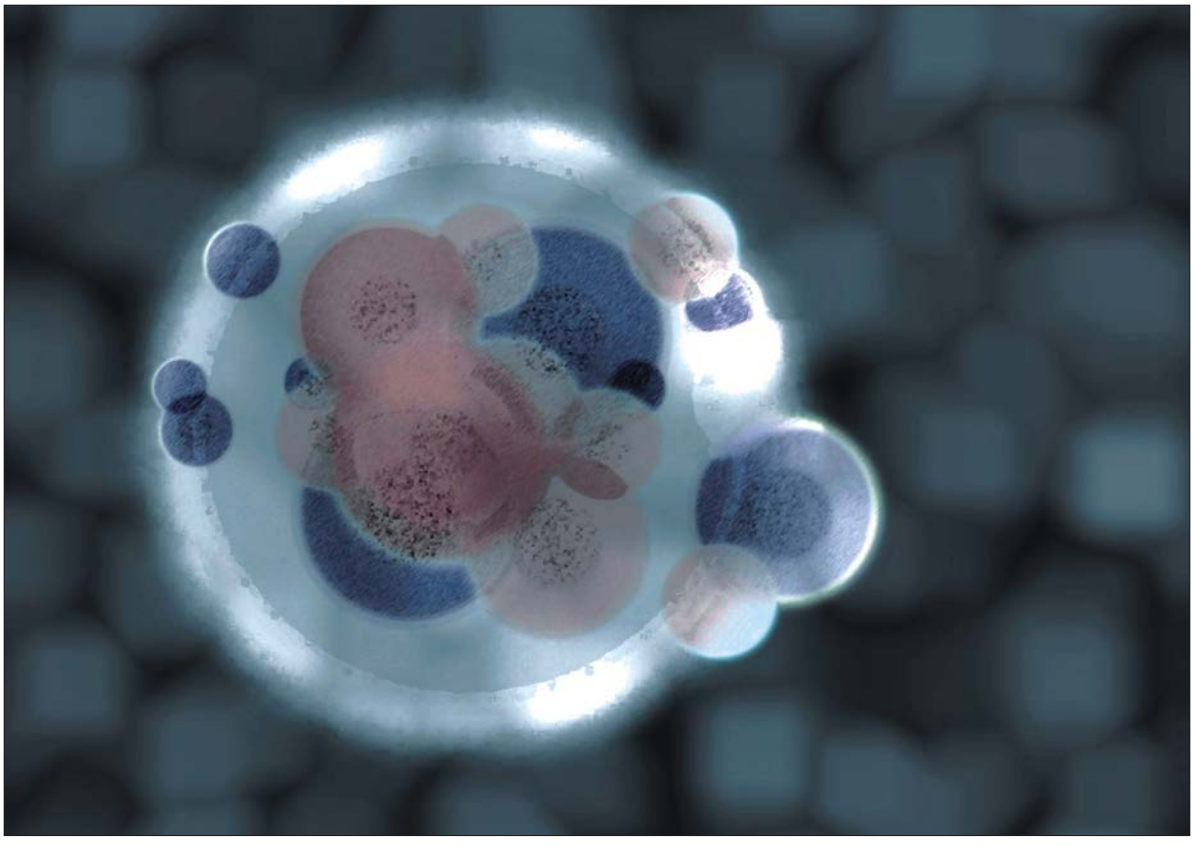

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Biological Properties of Lectin from Sea Cucumber (*Holothuria scabra* Jaeger)

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Abstract: A lectin isolated and purified by gel chromatography from the internal organs of *Holothuria scabra* Jaeger was assayed for different biological properties. The lectin was found to have hemolytic and mitogenic activities. It was also found to be a good inhibitor of seed germination and inhibited the germination of radish seeds. LC_{50} against *Artemia salina* is lower than commercial therapeutic agents. No antimicrobial, antiprotozoal, clastogenic, insecticidal and molluscicidal activities were observed.

Key words: Lectin, *Holothuria scabra*, sea cucumber

INTRODUCTION

Lectins constitute a group of proteins or glycoproteins of non-immune origin, which bind reversibly to carbohydrates and agglutinate cells or precipitate polysaccharides and glycoconjugates[1]. They were later redefined as proteins possessing at least one non-catalytic domain which binds reversibly to a specific mono or oligosachharide[2]. But antibodies and proteins with enzymatic activity related to carbohydrates cannot be considered as lectins[3]. Because of their chemical properties, they become a useful tool in several fields of biological research (immunology, membrane structure, cell biology, cancer research, pharmacology and genetic engineering).

Lectins are present in a wide range of organisms from bacteria to animals[4]. Various lectins have been found in the hemolymph and tissue extracts of marine invertebrates[5-7]. Different biological roles have been proposed for lectins and the most thoroughly explored potential functions are those involved with invertebrate immunity. This has been demonstrated by studying the regulation of the synthesis of these proteins by inducing invertebrates to injury or by introducing microorganisms to them[8].

Lectins also acts as humoral factors in the defense mechanism, the same way as immunoglobulins in vertebrates. It has been observed that lectin activate phagocytes by the binding of lectin to foreign cell or by increase in lectin production upon injection of foreign substances[6]. They are also involved in processes such as non-self recognition, inflammation, cell-cell or cell-extracellular matrix interactions, fertilization, development and regeneration[9]. Lastly, they may also be involved in signal transduction, the organization and transport (sugar) of macromolecules or multi-enzyme complexes and even in promoting the transport of calcium or sugars.

Some of the lectins isolated from marine invertebrates were found to exhibit different biological activities. Mitogenic activity was observed in lectins from *Cicachyrella alloclada* (marine sponge)[10] and *Toxopneutes pileolus* Lamarck (sea urchin)[11] while cytotoxic activity was exhibited by lectins from *Haliclona cratera* (marine sponge)[12] and *Cucumaria echinata*[13]. In addition, lectin from *Styela plicata* mimicked the properties of interleukin 1, an inflammatory cytokine[14] while hemolymph lectin of *Modiolus modiolus* L. exhibited antibacterial activity against marine bacteria[15].

This study was conducted to determine the biological properties of a lectin from the internal organs of sea cucumber (*Holothuria scabra* Jaeger).

MATERIALS AND METHODS

Isolation of lectin: Internal organs from fresh composite samples of *Holothuria scabra* collected from shallow waters of Bauan, Batangas, Philippines in the summer of 2002 and identified by the Bureau of Fisheries and Aquatic Resources (BFAR) was homogenized in an Osteorizer blender with 0.1M Tris Buffer Saline (TBS) at 1:5 (w/v) ratio. Extraction was continued by stirring the homogenate at 10°C for 6 h and then filtering the extract through a cheesecloth. The collected supernatant

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was then centrifuged at 10,000 rpm for 10 min at 10°C. Clarified extract was precipitated at 90% saturation with ammonium sulfate. The resulting precipitate was collected after centrifugation at 10,000 rpm at 10°C for 10 min. The collected precipitate was dissolved in minimum amount of buffer and was then reprecipitated at 0-60% saturation with ammonium sulfate. The precipitate was collected through centrifugation. It was dissolved in minimum amount of buffer and dialyzed against a dilute solution of TBS using a Sigma dialysis tubing with a molecular weight cut-off of 12,000 at 10°C. Polyethylene glycol was added to concentrate the dialyzed sample.

The concentrated sample was applied to Sephadex G-200 column and eluted with 0.01M TBS pH 7.5 Eluted fractions of 2.5 mL were monitored for absorbance at 280 nm and assayed for agglutination activity. Fractions showing agglutinating activity were pooled, lyophilized and used for the different biological assays.

Agglutination assay: Agglutination assay using the gel filtration eluates was carried out in multiwell microtiter plates using human erythrocytes (Type A). A 50 μL portion of the lectin solution was serially diluted two fold in Tris buffer solution. The solution was added with 50 μL of 2% (v/v) erythrocyte suspension in the same buffer. The plates were incubated for one hour at room temperature and were examined visually for agglutination. A positive test was indicated by the formation of a uniform layer over the surface of the well. On the other hand, the formation of a discrete button at the bottom of the wall indicated negative results.

Antimicrobial activity: The effect of the purified lectin on the growth of microorganisms was evaluated by the modified filter paper disc agar-plate method using the following microorganisms: *Staphyloccus aureus*, *Bacillus cereus, Escherichia coli* and *Saccharomyces cerevisiae*. Filter paper disks were soaked in lectin solution, antibiotic solution (Amoxicillin and Sumapen) that served as positive controls and TBS buffer that served as negative control and then placed on top of the dried agar inoculated with each microorganism. The plates were incubated at room temperature for 24 h and were then observed for clearing or zones of inhibition.

Seed germination inhibition activity: Radish (*Raphanus sativus* L.) seeds were used to determine the inhibitory effect of lectin on seed germination. Radish seeds were dipped in lectin solution and then allowed to germinate in a vial. Controls using distilled water and TBS solution were also done and same conditions were applied to all treatments.

Hemolytic assay: The hemolytic assay was determined by visual examination of the hemolysis of the erythrocytes under the same conditions as for the hemagglutination assay.

Molluscicidal activity: Young golden snails (*Pomacea canaliculata* L.) collected from the field were used for the moluscicidal activity. Each treatment replicated thrice following the soaking method of the World Health Organization[16]. Twenty young snails were used in each replicate for the bioassay using concentrations of 50, 100 and 500 ppm. Mortality rate was observed after 24 and 48 h of treatment.

Insecticidal (larvicidal) activity: The effect of the purified lectin on mosquito larvae was also evaluated. Mosquito larvae (*Culex* sp.) were placed in vials containing distilled water with different concentrations of lectin solutions. Insecticidal activity was measured in terms of the mortality of the larvae in 6 h interval for 24 h.

Mitogenic activity: Human peripheral lymphocytes were cultured using a method developed by Toyoshima *et al.*[17]. Normal human venous blood was withdrawn using sterilized syringe and was transferred to a glass cylinder containing EDTA as anticoagulant. The erythrocytes were allowed to sediment by gravity at 37°C. The leukocyte rich plasma was withdrawn and a 0.45 mL aliquot was transferred to a sterile culture bottle to which 0.75 mL of NCTC-109 (Sigma), 0.15 mL of calf serum and 0.15 mL of lectin solution have been added. The culture was incubated at 37°C for 72 h. Positive and negative controls were employed. Cells were harvested 72 h after incubation. A morphological assay was done using Giemsa-stained preparations and viewed through HPO of a research microscope. Photomicrographs were taken.

Bioactivity assay: Brine shrimp eggs were placed in sea water solution inside an improvised brine shrimp hatching tank consisting of two chambers, a dark chamber where the eggs are placed and a nauplii-collecting chamber. After 48 h, 100 μL of salt solution containing the nauplii were dispensed to individual wells of a 96-well microtiter plate. Approximately 10-15 shrimps were placed in each well provided with one hundred microliter (100 μL) of lectin solutions (500, 100 and 10 ppm). The set-up was incubated in the dark for 48 h. Percentage of dead shrimps were counted under the microscope. Distilled water served as negative control while Taxol (paclitaxel) (Bristol-Myer Squibb) and Cytosine β-o-Arabinofuranoside (Ara-C) (Sigma Chemical Co.) were used as positive control. Data were analysed using Reed

Muench method. Lethal concentration (LC_{50}) was determined using linear regression.

Antiprotozoal assay: Two concentrations of lectin solution (500 and 200 ppm) were used for the anti-protozoal activities against *Acantamoeba* sp. and *Tetrahymena pyriformis* using microplate-based *in vitro* bioassays. This was done by mixing cultured cells of the test organisms (100 μL) with the lectin solution (100 μL) in microtiter plate well. The mortality of the cultured cells was examined upon addition of lectin solution and after 24 h.

Clastogenic activity: Two concentrations of lectin solutions were prepared: 500 and 200 ppm. Germinating bulbs of *Allium cepa* with roots about 2-4 mm in length were immersed separately in test tubes containing the prepared solutions for 3 days. Distilled water was used as control. The number and length of the root were recorded after which, both control and treated roots were fixed in Carnoy's fluid (containing alcohol, chloroform and acetic acid in 2:1:1) and stained with acetocarmine. The slides were observed under a compound microscope.

RESULTS AND DISCUSSION

Antimicrobial assay: No antimicrobial activity was observed for the isolated lectin against *Staphyloccus aureus*, *Bacillus cereus, Escherichia coli* and *Saccharomyces cerevisiae*. This could possibly mean that the isolated lectin do not possess biological activity against the test organisms. It could also be due to the high population of the microorganisms used since some of the positive controls also failed to inhibit the growth of the test organisms.

Marine invertebrates are reported to possess ligand-binding lectins that may act in defense reactions such as opsonisation or phagocytosis. This has been demonstrated in shellfish lectins[18], the lectin from the serum of *Crassostrea virginica*[19] and a sialic-acid binding lectin from horse mussel (*Modiolus modiolus*)[15]. Achatinin, a lectin from the hemolymph of *Achatina fulica* snail has also bacteriostatic effect on the gram-negative bacteria *E. coli*[20]. In contrast, a sialic acid-binding lectin from the body walls of brown sea cucumber showed no activity against *E. coli*[21].

Recognition of pathogenic microorganisms by lectins is assumed to be due to lectin-carbohydrate interaction. Inhibition of spore germination and fungal growth is presumably due to disruption of chitin synthesis by lectins that are specific for N-acetyl-glucosamine[22]. This in turn can trigger the subsequent saprophytic, symbiotic or pathogenic relationships[23]. Other mechanisms for the antimicrobial activity include inactivation of the ribosomes as shown by ricin and *Eranthis hyemalis* lectin[24] and immobilization of virulent strains as manifested by potato lectin[25] and *Datura stramonium* lectin[26].

Seed germination inhibition activity: Radish (*Raphanus sativus* L.) seeds were used to determine the seed germination inhibiting activity of the isolated lectin. Results showed (Table 1) the inhibition of germination of radish seeds treated with lectin solution. This confirms the seed germination inhibition activity of the isolated lectin since seeds treated with distilled water and TBS germinate normally. There are still no studies on the use of lectin as an inhibitor of seed germination. In general, most of the seed germination inhibitors are secondary metabolites[27].

It is possible that the isolated lectin react with a substance present in the seed that result in the inhibition of seed germination. Bewley and Black[28] proposed the classical triphasic time course of seed imbibition. This comprised of initial rapid absorption of water by the dry seed (Phase I), followed by a period of variable length where seed water content is relatively constant or only slowly increasing (Phase II) and a resumption of water uptake associated with expansion and growth of the embryo (Phase III). Germination is usually completed between Phase II and III. The action of the lectin to inhibit germination could take place before this. It is possible that the lectin could prevent the reformation of keto-acids from amino acids few minutes after water enters the seeds. Keto-acids are important intermediates in respiratory pathways and usually unstable and absent in dry seed[28].

An increase in the demand of oxygen occurred in Phase I due to the activation and hydration of mitochondrial enzymes involved in the citric acid and electron transport chain[28] and it is possible that an action occurred which results in the prevention of oxygen consumption upon addition of lectin. Lastly, is the possible inhibition or inactivation of the enzymes involved in germination by the lectin, thus resulting to non-germination of the seed.

With this result, it is possible that the isolated lectin would be effective as an active ingredient of herbicide.

Table 1: Percentage germination of radish seeds treated with lectin

Condition/experimental set-up	Germination (%)
Distilled water	100
Phosphate buffer	100
Lectin solution (200 mg mL^{-1})	5
Lectin solution (500 mg mL^{-1})	0

Further studies can be done to confirm the seed germination inhibition activity of the isolated lectin by using other assay methods.

Hemolytic assay: Hemolysis is the term to used to describe the premature destruction of erythrocytes. Holothurins found in sea cucumbers were found to exhibit hemolytic activity against human erythrocytes[29]. The isolated lectin showed hemolytic activity when incubated with human and animal erythrocytes. Addition of calcium ions showed increased in hemolytic activity which could only mean that the activity is calcium dependent. This is the same with the CEL-III lectin from *Cucumaria echinata*[30], which is known for its hemolytic activity. Since many C-type lectins from invertebrates are considered to play important roles in their immunity, the isolated lectin appears to be an interesting example of a toxic protein which might act directly against foreign microorganisms. However, no hemolytic activity was detected in lectins isolated from *Stichopus japonicus*, which is a closely related organism to *Holothuria scabra* and *Cucumaria echinata*.

It is possible that hemolysis is caused by ion permeable pores formed by the lectin and inserted into the erythrocyte membrane. Damaging the membrane by pore formation is the usual mechanism common to many cytolytic protein toxins of microbial origin[31]. It is also possible that the hemolytic activity was due to the susceptibility of erythrocytes to hemocytes, which have the necessary machinery for this kind of activity as shown by a lectin from *Scylla serrata*[7]. Hemolysins have been reported in some studies[31,32] but they do not have the characteristics of the calcium-dependent lectins.

Molluscicidal activity: No molluscicidal activity was observed at increasing concentration of lectin and increasing time against golden snail (*Pomacea canaliculata* L). The golden snails were not affected by exposure to the lectin after 48 h.

Insecticidal (larvicidal) activity: Previous study by Merca and delos Reyes[33] showed that lectin from spores of *Bacillus thuringiensis* subsp. Morrisoni (serotype H 8a:8b) PG-14 possess weak mosquitocidal activity against 3-day old *Aedes albopictus* larvae. Mosquito larvae (*Culex* sp.) were used to test the insecticidal activity of the isolated lectin

The *Holothuria scabra* lectin did not show any insecticidal activity against mosquito larvae as 0% mortality was observed in all concentrations except at 500 mg mL^{-1} wherein 8% mortality was observed after 24 h. It is possible that the isolated lectin does not exhibit insecticidal activity or that a much higher concentration is needed for the lectin to be effective against mosquito larvae. No study has been done on the larvicidal activity of lectin isolated from marine invertebrates.

Mitogenic activity: The major constituent of the immune system is lymphocyte. Giemsa stained preparation from cultures containing lectin from *Holothuria scabra* internal organs were found to increase in cell size similar to the positive controls (*Phaseolus vulgaris* and Concanavalin A). This suggests the mitogenic activity of the lectin although the activity is not very strong since about 10% only of the lymphocytes increased in cell size when *H. scabra* lectin was added. Lectin isolated from the body wall of brown sea cucumber, *Holothuria* sp. was found to be mitogenic[21].

Aside from increased cell size, the lymphocytes treated with mitogenic agents can be differentiated in terms of the basophilia of the cytoplasm, the structure of the nucleus and the presence of nucleoli. Mature lymphocytes have a nucleus about the size of a red blood cell. The nucleus is made up of masses of chromatin. The periphery of the nucleus is usually defined rather sharply and the cytoplasm forms a narrow rim around the nucleus. Treated lymphocytes on the other hand, is a large cell (>0 μm) with a nucleus that is made up of finely granular chromatin. The nucleoli may or may not be found and the cytoplasm of the cell is deeply basophilic. Sometimes, the cell appears with almost no cytoplasm but contained the characteristic nucleus[34,35].

Bioactivity assay: Brine shrimp lethality assay was used to determine the bioactivity of the isolated lectin. This assay has been used for screening plant extracts with antitumor activity. The isolated lectin had an LC_{50} of less than 30 ppm and can be considered to contain cytotoxic agents since the standard set by the National Cancer Institute of the US for a bioactive compound to be an effective antitumor agent is equal or less than 30 ppm. It is more potent and toxic than Taxol and Ara C, the commercial chemotherapeutic drugs used in the experiment which has an LC_{50} higher than 30 ppm.

Antiprotozoal: Antiprotozoal assay showed no activity of the isolated lectin against *Acantamoeba* sp. and *Tetrahymena pyriformis*. It is possible that the isolated lectin tested played no role in defense against protozoans.

Table 2: Mean±SD mitotic indices of *Allium cepa* at different conditions

Conditions	Mean±SD
Control	17.36±0.15
Lectin solution (200 ppm)	17.36±0.11
Lectin solution (500 ppm)	17.44±0.15

No studies have been reported yet on the effect of an isolated lectin against any protozoans. Although lectins are reported to be induced in mollusks by exposure to bacteria, there is still little information on the relationships between lectins and protozoan parasites of mollusks. Oysters (*Crassostroea virginica*) were exposed to two protozoans (*Haplosporidium nelsoni* and *Perkinsus marinus*) and results showed that lectins played no role against either *H. nelsoni* and *Perkinsus marinus*[36].

Clastogenic activity: No significant difference in the mitotic index (Table 2) was observed for the lectin treated *Allium cepa*. The length of the roots in both set-ups was comparatively the same. In addition, no chromosomal aberrations were observed in both set-ups. It is possible that the lectin does not have clastogenic activity or the concentration used is too small to induced clastogenic effect which could lead to chromosomal aberrations. It is therefore concluded that at the concentration used, the isolated lectin is not a mutagen.

The biological properties of an isolated lectin from the internal organs of *Holothuria scabra* was determined using different biological assays. Results showed that the isolated lectin exhibited hemolytic and mitogenic activities. It is also an inhibitor in seed germination and bioactive against brine shrimp. No antimicrobial, antiprotozoal, clastogenic, insecticidal and molluscicidal activities were observed.

REFERENCES

1. Goldstein, I.J., R.C. Hayes, M. Monsugny, T. Osawa and N. Sharon, 1980. What should be called a lectin? Nature, 285: 66.
2. Peumans, W.J. and E.J.M. Van Damme, 1995. Lectins as plant defense proteins. Plant Physiol., 109: 347-352.
3. Cummings, R.D., 1997. Lectins as Tools for Glycoconjugate Purification and Characterization. In: Glycoscience, Status and Perspectives. (Gabius, H.J. and S. Gabius, Eds.) Champman and Hakk GmbH, Weinham, pp: 191-199.
4. Lis, H. and N. Sharon, 1981. Lectins in higher plants. Plant Biochem., 6:371-447.
5. Brown, R., L.R. Almodovar, H.M. Bhatia and W.C. Boyd, 1968. Blood group specific agglutinins in invertebrates. J. Immunol., 100: 214-216.
6. Matsui, T., Y. Ozeki, M. Suzuki, A. Hino and K. Titani, 1994. Purification and characterization of two Ca^{2+}-dependent lectins from coelomic plasma of sea cucumber, *Stichopus japonicus*. J. Biochem., 116: 1127-33.
7. Mercy, S.P.D. and M.H. Ravindarnath, 1994. Hemolysis and clearance of erythrocytes in *Scylla serrata* are related to the agglutination by the native sialic acid-specific lectin. Comp. Biochem. Physiol., 109A: 1075-1083.
8. Kubo, T., K. Kawasaki and S. Natori, 1993. Transient appearance and localization of a 26 kDa leactin, a novel member of the *Periplaneta* lectin family, in regenerating cockroach legs. Dev. Biol., 156: 381-390.
9. Saito, T., M. Hatada, S. Iwanaga and S. Kawabata, 1997. A newly identified horseshoe crab lectin with binding specificity to O-antigen of bacterial lipopolysaccharides. J. Biol. Chem., 272: 30703-30708.
10. Atta, A.M., M. Barral-Netto, S. Peixinho and M.L. Soussa-Atta, 1989. Isolation and functional characterization of a mitogenic lectin from the marine sponge *Cinachyrella alloclada*. Braz. J. Med. Biol. Res., 22: 379-385.
11. Nakagawa, H., C. Yamaguchi, H. Sakai, K. Kanemaru, H. Hayashi, Y. Araki and Y. Tomihara, 1999. Biochemical and physiological properties of pedicellarial lectins from the toxopneustid sea urchins. J. Natl. Toxins, 8: 297-308.
12. Pajic, I., Z. Kljajic, N. Dogovic, D. Sladic, Z. Juranic and M.J. Gasic, 2002. A novel lectin from the sponge *Haliclona cratera*: Isolation, characterization and biological activity. Comp. Biochem. Physiol. C Toxicol. Pharmacol., 132: 213-21.
13. Oda, T., M. Tsuru, T. Hatakeyama, H. Nagatomo, T. Muramatsu and N. Yamasaki, 1997. Temperature and pH-dependent cytotoxic effect of the hemolytic lectin CEL-III from the marine invertebrate, *Cucumaria echinata* on various cell lines. J. Biochem., 121: 560-567.
14. Nair, S.V., M. Burandt, A. Hutchinson, Raison, R.L. and D.A. Raftos, 2001. A C-type lectin from the tunicate, *Styela plicata*, that modulates cellular activity. Comp. Biochem. Physiol. Toxicol. Pharmacol., 129: 11-24.
15. Tunkijjanukij, S. and J.A. Olafsen, 1998. Sialic acid-binding lectin with antibacterial activity from the horse mussel: Further characterization and immunolocalization. Dev. Comp. Immunol., 22: 139-150.
16. Mott, K.E., 1983. Plant Molluscicides. John Wiley and Sons Ltd., NY, pp: 321.
17. Toyoshima, S., O. Toshiaki and A. Tonomura, 1970. Some properties of the phytohemagglutinin from *Lens culinaris* seeds. Biochem. Biophy. Acta, 221: 514-521.
18. Olafsen, J.A., 1996. Lectins, models of natural and induced molecules in invertebrates. Advances Comp. Environ. Physiol., 24: 49-76.

19. Fisher, W.S., 1991. Lectin content and specificity in serum and mucus of oysters. J. Shellfish Res., 10: 274-280.
20. Biswas, C., D. Sinha and C. Mandal, 2000. Investigation on intraction of achatinin, a 9-O-acetylsialic acid binding lectin, with lipopolysaccharide in the innate immunity of *Achatina fulica* snails. Mol. Immunol., 237: 745-754.
21. Gana, A.E. and F.E. Merca, 2002. Isolation and purification of a cytotoxic lectin from brown sea cucumber by affinity chromatography. Philipp. Agric. Scientist, 85: 236-247.
22. Lyumibova, N.V. and E.G. Sal'kova, 1989. Lectin-carbohydrate interaction in plant pathogen relationship. Applied Biochem. Microb., 24: 493-499.
23. Ayouba, A., H. Causse, E.J.M. Van Damme, W.J. Peumans, C. Cambillau and P. Rouge, 1994. Interaction of plant lectins with the components of the bacterial cell wall peptidoglycan. Biochem. Syst. Ecol., 22: 153-159.
24. Kumar, M.A., D.E. Timms, K.E. Neet, W.G. Owen and W. Peumans, 1993. Characterization of the lectin from the bulbs of *Eranthis hyemalis*, winter aconite as an inhibitor of protein synthesis. J. Biol. Chem., 268: 25176-183.
25. Broekart, W.F. and W.J. Peumans, 1986. Lectin Release From Seeds of *Datura stramonium* and Interference of *Datura stramonium* Lectin With Bacterial Motility. In: Lectins, Bio., Biochem., Clinical Biochem., (Tcbog Hansen and E. Van Driessche, (Eds)). Berlin,. 5: 57-65.
26. Sequira, L. and T.L. Graham, 1977. Agglutination of a virulent strain of *Pseudomonas solanacearum* by potato lectin. Physiol. Pathol., 11: 43-54.
27. Putnam, A.R., 1988. Allelochemicals from plants as herbicides. Weed Technol., 2: 510-518.
28. Bewley, J.D. and M. Black. 1985. Seeds Physiology of Development and Germination. New York: Plenum Press, pp: 136-138.
29. Pocsidio, G.N., 1983. Holothurin of some Philippine holothurians and its hemolytic activity. Phillip. J. Sci., 112: 13-28.
30. Hatakeyama, T., H. Kohzaki, H. Nagatomo and N. Yamasaki, 1994. Purification and characterization of four Ca^{2+}-dependent lectins from the marine invertebrates *Cucumaria echinata*. J. Biochem., 116: 209-214.
31. Ryoyama, K., 1973. Studies on the biological properties of coelomic fluid of sea urchin. Biophys. Biochim. Acta, 320: 157-165.
32. Camicatti, C., 1987. Membrane damage by coelomic fluid from *Holothuria polii* (Echinodermata). Experientia, 43: 611-614.
33. Merca, F.E. and A.M. De Los Reyes, 1997. Isolation and purification of lectin from spores/crystals of *Bacillus thuringiensis* subs Morrisoni (serotype H8a;8b) PG 14. Philipp. J. Sci., 126: 1-18.
34. Daland, G.A., 1956. A Color Atlas of Morphologic Hematology. Harvard University Press, Massachusetts, pp: 120-123.
35. Bevelandar, G. and J.A. Ramaley, 1979. Essentials of Histology. 8th Ed. The C.V. Mosby Co., London, pp: 59.
36. Chintala, M.M., S.E. Ford, W.S. Fisher and K.A. Ashton-Alcox, 1994. Oyster serum agglutinins and resistance to protozoan parasites. J. Shellfish Res., 13: 115-121.