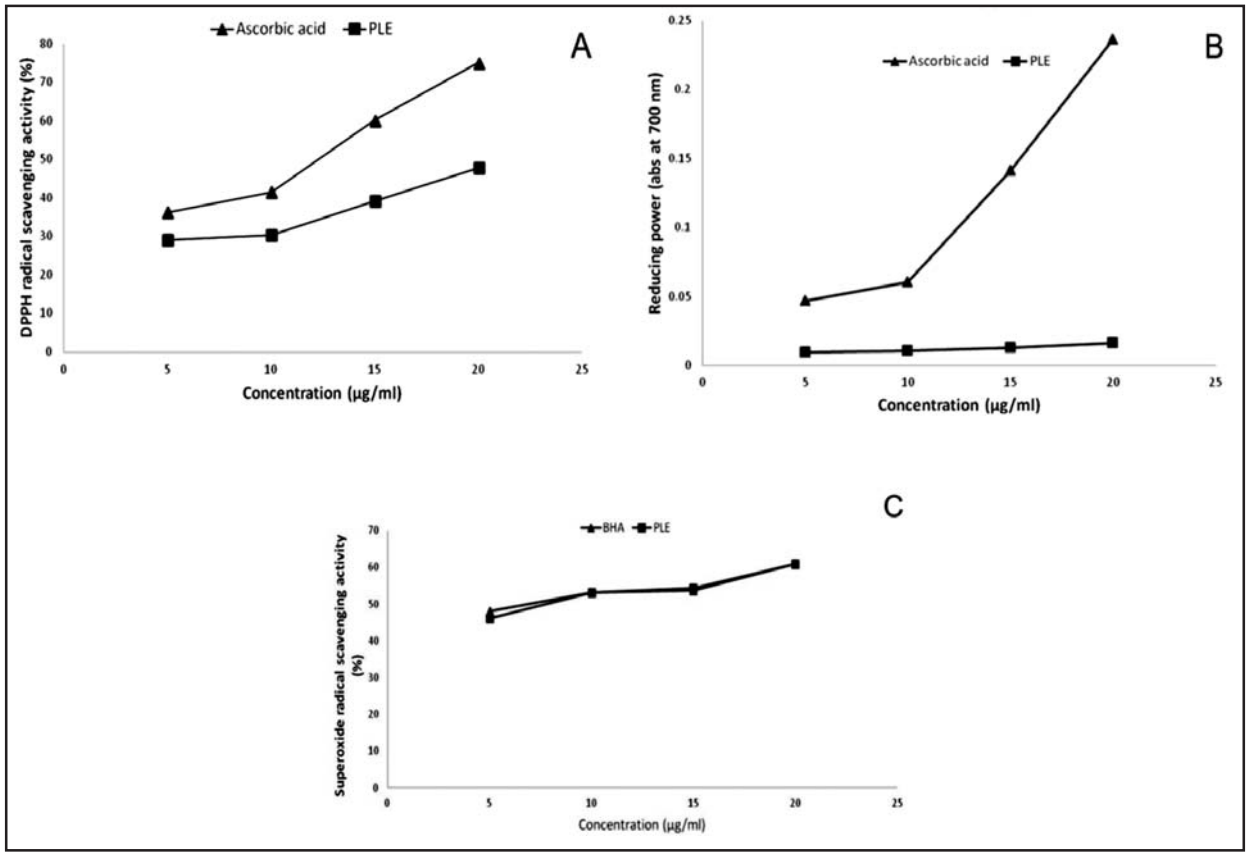

Figure 1. DPPH scavenging activity (A) and reducing power (B) with ascorbic acid as standard; and superoxide radical scavenging activity (C) of PLE with BHA as standard.

activity of PLE was almost similar with that of BHA, a known synthetic antioxidant. This activity of PLE can be explained by the presence of sulfates detected on PLE which was in similar observation with the study of Zhang *et al.* (2011) on the superoxide radical scavenging activity of *Auricular auricular* due to its sulfation.

Another contributory aspect in the antioxidant activities of PLE is the presence of chondroitin sulfates due to its carboxylic acid group (-COOH) of the glucuronic acid residue which can bind to free radicals and terminate their reactions, thus, preventing oxidation (Campo *et. al.,* 2006).

RAW 264.7 macrophages were incubated with various amounts (0.01-200.0 µg/ml) of PLE. As reflected in Fig. 2A, NO production was increasing in a dose-dependent manner. As the concentration of PLE is increased, there was a corresponding increase in the production of NO. In a study conducted by Friedl *et al*. (2001), the polysaccharide components from ginseng (*Panax ginseng*) roots were responsible for NO stimulation and not due to its other components (e.g. triterpenes) which did not show any NO stimulation until further confirmatory experiments. Similarly, the polysaccharide content of PLE was responsible for its stimulatory activity toward NO production. As shown in the graph (Figure 2), at 200.0µg/ml PLE, there was a drop in NO production. This behavior of PLE showed that, inclusive only at 0.01-100.0 µg/ml concentrations, there was NO production, and possiblyconcentration beyond 100.0 µg/mlofPLE would have an inhibitory effect on NO production.As an overview, the increased production of NO is beneficial for macrophages (first line of defense against

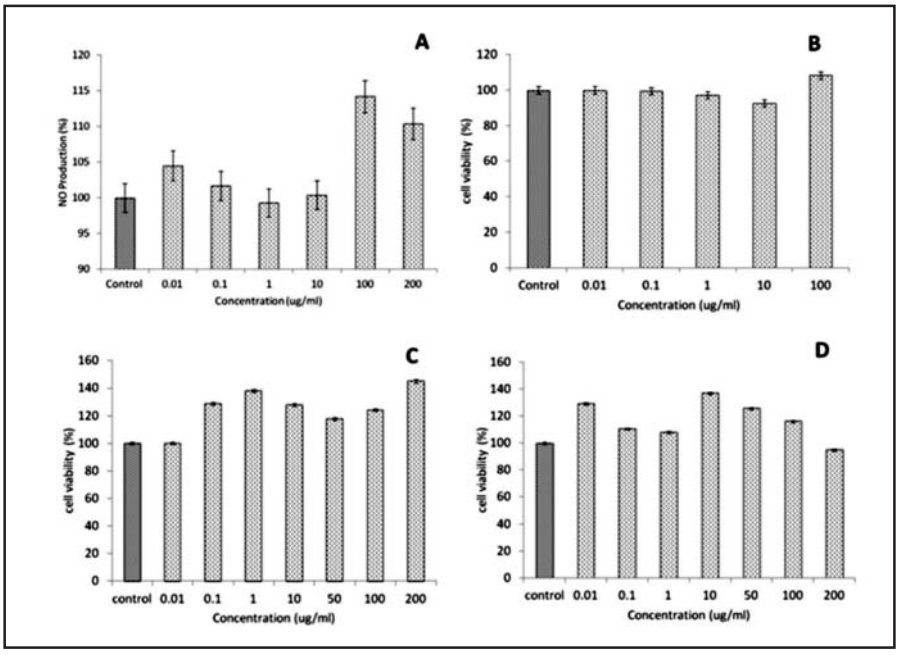

Figure 2. NO production of RAW 264.7 cells after treated with PLE (A), Cell viability test of PLE on HaCat (B), AGS (C) and HT29 (D).

infections), since macrophages are known to produce NO, and one of the most prominent functions of NO is its participation in antimicrobial and antiviral defense (Nathan & Hibbs, 1991). A sustainable NO production from macrophages empowers them with cytostatic or cytotoxic activity against viruses, bacteria, and tumor cells (Lowenstein *et al*., 1996). Also, nitric oxide synthase inhibitors (iNOS) have been shown to aggravate infectious diseases (Chan *et al.,* 1995). The findings in this study suggested that iNOS stimulation by PLE might contribute to a decreased susceptibility to infections due to an enhanced NO release.

The cell proliferation of PLE on human keratinocytes using varied concentrations (0.1, 1.0, 10.0, 50.0, 100.0, and 200.0 µg/ml) were investigated using HaCaT cells (keratinocyte cell-line that do not require feeder layer or complex media for growth and are generally more convenient to handle compared with the primary keratinocyte cultures) (Howling *et al.*, 2001). Figure 2.B. shows the behavior of PLE towards HaCaT cells compared with the control (untreated with PLE), wherein, at 0.1-100.0 µg/ml, there was no effect to lowering on HaCaT cell growth. However, at the final concentration used (200.0 µg/ml), there was a little increase on HaCaT proliferation. This observation showed the dual property of PLE (antiproliferative and proliferative) at specific concentrations. To account for these findings, considerations such as; PLE is a protein-polysaccharide complex (Data not shown), considered as a proteoglycan, which is a major constituent in the extracellular matrix (ECM) of cells, and through their core proteins or via GAG chains, they are able to interact with collagens, growth factors, growth factor receptors and adhesion molecules and through these interactions may participate in the regulation of many cellular events. Thus, it is importantin cell to cell communication. In relation to this, the not so pronounced antiproliferative effects of PLE at concentrations 10.0-100.0 µg/ml can be explained by the possibility that, there was an interaction which occurred between the growth factors and some metals (e.g. calcium) that were present on *L. tesellatus* eggs (Data not shown) on these concentrations of PLE. This interaction may have reduced the availability of the growth factors on PLE to the cells. This result is in corre-

lation with the study of Howling *et al*. (2001) on the effect of chitin and chitosan on the proliferation of human keratinocytes *in vitro*. On the other hand, the increase on HaCaT proliferation at 200.0 µg/ml may be due to the interaction of cellular components with heparin growth factors, since, heparin disaccharides were detected on PLE as determined by HPLC data. Similarly, in a study conducted by Kratz *et al*. (1997), they found out that, stimulation of wound healing in the human skin caused by heparin-chitosan complexes may be an effect caused by stabilization and activation of growth factors that bind to immobilized heparin. The dual behavior of PLE as antiproliferative and proliferative agent towards HaCaT cells was dependent on its concentration in order to exhibit such specific effects.

PLE was evaluated for its effects on HT 29 (Figure 2C) and AGS (Figure 2D) cell lines. Various concentrations (0.01-200.0 µg/ml) of PLE were used on both cell line assays. As shown by the graphs in Figure 2, there was no growth inhibitory effect observed on PLE toward the cancer cells. Nearly, in a study conducted by Tabarsa*et al.* (2012) on the immunomodulatory activities of water-soluble sulfated polysaccharides from *Ulvapertusa*, they found out the low in vitro anticancer activity of these polysaccharides against AGS cells. With their observation, they claimed the probability of the polysaccharide samples as strong immunostimulators. In addition, in their study, there was also increased NO stimulation observed on the test macrophages using the *U. pertusa* sulfated polysaccharides, which strengthened its immunostimulating effect.

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REFERENCES

Alley, M.C., D.A. Scudiero, P.A. Monks, M.L. Hursey, M.J. Czenwinski, D.L. Fine, B.J. Abbott, J.G. Mayo, R.H. Shoemaker, and M.R. Boyd. 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Research* 48: 589-601

Campo, G.M., A. Avenoso, S. Campo, A.M. Ferlazzo, and A. Calatroni. 2006. Antioxidant activity of chondroitin sulfate. *Advances in Pharmacology*. 53: 417-431.

Cesaretti, M., Luppi, E., Maccari, F., and N. Volpi. 2003. A 96-well assay for uronic acid carbazole reaction. *Carbohydrate Polymers*. 54: 59-61.

Chen, H., M. Zhang, and B. Xie. 2004.Quantification of uronic acids in tea polysaccharide conjugates and their antioxidnat properties. *J. Agric. Food Chem.* 3333-3336

Chen, X.Q., Q. Liu, X.Y. Jiang, and F. Zeng. 2005. Microwave-assisted extraction of polysaccharides from *Solanum nigrum*. *Journal of Central South University of Technology.* 12: 556-560.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28: 350-356.

Friedl, R., J. Moeslinger, B. Kopp, and P.G. Spieckermann. 2001. Stimulation of nitric oxide synthesis by the aqueous extract of *Panax ginseng* root in RAW 264.7 cells.*British*

Journal of Pharmacology, 134: 1663-1670.

Hazra, B., S. Biswas, and N. Mandal. 2008. Antioxidant and free radical scavenging activity of *Spondiaspinnata*. *BMC Complementary and Alternative Medicine*. 8: 5-10

Lee, H.Y., and J. Surh. 2011. Seasonal variations in the nutritional compositions and heavy metals in two demersal fish, *Liparis tessellatus* (cubed snailfish) and *Hemitripterus villosus* (shaggy sea raven). *Korean Journal of Food Science and Technology*, 43, 282-290.

Liu, X., Z. Sun, M. Zhang, X. Meng, X. Xia, W. Yuan, F. Xue, and C. Liu. 2012. Antioxidant and antihyperlipidemic activities of polysaccharides from sea cucumber *Apostichopus japonicus*. *Carbohydrate Polymers.* 90:1664-1670.

Lowenstein, C.J., S.L. Hill, A. Lafond-Walker, J. Wu, G. Allen, M. Landavere, N.R. Rose, and A. Herskowitz. 1996. Nitric oxide inhibits viral replication in murine myocarditis. *Journal of Clinical Investigation*, 97: 1837-1843.

Nathan, C.F., and J.B. Hibbs. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Current Opinions in Immunology*, 3, 65-70.

Oyaizu, M. 1986. Studies on products of browning reaction prepared from glucosamine. *Japan Journal of Nutrition*, 307-314.

Senni, K., J. Pereira, F. Gueniche, C. Delbarre-Ladrat, C. Sinquin, J. Ratiskol, G. Godeau, A.M. Fischer, D. Helley, and S. Colliec-Jouault. 2011. Marine polysaccharides: A source of bioactive molecules for cell therapy and tissue engineering. *Marine Drugs*, 9, 1644-1681.

Sokolovskii, A.S., and T.G. Sokolovskaya. 2005. A record of the cubed snailfish, *Liparis tessellatus* (Pisces: Liparidae), in Peter the Great Bay, Sea of Japan. *Russian Journal of Marine Biology*, 31, 126-127.

Tabarsa, M., J.H. Han, C.Y. Kim, and S.G. You. 2012. Molecular characteristics and immunomodulatory activities of water soluble sulfated polysaccharides from *Ulva pertusa*. *Journal of Medicinal Food*, 15, 135-144.

Xie, J.H., M.Y. Xie, M.Y. Shen, S.P. Nie, C. Li, and Y.X. Wang. 2010. Optimisation of microwave-assisted extraction of polysaccharides from *Cyclocarya paliurus* (Batal.) Iljinskaya using response surface methodology.*Journal of the Science of Food and Agriculture*, 90, 1353-1360.

Yin, X., Q. You, and Z. Jiang. 2011. Optimization of enzyme-assisted extraction of polysaccharides from *Tricholoma matsutake* by response surface methodology. *Carbohydrate Polymers*, 86, 1358-1364.

Zhang, D., H. Wu, Z. Xia, C. Wang, J. Cai, Z. Huang, L. Du, P. Sun, and J. Xie. 2012. Partial characterization, antioxidant and antitumor activities of three sulfated polysaccharides purified from *Bullactae xarata*. *Journal of Functional Foods*, 4, 784-792.

Zhou, D., L. Qin, B. Zhu, D. Li, J. Yang, X. Dong, and Y. Murata. 2011. Optimisation of hydrolysis of purple sea urchin (*Strongylocentrotus nudus*) gonad by response surface methodology and evaluation of in vitro antioxidant activity of the hydrolysate. *Journal of Scientific Food Agriculture*.