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Purification of a Galactose-specific Lectin with Antibacterial and Mitogenic Activity from *Musca domestica* Pupae

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Insects are a large, unexplored and unexploited source of potentially useful compounds for modern medicine. In many insects, lectins present in the haemolymph and play important roles in internal host defense mechanisms. The objective of this study was to evaluate the antibacterial and mitogenic activity of *Musca domestica* pupae lectin (MPL).The main fraction of lectin in buffer insect solution (BIS) was purified by affinity chromatography on D-galactose-Sepharose-4B and ultrafiltration. MPL agglutinated typsin treated rabbit blood cells (RBC) and such hemagglutination activity was inhibited by D-galactose. MPL showed strong broad spectrum antibacterial activity against both gram-negative and gram-positive, while gram-negative bacteria were more resistant than gram-positive bacteria. The purified MPL mediate agglutination of the tested bacteria, thereby indicating a possible mode of action in physiological situation. The lectin further showed mitogenic activity to mouse splenocytes. Our results suggested the lectin of *M. domestica* involves the defense of body against microbial invasion and stimulates splenocytes proliferation.

Key words: *Musca domestica*; Housefly; Lectin; Purification.

Musca domestica[1] (Housefly) is infected, even when living in environments containing many kinds of pathogenic microorganisms. There are many antibacterial proteins present in *Musca domestica*, which play vital roles in humoral defense against invaded microbes(Natori *et al.,* 1999; Franc *et al.,* 2000; Tzou *et al.,* 2002). *In vivo*, lectins are antibacterial proteins that recognize foreign materials (Gaidamashvili *et al.,* 2002; He *et al.,* 2011; Lavine *et al.,* 2002). *In vitro*, lectins have been investigated owing to the variety of their biological properties, including antibacterial (Nagaraj *et al.,* 2008), antitumor (Zhang *et al.,* 2010), immunostimulation (Lee *et al.,* 2010) and anti-HIV (Wang *et al.,* 2007) activities. In addition, lectin shows many practical applications. For example, immobilized lectins are used for affinity chromatography, and the efficacy of drug absorption in the gastrointestinal tract can be increased by coating with lectins (Pellegrina *et al.,* 2005). The lectin of *Musca domestica* is a D-galactose-binding lectin and this lectin agglutination rabbit blood cells and the tested bacterial. The evaluation of lectin from *Musca domestica* for various potentially exploitable biological properties, such as antibacterial and mitogenic activities, has not been reported. In this study, a galactose-specific lectin was isolated from *Musca domestica*, and assayed for various activities and biological properties.

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MATERIALS AND METHODS

Insect

The larvae of *Musca domestica* were obtained from Tianjin Disease Prevention and Control Center, Tianjin, China. The larvae were reared until the third instar stage, which was interrupted by inserting the tip of a stainless steel needle into the abdominal cavity. The injured insects were then cultivated to become the pupae.

Microorganisms

Escherichia coli ATCC11775, *Salmonella typhimurium* ATCC43971, *Klebsiella pneumoniae* ATCC13883, *Bacillus subtilis* ATCC 6051 and *Streptococcus pneumoniae* ATCC49619 were obtained from the Tianjin Disease Prevention and Control Center, Tianjin, China. *Staphylococcus aureus* were preserved in our laboratory.

Animal

Male BALB/c mice 6–8 weeks old, weighing 20–25 g were housed in open top cages and maintained on food and water. Room temperature was kept at 22 ± 2°C.

Isolation and purification of MPL

The collected pupae was about 30–50 g, which was ground and dipped into 200 ml buffer insect solution (BIS) (Komano, 1980) containing 50 µg/ml N-phenylthiourea. The extract was subsequently centrifuged for 20 minutes at 10000 rpm to remove the precipitation. Afterwards, the supernatant was concentrated and sterilized by ultrafiltration. Ultrafiltration membranes in different pore sizes were applied to fractionate the supernatant, and the main fraction was separated by a membrane with nominal molecular weight cut-off of 50 kDa (Sheng *et al.,* 2007). The main fraction was collected and added into 20 ml Sepharose-4B with slow stirring for 1 h at 4°C. The mixture was put into 1.5×22 cm columns and washed with 200 ml BIS with a flow rate of 1 ml/minute until no protein was detected in the elute by monitoring the absorbance at 280 nm. The proteins were then eluted with 0.2M D-galactose-BIS and each fraction was dialyzed extensively against BIS to remove D-galactose. Finally, the lectin was lyophilized and stored in the freezer at -20°C.

Haemagglutinating and inhibition of haemagglutinating assay of the MPL

Rabbit blood cells (RBC) were washed four times with tris-buffered saline (TBS: 20 mM tris(hydroxymethyl)aminomethane hydrochloric acid [Tris–HCl] buffer at pH 7.2 containing 150 mM NaCl). A final suspension of 2% (v/v) was prepared in TBS and treated with pronase (0.5 mg/ml), incubated at 37° for 1 hour and later washed four times with TBS before use. Haemagglutination assays were performed in standard microtitre plates with v-bottom wells by two-fold serial dilution method. The 50 µl aliquot of erythrocyte suspension was mixed with 50 µl of serially diluted MPL, incubated for 1 hour and visually examined for agglutination. Microscopic observation revealed that co-incubation induced agglutination of blood cells. To determine the agglutination-inhibition by simple sugars (D-glucose, D-Fucose, D-galactose, D-mannose, D-rhamnose), the MPL solution was diluted with different sugar solutions containing sugar in TBS, and incubated with erythrocyte suspension at 37°C for 1 hour. The minimum inhibitory concentration was read (Jayasree, 2001).

Gel electrophoresis

The purity of MPL eluted from the Sepharose-4B was tested using native polyacrylamide gel electrophoresis (PAGE) with 4–16% gradient (Keisuke *et al.,* 2008). Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12% separating gel; 5% stacking gel) was performed at room temperature in vertical gels under reducing agents (2-mercaptoethanol [ME]) to identify the affinity purified lectin. The gel was subsequently stained with Coomassie Brilliant Blue R-250 (CR-250).

Effect of temperature and pH on haemagglutinating activity of MPL

To study the effect of temperature on haemagglutinating activity, MPL (1 ml, 0.5 mg/ml) was incubated for 30 minutes at 4, 20, 37, 50, 60, 70°C, serially diluted and the haemagglutination assay was performed at room temperature. The effect pH was evaluated by measuring the haemagglutinating activity of MPL in the pH range (4–10) using the following buffers: 0.02 M acetate buffer (pH 4–5), 0.02 M phosphate buffer (pH 6–7), 0.02 M Tris–HCl buffer (pH 8–9) and 0.02 M glycine/NaOH buffer (pH 9–10). Lectin (0.3 ml, 0.5 mg/ml) was incubated with each buffer solution (0.3 ml) for 2 hours and the haemagglutinating activity was evaluated.

Structural analysis of MPL by atomic force microscopy

The size, pattern and surface properties of the protein particles were studied by atomic force microscopy (AFM), performed using an ambient air scanning probe microscope (JSPM-5200 Scanning Probe Microscope, JEOL, Japan).

Images were recorded with typical contact force loads of 0.5 nN using pyramidal Si3N4 probe tips mounted on triangular 0.03 (±0.01) nm^{-1} Au-coated cantilevers. 10 µl of 0.8 mg /ml purified protein was deposited on a freshly prepared thin layer of mica film and dried under nitrogen for 2 hours.

Antibacterial activity

Antibacterial activity was measured by the method as described (Olafsen *et al.,* 1992). Each bacterial cell suspension was diluted to 10^4 cells/ml. MPL was added into a 2 ml broth in serial concentrations (0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5 mg/ml), incubated for 6 hours at 37°C. The growth of bacteria was expressed as the turbidity, as measured at A620. Comparison of antibacterial activity against different bacteria was performed using the ratios of different concentrations of MPL giving 50% inhibition of bacterial growth.

Assay of mitogenic activity

Four BALB/C mice (20–25 g) were sacrificed by cervical dislocation and the spleens were aseptically removed. Splenocytes were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve, and re-suspended to 5×10^6 cells/ml in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units penicillin/ml and 100 µg streptomycin/ml (Zheng *et al.,* 2007). The splenocytes (5×10^5 cells/100 µl/well) were seeded into a 96-well culture plate and serial concentrations of MPL in 20 µl medium were added. Following incubation of the splenocytes at 37°C in a humidified atmosphere of 5% CO^2, in the presence or absence of MPL for 24 hours, the proliferative lymphocyte response was measured by the MTT method (Mossman, 1983). The positive controls were incubated with 20µl Concanavalin A (ConA).

Statistical analysis

Data were represented as means ± standard deviation (SD) of three separate experiments performed in triplicate. The data were compared using Student's t-test. Differences were considered statistically signiûcant when the p-values were below 0.05 ($P<0.05$).

RESULTS

Isolation of MPL from Musca domestica pupae

Affinity chromatography using Sepharose-4B was able to absorb MPL effectively from housefly haemolymph. All proteins without D-galactose specific-character were eluted out from the column rapidly. The fractions eluted with 0.2M D-galactose in BIS, as indicated by the arrow in Fig.1, showed obvious haemagglutinating activity.

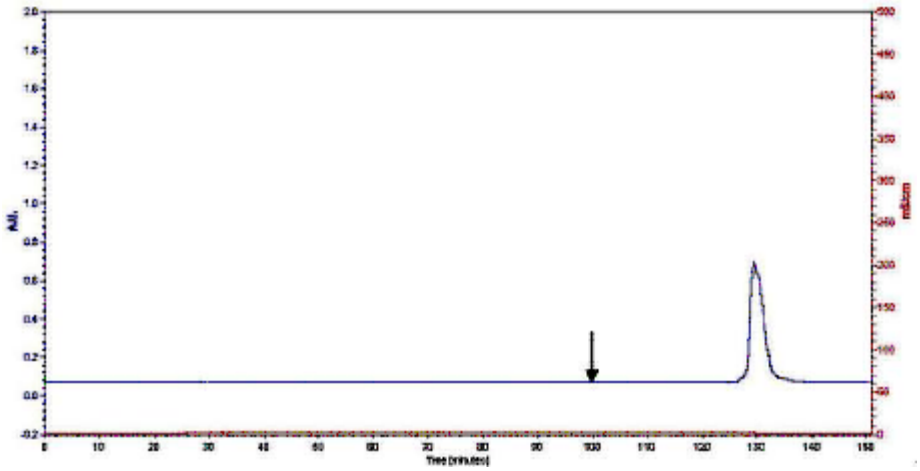
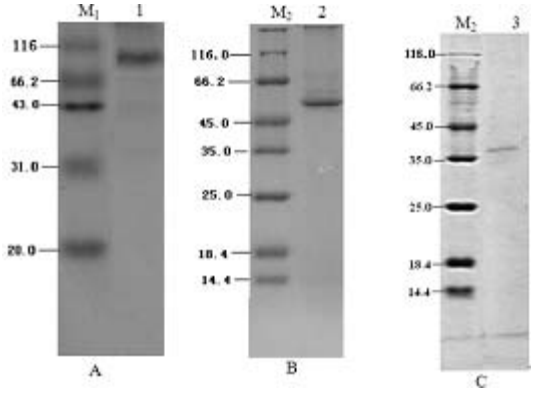


Fig. 1. Affinity chromatography (Sepharose-4B) of lectin from *Musca domestica* pupae



(A) Native polyacrylamide gel electrophoresis (Native PAGE) with 4–16% gradient. 1: MPL without 2-mercaptoethanol (running time 2 hour at a constant 60V).
(B) Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12% separating gel; 5% stacking gel). 2: MPL with 2-mercaptoethanol (running time 3 hours at a constant 80V).
(C) Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12% separating gel; 5% stacking gel). 3: MPL without 2-mercaptoethanol (running time 2.5 hours at a constant 80V).
M_1: Aldolase bovine (160.0 kDa), albumin bovine (67.0 kDa), albumin egg (45.0 kDa), chymotrypsinogen A (25.0 kDa), myoglobin equine (17.6 kDa).
M_2: From top downward: â-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), beta-lactoglobulin (18.4 kDa), restriction endonuclease Bsp-987 (25.0 kDa), beta-lactoglobulinand alactalbumin (14.4 kDa).

Fig. 2. Native-PAGE and SDS-PAGE electrophoresis

like neurotoxins produced by the anaerobic bacteria *Clostridium tetani* and *Clostridium botulinum* are specifically bound by the gangliosides (Antonyuk *et al.,* 2010).

Atomic force microscopy(AFM) determination of the MPL sample

The morphology of MPL was characterized by AFM (Fig. 3). Globular proteins, linked with something resembling fibre, were shown in the scope at 200 nm, with the single protein measuring 75 nm or so.

Dependence of MPL on pH and temperature

The MPL haemagglutinating activity was stable between pH 6 and pH 8.5. When the pH was at 3, there was no activity at all. The haemagglutinating activity was reduced by 40% when the pH was at 5, and by 20% when the pH was close to 9. The haemagglutinating activity was completely lost when the pH was raised to 12. These results demonstrate that the MPL was pH dependent.

Haemagglutination and inhibition of haemagglutinating activity

The MPL showed haemagglutinating activity at 10 µg/ml concentration. A variety of carbohydrates were used to detect the carbohydrate-specific character of MPL. Total inhibition was observed when D-galactose was used and the haemagglutinating activity decreased (Table 1). The result suggested that the carbohydrate specificity of the lectins plays an important role in their biological activity. The lectin-

Table 1. Inhibition of haemagglutination activity

Inhibiter	Minimum inhibitory concentration (mM)
Ribose	n.i
D-glucose	n.i
D-fructose	n.i
D-galactose	15.6
D-mannose	n.i
D-fucose	n.i
Maltose	n.i
N-acetyl-D-glucosamine	n.i
N-acetyln	n.i
euraminic acid	n.i
N-acetyl-D-galactosamine	n.i

n.i.= not inhibited

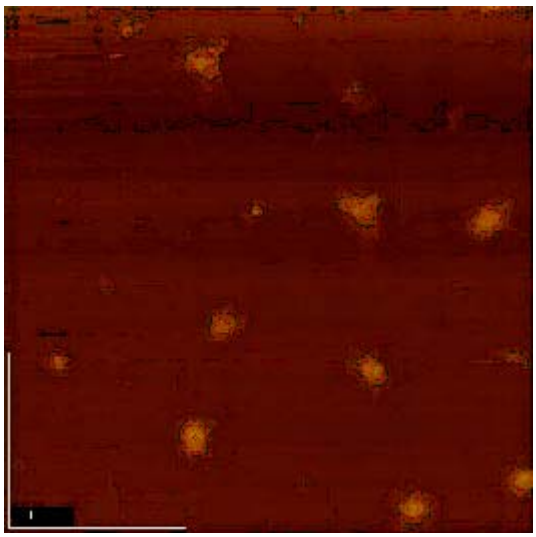


Fig. 3. AFM imaging of MPL

Additionally, MPL was fully active at temperatures of between 10! to 40!, with activity markedly decreased above 40!. Incubation of MPL at 80! led to irreversible denaturation. The results indicate that the MPL was temperature dependent.

AFM imaging by tapping mode of *Musca domestic* pupae lectin added to the surface of freshly cleaved mica. AFM= atomic force microscopy

Assay of antibacterial activity with MPL

Purified MPL exhibited strong antibacterial activity against both gram-positive and gram-negative bacteria. MPL 0.1 mg/ml was able to efficiently inhibit the growth of Staphylococcus species as indicated by a 50% decrease in turbidity, whereas MPL 0.5 mg/ml almost completely inhibited growth. MPL also effectively inhibited the growth of gram-negative and gram-positive bacteria (Fig. 4).

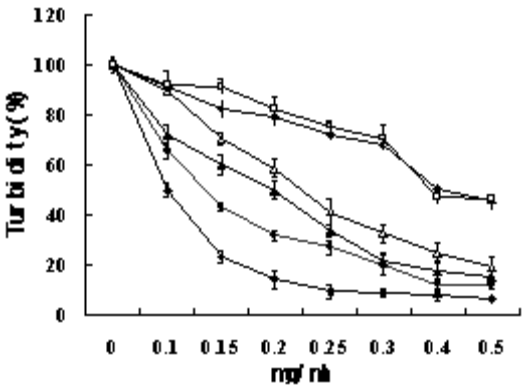


Fig. 4. Antibacterial activity of MPL against different bacteria stains

The antibacterial activity against both gram-positive and gram-negative bacteria was measured by turbidity at 620 nm after incubation for 6 hour with MPL in different concentrations. *Staphylococcus aureus* (Æ%), *Bacillus subtilis* (Ï%), *Streptococcus pneumoniae* (²%), *Escherichia coli* (³%), *Salmonella typhimurium* (Ç%), *Klebsiella pneumoniae* (¡%)

Splenocyte proliferation by MPL. To evaluate the overall effect of MPL on immune cells, the effect of MPL on splenic lymphocyte proliferation was assessed (Fig. 5). Treatment with MPL 5 µg /ml caused a significant increase in comparison with control (MPL 0 µg/ml). In the 10 and 50 µg /ml MPL groups, lymphocyte proliferation was significantly stimulated compared with control ($P<0.05$). Treatment with positive control (ConA 5 µg /ml) resulted in significant increase in the splenocyte proliferation index compared with treatment with either control or blank control (MPL 0 µg /ml).

MPL increased the proliferation of splenic lymphocyte from male BALB/c mice. Murine splenocytes (10^6 cells/ml) were stimulated by MPL (5–50 µg/ml) or Con A (5 µg/ml) for 24 hours

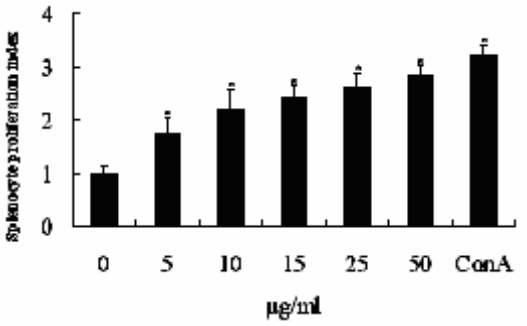


Fig. 5. Splenocyte proliferation with MPL

DISCUSSION

Bioactive proteins, such as lectins, have been isolated from plants, invertebrates and insects, especially those with specificity to monosaccharides D-galactose and D-mannose (Alencar *et al.,* 2003). Lectins that inhibit haemagglutinating activity by carbohydrate have been purified (De-Simone *et al.,* 2003). In this study, we purified a lectin from *Musca domestica*. In the haemolymph, the MPL activity was specific to D-galactose, so we purified MPL by affinity chromatography on Sepharose-4B, which contains the galactose polymer. Lectins recognize specific carbohydrates (Fujita 2002), and their haemagglutinating activity is inhibited by some monosaccharides.

Like other lectins such as pinto bean lectin (Wong *et al.,* 2006), purified MPL was tested for its ability to agglutinate RBC. Reduced RBC agglutination was observed when MPL was pre-incubated with D-galactose. The reduction of RBC agglutination may be due to the presence of cognate glycan on the cell surface.

The haemagglutinating activity of MPL is strikingly reduced above 40!; this thermal

instability is similar to that seen in marine sponge lectin (Kawsara *et al.,* 2009). Incubation of MPL at 80! led to irreversible denaturation. These results indicate that MPL is temperature dependent. In terms of pH, MPL activity was stable between pH 6 and pH 8.5. Interestingly, comparison of SDS-PAGE results in the presence or absence of 2-ME indicates that the MPL is a compact globular protein at least partly because of the intra-molecular disulfide bonds (Krondiris, 2002). Under reducing condition in which all S-S bonds are separate, the proteins are expected to assume 'stretch structure', thereby behaving as a larger molecule.

It has been previously reported that lectin from *Manila clam* exhibits strong antibacterial activit. MPL also effectively inhibited the growth of both gram-negative and gram-positive bacteria, indicating its broad spectrum antibacterial effect. These results are in accordance with the bacterial agglutination, residual haemagglutination assays and antibacterial activity seen *in vivo*. This clearly demonstrates that the MPL preferentially binds to the glycoconjugate moiety on the cell surface of the bacteria, thereby enhancing the phagocytosis rate and/or bactericidal activity. Lectin from Horse mussel has also exhibited strong antibacterial effect against Vibrio species (Tunkijjanukij *et al.,* 1998), and galactose-binding lectin from *Polyandrocarpa misakiensis* has shown strong antibacterial activity (Suzuki *et al.,* 1990).

At present, the mechanism underlying the immunomodulatory effect of the bioactive lectin protein remains uncertain, but there has been increasing evidence to support the idea that they mainly enhance lymphocyte proliferation. For example, the new immunomodulatory protein, derived from Jew's Ear mushroom, has been reported to activate murine splenocytes by markedly increasing their proliferation and interferon-ã secretion *in vitro* (Sheu *et al.,* 2004). Experimental data suggested that MPL was able to stimulate mice splenocytes for proliferation, though the proliferation index was less than that of ConA. The results suggested that MPL involves the defense of body against microbial invasion and stimulates splenocytes proliferation.

CONCLUSION

In conclusion, a lectin with sugar specificity, high molecular weight, haemagglutinating activity, potent antibacterial activity and mitogenic activity to splenocytes was isolated from *Musca domestica* pupae. The potent mitogenic activity is remarkable, and suggests potential for immune therapy in the future. The authors are presently investigating the immunomodulation activity of MPL.

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REFERENCES

1. Alencar NMN, Assreuy AMS, Alencar VBM, Melo SC, Ramos MV, Cavada BS., The galactosebinding lectin from Vatairea macrocarpa seeds induces in vivo neutrophil migration by indirect mechanism. *Int. J. Biochem. Cell. B* .2003; **35**: 1674–1681.
2. Antonyuk VO, Klyuchivska OYu, Stoika RS., Cytotoxic proteins of Amanita virosa Secr. mushroom: Purification,characteristics and action towards mammalian. *Toxicon*. 2010; **55**: 1297-1305.
3. De-Simone SG, Netto CC, Silva JFP., Simple affinity chromatographic procedure to purify β-galactoside binding lectins. J. Chromatogr. A. 2006; **838**: 135-138.
4. Franc NC, White K., Innate recognition system in insect immunity and development: new approaches in Drosophila. *Microbes. Infect*. 2000; **2**: 243-250.
5. Fujita T., Evolution of the lectin-complement pathway and its role in innate immunity. *Nat. Rev. Immunol*. 2002; **l2**: 346-353.
6. Gaidamashvili M, Staden JV., Interaction of lectin-like proteins of South African medicinal plants with *Staphylococcus aureus* and *Bacillus subtilis*, *J. Ethnopharmacol.* 2002; **80**:131-135.
7. He X, Zhang Y, Yu F, Yu Z., A novel sialic acid binding lectin with anti-bacterial activity from the Hong Kong oyster (*Crassostrea hongkongensis*). *Fish. Shellfish. Immun.* 2011; **31**: 1247-1250 .
8. Jayasree S., Biological properties of a natural agglutinin in the hemolymph of Indian white prawn, *Penaeus indicus H.* Milne Edwards. *Aquaculture*. 2001; **194**: 245-252.
9. Kawsara MA, Yuki F, Matsumoto R, Ichikawa T, Tateno H, Hirabayashi J., Isolation, purification, characterization and glycan-binding profile of a D-galactosidespecific lectin from the

marine sponge, *Halichondria okadai. Comp. Biochem. Phys. B.* 2009; **152**: 382-389.
10. Keisuke G, Takahashi KG, Kuroda T, Muroga K., Purification and antibacterial characterization of a novel isoform of the Manila clam lectin (MCL-4)from the plasma of the Manila clam, Ruditapes philippinarum. *Comp. Biochem. Phys*. B. 2008; **150**: 45-52.
11. Komano H., Purification of lectin induced in hemolyph of Sarcophaga peregrina larvae on injury. *J. Biol. Chem*. 1980; **255**: 2919-2924.
12. Krondiris JV, Sideris DC., Intramolecular disulfide bonding is essential for betanodavirus coat protein conformation. *J. Gen. Virol*. 2002; **83**: 2211-2214.
13. Lee CH, Kim JK, Kim HY, Park SM, Lee SM., Immunomodulating effects of Korean mistletoe lectinin vitro and in vivo. *Int. Immunopharmacol.* 2010; **9**: 1555-1561.
14. Lavine MD, Strand MR., Insect hemocytes and their role in immunity. *Insect. Biochem. Molec.* 2002; **32**: 1295-1309.
15. Mossman T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods*. 1983; **65**: 55–63.
16. Natori S, Shiraishi H, Hori S, Kobayashi A., The roles of Sarcophaga defense molecules in immunity and metamorphosis. *Dev. Comp. Immunol*. 1999; **23**: 317-32.
17. Nagaraj MG, Goswami U, Khan MI., T-antigen binding lectin with antibacterial activity from marine invertebrate, sea cucumber (Holothuria scabra): Possible involvement in differential recognition of bacteria. *J. Invertebr. Pathol.* 2008; **99**: 141-145.
18. Olafsen JA., Fletcher TC, Grant PT., Agglutinin activity in Pacific oyster (Crassostrea gigas) hemolymph following in vivo Vibrio anguillarum challenge. *Dev. Comp. Immunol*. 1992; **16**: 123-138.
19. Pellegrina CD, Rizzi C, Mosconi S, Zoccatelli G, Peruffo A., Chignola R., Plant lectins as carriers for oral drugs: is wheat germ agglutinin a suitable candidate. *Toxiol. Appl. Pharm*. 2005; **207**: 170-178.
20. Sheng JC, Yu F, Xin ZH, Zhao LY., Preparation, identification and their antitumor activities in vitro of polysaccharides from *Chlorella pyrenoidosa*. *Food. Chem.* 2007; **105**: 533-539.
21. Sheu F, Chien PJ, Chien AL, Chen YF, Chin KL., Isolation and characterization of an immunomodulatory protein (APP) from the Jew's Ear mushroom Auricularia polytricha. *Food. Chem.* 2004; **105**: 593-600.
22. Suzuki T, Takagi T, Furukohri T, Kawamura K., A calcium-dependent galactose-binding lectin from the Tunicate Polyandrocarpa misakiensis. *J. Bio Chem*. 1990; **265**: 1274-1281.
23. Tunkijjanukij S, Olafsen JA., Sialic acid-binding lectin with antibacterial activity from the Horse mussel: further characterization and immunolocalization. *Dev. Comp. Immunol*. 1998; **22**: 139-150.
24. Tzou PT, Gregorio ED, Lemaitre B., How Drosophila combats microbial infection: amodel to study innate immunity and host-pathogen interactions. *Curr. Opin. Immunol.* 2002; **5**: 102-110.
25. Wang JH, Yang DY, Zheng YT., A new lectin from the sea worm *Serpula vermicularis*: Isolation, characterization and anti-HIV activity. *Toxicol. Pathol*. 2007; **145**: 184-193.
26. Wong JH., Clarence CT, Wong TB., Purification and characterization of a galactose-specific lectin with mitogenic activity from pinto beans. BBA-Proteom. 2006; **1760**: 808-813.
27. Zhang G, Sun J, Wang H , Ng TB., First isolation and characterization of a novel lectin with potent antitumor activity from a Russula mushroom. *Phytomedicine*. 2010; **17**: 775-781.
28. Zheng SY, Li CX, Ng TB, Wang HX., A lectin with mitogenic activity from the edible wild mushroom Boletus edulis. *Process Biochem.* 2007; **42**: 1620–1624.