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ISOLATION OF A LECTIN FROM Gracilaria firma Chang & Xia AND DETERMINATION OF SOME OF ITS BIOLOGICAL ACTIVITIES

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ABSTRACT - A lectin was obtained from the red marine alga Gracilaria firma Chang & Xia by extraction with 0.02 M phosphate buffered saline (pH 7.2 with 0.15 M NaCl) followed by ammonium sulfate precipitation at 50 % saturation and isoelectric precipitation at pH 5.2. The lectin yield was 0.087 % (fresh weight basis). The lectin agglutinated rabbit erythrocytes with a specific activity of 133.33 HU mg-1 (hemagglutination units per mg protein). This ability was inhibited by D-(+)-glucosamine at a sugar concentration of 250 mM. The lectin was found to contain 0.17 % total carbohydrates using the phenol-sulfuric acid assay. Highest activity of the lectin was observed at 30 and 40 °C, and at pH 5.0 and 6.0. Assessment of its biological activities showed its ability to inhibit mungbean seed germination at 600 µg mL-1, its toxicity against brine shrimp naupilii at 220 µg mL-1, its inhibition of the cell division of onion root tip cells at 220 µg mL-1, and its activity against mosquito larvae at 700 µg mL-1. However, it did not exhibit antimicrobial activity against Escherichia coli and Staphylococcus aureus even at 1000 µg mL-1.

Keywords: hemagglutination, inhibition of cell division, larvicidal activity

INTRODUCTION

The carbohydrate-binding specificity of lectins is by far its most important feature that makes it useful in various applications such as cell separation techniques, pharmacology, biochemical isolation and purification procedures, immunology and histochemical studies. A wide range of living organisms can be studied with lectins since almost all biological membranes contain glycoconjugates (Andrade et al., 2004). Blood-type specific lectins have been used for identifying blood group substances (Sharon and Lis, 2004). The ability of lectins to agglutinate erythrocytes made it clearly useful for detection of disease-related alterations of glycan synthesis, blood group typing, quantification of aberrations of cell surface glycan presentation (e.g. malignancy) and cell markers for diagnosing infectious agents (viruses, bacteria, fungi and parasites) (Rudiger and Gabius, 2001).

Lectins can distinguish malignant from normal cells due to their ability to agglutinate malignant cells or induce proliferation of lymphocytes. As such, lectins may be considered as potent therapeutic agents for cancer. Aside from these, lectins were also found to have anti-viral activity due to their ability to bind carbohydrates present in viral envelopes which makes the virus incapable of binding to the host cell thereby halting the transmission of viral DNA. Lectins like griffithsin, cyanovirin-N and scytovirin from Griffithsia sp. (Mori et al., 2005), Nostoc ellipsosporum (Boyd et al., 1997) and

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Scytonema varium (Bokesch et al., 2003), respectively, are some of the lectins that were observed to inhibit HIV-1 infection by binding to mannose-rich glycans (Cardozo et al., 2007).

Lectins have been reported to be present in some species of plants, animals, microorganisms and viruses. They are structurally different from one another in terms of metal ion requirement, number of peptide chains, presence or absence of disulfide bonds and molecular weight (Beeley, 1985). However, most of the lectins that have been thoroughly studied were of plant and marine invertebrate origin and not much from algal sources. This is primarily due to the low concentrations of lectins from algal extracts that poses difficulty in obtaining sufficient amounts (Kawakubo et al., 1999). Nevertheless, many macroalgal species have been screened for the presence of lectins such as *Eucheuma serra* (Kawakubo et al., 1997), *Ptilota filicina* (Sampaio et al., 1998), *Eucheuma cotonii and Eucheuma amakusaensis* (Kawakubo et al., 1999), *Ptilota serrata* (Sampaio et al., 1999), *Pterocladiella capillacea* (Oliveira et al., 2002), *Ptilota plumosa* (Sampaio et al., 2002), *Enteromorpha prolifera* (Ambrosio et al., 2003), *Kappaphycus alvarezii* (Hung et al., 2009), *Tichocarpus trinitus* (Molchanova et al., 2010), *Bryopsis plumosa* (Han et al., 2011) and *Eucheuma denticulatum* (Hung et al., 2015). However, only a few of these lectins have been thoroughly characterized in terms of physicochemical properties mainly due to the difficulty in their isolation and obtaining them in sufficient amounts for further studies (Calvete et al., 2000).

Goldstein et al. (1980) defined lectins as sugar-binding proteins of non-immune origin that agglutinate cells or precipitate glycoconjugates. They may be soluble in the aqueous medium of the cell or membrane-bound. The biochemical properties of lectins such as molecular weight, pH at which they are stable, sugar specificity, and dependence on certain metal ions vary among the lectins that were isolated from various sources. For lectins of algal origin, they have been found to have molecular weights ranging from 3.5 to 71 kDa and their isoelectric pH ranges from 4.0 to 6.0. Their hemagglutination activities usually are independent of divalent cations and they are typically more specific towards complex carbohydrates and/or glycoproteins (Molchanova et al., 2010). Most lectins that have been thoroughly studied were found to have a number of immunological and histochemical applications due to their carbohydrate-binding specificity (Cardozo et al., 2007). However, there is a dearth of reports regarding other biological activities of lectins especially those of lectins from algal sources. One of the few algal lectins found to have significant acaricidal effects against cattle tick *Boophilus microplus* (Acari: Ixodidae) is the *Gracilaria cornea* lectin (Lima et al., 2005).

Gracilaria belongs to class Rhodophyta and is a valued marine alga being a natural source of agar. However, it is not yet much cultivated due to its low growth rate. In the Philippines, locally cultivated species of *Gracilaria* include *G. arcuata* Zanardini var. *snackeyi* Weber-van Bosse, *G. blodgetii* Harvey, *G. edulis* (S.G. Gmelin) P.C. Silva, *G. eucheumatoides* Harvey, *G. firma* Chang & Xia, *G. heteroclada* J.F. Zhang & B.M. Zia, *G. manilaensis* Yamamoto & Trono, *G. salicornia* (C. Agardh) E.Y. Dawson, *G. tenuistipitata* var. *liui* Zhang & Xia, *G. textorii* (Suringar) De Toni, *G. canaliculata* Sonder, *G. cylindrica* Børgesen, *G. fastigiata* J.Agardh, *G. gigas* Harvey, *G. sullivanii* Yamamoto & Trono, and *G. turgida* E.Y. Dawson. They are cultivated primarily for processing as animal feed, fertilizer, human food, for industrial uses such as for waste water purification, and medicinal uses such as antidiarrheal and antimicrobial. Still little is known regarding the protein content of such species thus opening a wide opportunity for research endeavors on it. (Trono, 1997; Trono, 2004).

While many lectins have been isolated from a number of macroalgal species, very few have been reported from *Gracilaria* species. Among these are the lectins from *Gracilaria verrucosa* (Kakita et al.,1997), *Gracilaria ornata* (Leite et al., 2005) and *Gracilaria cornea* (Lima et al., 2005).

This study reports on the isolation of a lectin from a Philippine-grown red algae *Gracilaria firma* Chang & Xia, and determination of some of its biological activities.

MATERIALS AND METHODS

Preparation of the Algal Source. Samples of Gracilaria firma were obtained from a seaweed farm in Poblacion 2, Calatagan, Batangas, Philippines. These were washed with sea water, chopped into small pieces and were then homogenized with phosphate buffered saline (PBS) (0.02 M phosphate buffer pH 7.2 containing 0.15 M NaCl) in a 1:2 (w/v) ratio. The resulting homogenate was incubated at 4 °C for 18 hours followed by stirring for four hours at 10 °C and squeezing through cheesecloth. The resulting filtrate was centrifuged using a Beckman Coulter Allegra® X-30 benchtop refrigerated centrifuge at 10,000 rpm for 20 minutes at 4 °C to get the crude lectin extract.

Isolation of the Lectin. The isolation of the lectin was based on the method of Benevides et al. (1998) with some modifications. Solid ammonium sulfate was added to the clear crude extract with constant stirring to reach 50 % saturation while in an ice bath. Stirring was continued for four hours before putting the mixture in the refrigerator for the precipitate to settle. The precipitate was recovered by centrifugation using a Beckman Coulter Allegra® X-30 benchtop refrigerated centrifuge at 10,000 rpm for 20 minutes at 4 °C and then redissolved using a minimal amount of PBS. The resulting solution was then dialyzed using Sigma dialysis tubing with a 12,000 molecular weight cut-off against PBS for 24 hours. The dialyzed solution was then mixed with 0.1 M acetate buffer pH 5.2 at a 1:2 (v/v) ratio to precipitate the lectin. Centrifugation using a Beckman Coulter Allegra® X-30 benchtop refrigerated centrifuge at 10,000 rpm for 20 minutes at 4 °C was done to get the precipitate which was then redissolved in the minimum amount of PBS.

Determination of the Protein Profile of the Lectin Positive Fractions. The homogeneity of the different lectin positive fractions was determined using native polyacrylamide gel electrophoresis (PAGE) following the method of Laemmli (1970). Electrophoresis was done using a Bio-Rad Mini-PROTEAN® Tetra Cell set-up using a discontinuous polyacrylamide slab gel system consisting of 8 % polyacrylamide resolving gel and 4 % polyacrylamide stacking gel. The electrophoretic run was done at a constant voltage of 110 V. Protein bands on the gel were visualized using the silver staining method of Switzer et al. (1979) with modifications. The gel was rinsed with distilled water then placed for 20 minutes in a gel fix solution consisting of 12 % acetic acid and 30 % ethanol. The gel was then washed with 30 % ethanol, distilled water, freshly prepared 0.02 % $Na_2S_2O_3$ solution and distilled water prior to staining for 20 minutes with a solution of 0.1 % $AgNO_3$ and 0.08 % formaldehyde. The gel was again washed with distilled water twice and the bands were developed using a freshly prepared solution of 2 % Na_2CO_3 and 0.04 % formaldehyde. Color development was stopped by washing the gel with 5 % acetic acid.

Protein Content Determination. The amount of soluble proteins obtained at each stage of purification was determined using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as standard. Absorbance of the solutions was read at 595 nm using a Thermo Scientific MultiskanTM GO Microplate Spectrophotometer.

Carbohydrate Analysis. The presence of carbohydrates in the lectin was determined using the Molisch test as described by Shriner et al. (2004). Ten drops of the lectin sample were mixed with two drops of Molisch reagent (10 % naphthol in ethanol). Two milliliters of concentrated sulfuric acid was then poured slowly and steadily to the side of the tube. The formation of a purple color at the interface of the two layers was taken as the positive result. The total carbohydrate content of the lectin was determined using the phenol-sulfuric acid method by Dubois et al. (1956) with D-glucose as standard. One milliliter of 5 % phenol and 5 mL of concentrated sulfuric acid were added to 1.0 mL of the lectin sample or standard. The mixture was allowed to stand for 20 minutes for color development. Absorbance of the solutions was read at 490 nm.

Preparation of Blood Samples. Veterinarians from the Veterinary Teaching Hospital, College of Veterinary Medicine, University of the Philippines Los Baños, College, Laguna, Philippines collected the blood samples from a healthy rabbit. The required permit for this procedure was obtained from the Institutional Animal Care and Use Committee (IACUC) of the same institution. Human blood samples of type A, B, AB and O were drawn from healthy donors by a licensed nurse. The protocol for the blood sample preparation was based on the method by Benevides et al. (1998) with modification. One milliliter of the blood sample was mixed with 10 mL 0.02 M PBS (pH 7.2 with 0.15 M NaCl) instead of Tris-buffered saline as originally described, and was centrifuged at 1000 rpm for five minutes at room temperature using a Becton Dickinson Clay Adams® Compact II Centrifuge. A pipettor was used to remove the supernatant. Re-suspension and centrifugation of the cells were repeated thrice and the resulting packed cells were diluted to make a 2 % blood suspension in PBS.

Hemagglutination Assay and Hapten Inhibition Test. The protocol for the hemagglutination assay was based on the method of Benevides et al. (1998) with modifications. Fifty microliters of the lectin was placed in microtiter plates containing 50 μL PBS using two-fold serial dilution and then added with 50 μL of the 2 % (v/v) rabbit erythrocyte suspension. Fifty microliters of PBS in the last well was used as control. Plates were incubated for one hour at room temperature and were visually examined. A positive test for agglutination is indicated by the formation of a uniform dispersion of red blood cells which appears like a reddish solution while the formation of a discrete button at the bottom of the well indicates a negative result. The hemagglutination titer (HU) is the reciprocal of the highest dilution that caused agglutination while specific activity is expressed as titer units per milligram of proteins (HU mg-1). The effect of soluble sugars on the agglutination reaction and possible sugar specificity of the lectin were determined by the inclusion of the following standard sugars at initial concentrations of 200 and 1000 mM in the hemagglutination assay: D-glucose, D-mannose, D-maltose, D-fructose, L-fucose, D-glucosamine, D-galactose, D-sucrose, and N-acetyl-D-galactosamine. The inhibition assay was carried out in microtiter plates wherein to each well containing 50 μL of a two-fold serially diluted sugar solution, an equal volume of the lectin solution was added followed by the addition of 50 μL of 2 % (v/v) rabbit erythrocyte suspension. The plates were incubated for one hour at room temperature and were then examined visually.

Effect of Temperature and pH on Lectin Activity. The effect temperature and pH on the hemagglutination activity of the lectin was determined following the methods of Molchanova et al. (2010) with modifications. For the effect of temperature, a solution of the lectin was incubated at 10, 30, 40, 50, 60, 70 and 80 °C for 30 minutes. These solutions were then cooled before subjecting them to the hemagglutination assay as previously described. For the effect of pH, the following buffers, all made up with 0.15 M NaCl, were used as diluents in the hemagglutination assay as previously described: 0.01 M glycine-HCl buffer (pH 2.0, 3.0), 0.01 M acetate buffer (pH 4.0, 5.0), 0.01 M phosphate buffer (pH 6.0, 7.0), and 0.01 M Tris-HCl buffer (pH 8.0, 9.0). The pH of the buffer solutions was determined using a pH meter, Eutech CyberScan hand-held pH 110.

Determination of the Biological Activities of the Lectin

Seed Germination Inhibition Assay. Inhibition of seed germination by the lectin was determined using mungbean (Vigna radiata) seeds following the method of Mojica and Merca (2005) with modifications. The seeds were dipped in the lectin solution at concentrations of 150, 300 and 600 μg mL-1 and were then allowed to germinate in a six-well cell culture plate layered with folded pieces of paper towel moistened with 2 mL of the test solution. Germination was allowed to proceed for two days at room temperature. Controls such as distilled water and PBS were also tested employing the same conditions as those for the lectin solutions. Germination of the seed is indicated by the emergence from the seed of the radicle that is at least 3 mm-long after cracking of the seed coat. Percent germination was noted for each replicate.

Toxicity Assay. The toxicity of the lectin was determined using the brine shrimp (Artemia salina) lethality assay following the method described by Meyer et al. (1982) with modifications. Brine

shrimps were allowed to hatch on an improvised hatching vessel using a brine solution that is 2.1 % (w/v) rock salt in distilled water. The vessel was filled with about three-fourths full of the brine solution to which half a teaspoon of baking soda was added to act as buffering agent. Brine shrimp eggs were deposited in the medium and hatching was allowed to proceed with aeration and constant illumination using a 40-watt incandescent bulb for 24 hours. For this assay, the lectin was redissolved in distilled water. The lethality assay was done by pipetting ten one-day-old naupilii to each well of a white ice tray that contained 2.5 mL of the lectin solution (110 or 220 µg mL-1). The brine solution was used as diluent and negative control. The trays were incubated at room temperature with continuous illumination. The number of dead naupilli was noted after 12 and 24 hours.

Mitotic Index Assay. The anti-mitotic activity of the lectin was investigated using multiplier (native) onions (Allium cepa L.) following the method described by Fiskesjo (1985) with modifications. The dry loose skins of the onion bulbs were removed and the bottom plates were gently scraped without damaging the root primordia. The bottom of the bulbs was soaked in distilled water for 24 hours to allow growth of the roots to a length of about 1-2 cm. The bulbs were then soaked in the test solutions (110 and 220 µg mL-1 lectin in distilled water with distilled water as negative control) for another 24 hours. Colchicine treatment of the roots as described by Fiskesjo (1985) was not done since the test did not look into the details of chromosome configurations. The treated roots were cut and soaked in a 3:1 solution of absolute ethanol : glacial acetic acid fixing solution for 12 hours instead of a five-minute fixation with 9:1 45 % CH3COOH : 1 N HCl fixing solution as originally described. The fixed roots were cut in the area slightly above the root cap and were stained using the acetocarmine solution instead of a 2 % orcein solution in 45 % CH3COOH as originally described. Slides were viewed using a Nikon Alphapot 2 photomicroscope under 10X low power objective (LPO) magnification. For each slide, the number of dividing cells was noted for each 500 cells counted.

Larvicidal Activity. The larvicidal activity of the isolated lectin was determined following the method described by Mojica and Merca (2005) with modifications. Ten mosquito larvae were placed in plastic sauce cups containing the test solutions (700 µg mL-1 lectin solution; 0.01 M PBS pH 7.2 and distilled water as negative controls). The number of dead larvae was noted after 24 hours of treatment.

Antimicrobial Activity. The antimicrobial activity of G. firma lectin against microorganisms was determined using the filter paper disc (Robert et al., 2009), agar well (Ahmed et al., 2008) and cylinder cup (Ganga Rao et al., 2012) methods. Escherichia coli (gram-negative bacterium) and Staphylococcus aureus (gram-positive bacterium) were used as test organisms. Lectin solution at 130 µg mL-1 was used for the filter paper disc and agar well methods while lectin solutions at 500 and 1000 µg mL-1 were used for the cylinder cup method. Antibiotics such as spectinomycin and streptomycin were used as positive controls while PBS was used as negative control. Test microorganisms were inoculated on a nutrient broth and were allowed to grow for 24 hours. One milliliter of the microorganism suspension was then mixed with 100 mL of the previously sterilized nutrient agar. About 20 mL of the inoculated nutrient agar was poured on sterilized flat-bottomed petri dishes and was allowed to solidify. Sterilized filter paper discs were soaked in the test solutions before laying them on top of the solidified agar while a 50-µL aliquot of the test solutions was pipetted to the agar wells and to the cylinder cups. The plates were incubated for 24 hours at room temperature and were then observed for clearing or zones of inhibition.

RESULTS

The aqueous extract of *G. firma* was subjected to ammonium sulfate precipitation at 50 % saturation followed by isoelectric precipitation at pH 5.2 resulting in a lectin yield of 0.087 % (fresh seaweed weight basis). As seen in Table 1, the isolation procedure employed gave a lectin with a specific activity of 133.33 HU mg^{-1} and a purification fold of 3,333. The homogeneity of the isolate was verified from the electrophoretogram that was obtained using native PAGE which showed only one band upon staining with $AgNO_3$ (Figure 1).

The *G. firma* lectin was able to agglutinate rabbit erythrocytes but not any of the human blood samples (Types A, B, AB and O) used. The hemagglutination activity of the lectin was observed to be highest at pH 5 and 6 (Figure 2) and at 30 and 40 °C (Figure 3). The results of the Hapten inhibition assay using simple sugars and disaccharides are presented in Table 2. Results showed that the hemagglutination activity of the *G. firma* lectin was inhibited only in the presence of D-(+)-glucosamine at 250 mM.

The presence of a carbohydrate moiety in the *G. firma* lectin was revealed by the Molisch test when a distinct purple interface was observed. Also, the phenol-sulfuric acid assay gave a total carbohydrate content of 0.17 %.

Table 1. Purification of the lectin present in *Gracilaria firma* Chang & Xia.

FRACTION	TITER[a] (HU)	TOTAL VOLUME (mL)	PROTEIN CONTENT (mg mL^{-1})	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY[b] (HU mg^{-1})	PURIFICATION FOLD
Crude extract	16	2,500	0.17	425.00	0.04	---
50 % $(NH_4)_2SO_4$ precipitate	256	30	0.60	18.00	14.22	356
Isoelectric precipitate at pH 5.2	32	4	0.06	0.24	133.33	3,333

[a] inverse of the highest dilution that can still agglutinate rabbit erythrocytes
[b] hemagglutination units per mg protein

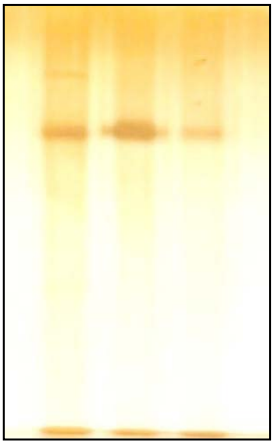

Figure 1. Native polyacrylamide gel electrophoretogram of the lectin from *Gracilaria firma* at different stages of its isolation. (1) crude extract; (2) 50 % $(NH_4)_2SO_4$ precipitate; (3) isoelectric precipitate at pH 5.2.

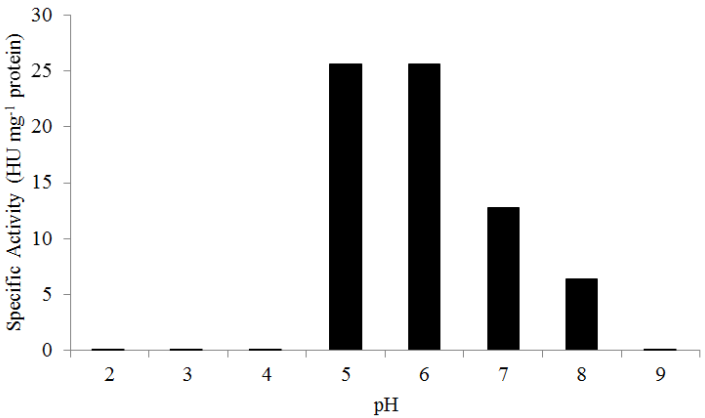

Figure 2. Effect of pH on the hemagglutination activity of the *Gracilaria firma* lectin. Lectin concentration = 250 µg mL^{-1} ; n = 2

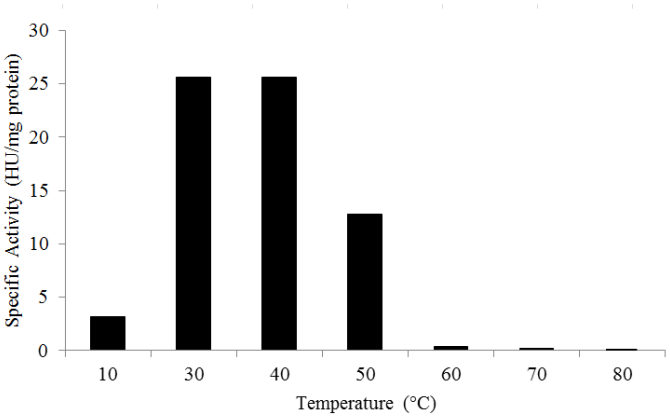

Figure 3. Effect of temperature on the hemagglutination activity of the *Gracilaria firma* lectin. Lectin concentration = 250 μg mL^{-1} ; n = 2.

The *G. firma* lectin was subjected to several biological activity assays. Table 3 shows that it was capable of inhibiting the germination of mungbean (*Vigna radiata*) seeds at 150, 300 and 600 μg mL^{-1} with the inhibition increasing with increasing lectin concentration. Total inhibition of seed germination was observed at 600 μg mL^{-1} of the lectin. Toxicity of the lectin against brine shrimp (*Artemia salina*) naupilli was also observed (Table 4). There was a marked increase in the average percent mortality from 6.67 ± 8.89 % of the control brine medium to 30.00 ± 10.00 % when the brine shrimp naupilli were placed in a 110 μg mL^{-1} lectin solution after 12 hours of incubation while all naupilli did not survive the incubation using 220 μg mL^{-1} lectin solution. The same trends were observed when the incubation period was extended to 24 hours. There was also an observed significant decrease in the average percent cell division of onion (*Allium cepa*) root tip cells upon treatment with the lectin solutions (Table 5). From an average of 8.30 ± 2.30 % cell division of onion root tip cells in distilled water, a significant decrease to 4.90 ± 0.80 % was observed when the root tips were treated with 110 μg mL^{-1} lectin solution. There was a further decrease in the average percent cell division when a 220 μg mL^{-1} lectin solution was used though not significant. Cells were considered dividing when it showed densely stained chromosomes during any of the stages of mitosis namely prophase, metaphase, anaphase and telophase. Larvicidal activity against mosquito (*Aedes aegypti*) larvae was also found to be significant when the larvae were allowed to thrive on a lectin solution at a concentration of 700 μg mL^{-1}. After 24 hours of treatment, an average mortality rate of 53.33 ± 13.33 % was observed whereas no mortality was observed when the larvae were treated with PBS or distilled water. The lectin did not exhibit antimicrobial activity against *Escherichia* coli and *Staphylococcus aureus* based on the results of the disc diffusion, agar well and cylinder cup antimicrobial assay methods.

Table 2. Hapten inhibition assay of the *Gracilaria firma* lectin.

CARBOHYDRATE	INHIBITORY CONCENTRATION (mM)
D-(+)-glucose	NI
D-(+)-fructose	NI
D-(+)-galactose	NI
D-(+)-mannose	NI
L-(−)-fucose	NI
Maltose	NI
Sucrose	NI
D-(+)-glucosamine	250
N-acetyl-D-galactosamine	NI

NI – no inhibition of hemagglutination

Table 3. Effect of the *Gracilaria firma* lectin on the germination of mungbean seeds.

TREATMENT	PERCENT GERMINATION [a]
Distilled water	100.00 ± 0.00^{A}
0.02 M PBS pH 7.2	98.33 ± 2.78^{A}
150 μg mL^{-1} lectin	60.00 ± 10.00^{B}
300 μg mL^{-1} lectin	28.33 ± 24.44^{C}
600 μg mL^{-1} lectin	0^{D}

[a] Each value is the mean ± average deviation of six replicates with each replicate containing ten seeds. Means within the same column with the same superscript are not significantly different in Tukey's Studentized Range test at $\alpha = 5$ %; $n = 5$; F value = 53.36; DF = 4; p value < 0.0001.

Table 4. Mortality of brine shrimp naupilii treated with *Gracilaria firma* lectin.

TREATMENT	PERCENT MORTALITY [a]	
	AFTER 12 HOURS	AFTER 24 HOURS
Brine medium [b]	6.67 ± 8.89 [A]	16.67 ± 15.56 [A]
110 μg mL^{-1} lectin	30.00 ± 10.00 [B]	46.67 ± 7.78 [B]
220 μg mL^{-1} lectin	100.00 ± 0.00 [C]	100.00 ± 0.00 [C]

[a] Each value is the mean ± average deviation of six replicates with each replicate containing ten brine shrimp naupilii. Means within the same column with the same superscript are not significantly different in Tukey's Studentized Range test at α = 5 %. For both 12 and 24 hours, n= 3; DF = 2; $p < 0.0001$. F value = 138.48 (for 12 hours) and 52.28 (for 24 hours);
[b] 2.1 % rock salt in distilled water

Table 5. Cell division of onion root tip cells treated with the *Gracilaria firma* lectin.

TREATMENT	PERCENT CELL DIVISION [a]
Distilled water	8.30 ± 2.3 [A]
110 μg mL^{-1} lectin	4.90 ± 0.80 [B]
220 μg mL^{-1} lectin	3.70 ± 0.43 [B]

[a] Each value is the mean ± average deviation of six replicates. A total of 500 cells were counted for each replicate. Means within the same column with the same superscript are not significantly different in Tukey's Studentized Range test at α = 5%; n = 3; F value = 9.72; DF = 2; p = 0.0020.

DISCUSSION

In this study, simple protein isolation techniques were employed to isolate the lectin from G. firma that included (1) homogenization of the sample using PBS (0.02 M phosphate buffer containing 0.15 M NaCl) at a 1:2 (w/v) ratio, (2) ammonium sulfate precipitation at 50 % saturation, and (3) isoelectric precipitation at pH 5.2 using 0.1 M acetate buffer pH 5.2. The entire process resulted in a dramatic increase in the specific activity (from 0.04 HU mg-1 protein to 133.33 HU mg-1 protein) and purification fold (up to 3,333) of the lectin from the crude extract up to the isoelectric precipitate. Ammonium sulfate precipitation is a salting-out process that can separate proteins in a mixture depending on their solubilities in the aqueous medium. It is based on the preferential solvation of the salt by the water molecules that are closely associated with the protein surface (Rupley et al., 1983). Isoelectric precipitation, on the other hand, is an isolation technique where a protein can be precipitated out of the solution when its isoelectric point (pI) is equal to the pH of the solution. At this pH, the protein is least soluble because there are no net positive or negative charges that can interact with water, thus the protein can coagulate with minimal repulsion (Scopes, 1994). Other lectins from Gracilaria species such as G.

verrucosa (Kakita et al., 1999) and G. ornata (Leite et al., 2005) were also obtained using ammonium sulfate precipitation but in combination with other methods namely ion-exchange chromatography, gel filtration chromatography and affinity chromatography.

The purple-colored interface obtained in the Molisch test suggests the glycoprotein nature of the G. firma lectin. In this test, the carbohydrate present in the sample is dehydrated by sulfuric acid to form furfural or hydroxymethylfurfural which in turn condenses with two molecules of α-naphthol to form the purple-colored product. When the lectin was subjected to the phenol-sulfuric acid assay a total carbohydrate content of 0.17 % was obtained. This value is relatively low compared to those previously reported for lectins from other algal sources. The carbohydrate content of the lectin from G. ornata was found to be 2.9 % (Leite et al., 2005), that of the Tichocarpus crinitus lectin was 6.9 % (Molchanova et al., 2010) while the lectin from Ulva pertusa had 1.2 % carbohydrate content (Wang et al., 2004). On the other hand, Lima et al. (2005) reported a relatively large amount of carbohydrate content of the G. cornea lectin which was 52.2 % while Oliveira et al. (2002) reported that the lectin from Pterocladiella capillacea did not contain any carbohydrate.

The G. firma lectin was able to agglutinate rabbit erythrocytes but not any of the human blood types A, B, AB and O. Rabbit erythrocytes, rather than human erythrocytes, were found to be the most suitable in detecting the presence of hemagglutinins from a variety of marine algal species as reported by Freitas et al. (1997) and Chiles and Bird (1989). Mature rabbit erythrocytes have at least seven major proteins with molecular weights ranging from 23 to 250 kDa but lack the major sialoglycoproteins and other glycoproteins that are detected in human erythrocytes by periodic acid/Schiff staining (Light and Tanner, 1977). Hemagglutination experiments using concanavalin A and the Phaseolus vulgaris lectin, also by Light and Tanner (1977), revealed their preferential binding with four membrane proteins/glycoproteins having molecular weights of 24, 34, 43 and 90 kDa. It is possible that the observed hemagglutination of the rabbit erythrocytes by G. firma lectin was due to its interaction with these proteins or glycoproteins.

The G. firma lectin was found to exhibit optimum hemagglutination activity at pH 5.0 and 6.0, and at 30 and 40 °C. Significant decrease in the specific activity of the lectin was observed at lower or higher pH values as well as at higher temperatures. This is most probably due to the changes in the ionization states of amino acid residues during extremes of pH or thermal-induced alteration of the lectin's native structure, both of which can lead to the denaturation of the lectin. The results of this study on the effects of pH and temperature on the hemagglutination activity of the G. firma lectin are similar with those of most lectins isolated from macroalgal species. For instance, Wang et al. (2004) reported that the lectin from Ulva pertusa had an optimum activity between pH 6 and 8 while that of the lectin from Tichocarpus crinitus was at pH 7 to 8 as reported by Molchanova et al. (2010). On the other hand, the G. ornata lectin was found to be stable up to 50 °C (Leite et al., 2005) while the G. cornea lectin was reported to be stable up to 40 °C (Lima et al., 2005).

The sugar specificity of the lectin may be determined through the Hapten inhibition test. The sugar that can cause inhibition of the hemagglutination activity of the lectin could possibly be its sugar specificity. This phenomenon can be attributed to the competition between the added carbohydrate and the carbohydrate moieties in the erythrocyte membrane for binding sites on the lectin molecule. The sugars used were the three sugar determinants in human blood namely N-acetyl-D-galactosamine, D-galactose, and L-fucose, other simple sugars such as D-glucose, D-fructose, D-mannose, disaccharides such as sucrose and maltose, and an amino sugar D-glucosamine. These sugars were used not only to

establish the sugar specificity of the G. firma lectin but also to have an idea regarding the possible sugar moieties present in the membranes of rabbit erythrocytes which has not yet been fully investigated. The use of simple sugars can provide information regarding the complexity of the sugar specificity of the G. firma lectin since the non-agglutination of these simple sugars may suggest the requirement for complex sugars. Only D-(+)-glucosamine was able to inhibit hemagglutination at a sugar concentration of 250 mM, indicating that G. firma lectin may have specificity for this amino sugar. The inability of the three sugar determinants in human blood to inhibit the agglutination of rabbit erythrocytes by the G. firma lectin somehow confirms the observed non-agglutination of any of the human erythrocyte samples by this lectin.

To date, many algal lectins have been studied in terms of physicochemical properties, which mainly focused on hemagglutination activity and factors that can affect it. Only a few lectins have been further studied in terms of biological activity such as antiviral activities (Adams et al., 2004; Tsai et al., 2004; Mori et al., 2005) and their ability to differentiate normal cells from malignant cells (Pinto et al., 2009. In this study, the G. firma lectin was subjected to several tests to determine potential biological activities which could be utilized for some pharmaceutical and agricultural applications.

The G. firma lectin inhibited the germination of mungbean seeds when used at concentrations of 150, 300 and 600 μg mL-1. As far as algal lectins are concerned, there are still no reports on their use as an inhibitor of seed germination. However, inhibition of germination by a lectin from the internal organs of black sea cucumber (Holothuria scabra Jaeger) was reported by Mojica and Merca (2005) wherein the lectin inhibited the germination of radish seeds at concentrations of 200 and 500 mg mL-1. Lectins could probably inhibit germination in several ways. First, the lectin may have interfered with the germination process at the molecular level. Bewley and Black (1985) suggested that lectins can block the re-formation of keto-acids from amino acids a few minutes before water enters the seeds. Keto acids, which are usually unstable and absent in dry seeds, are essential intermediates of the respiratory pathways. Second, it can also be that lectins prevented the uptake of oxygen that is needed upon activation and hydration of mitochondrial enzymes involved in the citric acid cycle and electron transport chain (Bewley and Black, 1985). Lastly, it is also possible that lectins caused inhibition or inactivation of enzymes involved in germination (Mojica and Merca, 2005).

Toxicity against brine shrimp (Artemia salina) naupilli was also observed when the naupilii were treated with 110 and 220 μg mL-1 G. firma lectin solutions. Though the brine shrimp toxicity assay has been used extensively to assess the toxic effects of different substances, the mechanism involved is still largely unknown. However, results from this assay can indicate a wide range of pharmacological activities such as anticancer, antiviral, insecticidal and pesticidal (Kawsar et al., 2010). A number of researches on lectins from non-algal sources have reported the toxic effects of such compounds on brine shrimps. For instance, lectins from the marine invertebrate sea hare eggs and polychaete exhibited percent mortalities of 63.33 % and 33.33 %, respectively, at a lectin concentration of 32 μg mL-1 (Kawsar et al., 2010). The average percent mortality was also directly proportional to the lectin concentration. Similar results were reported for the Potca fish and mullbery seed lectins (Absar et al., 2005; Absar et al., 2008) and the snail lectin (Santos et al., 2010). Mojica and Merca (2005) also reported the lectin from the internal organs of a black sea cucumber Holothuria scabra Jaeger to be toxic to brine shrimp at a concentration of 100 mg mL-1.

The anti-mitotic activity of the G. firma lectin was investigated using native onions. The average percentage of dividing cells of onion roots treated with 110 and 220 μg mL-1 lectin solutions

varied significantly with those treated with distilled water. Arresting mitosis is a mechanism exhibited by drugs collectively known as "anti-mitotics" (eg. Taxol) to kill cancer cells, next to the mainstay systemic chemotherapy. Such drugs inhibit mitosis by disrupting the mitotic spindle assemblies (Gascoine and Taylor, 2009). At present, there are no reported studies on lectins being subjected to the mitotic index assay. This study appears to be the first attempt to investigate the anti-mitotic property of lectins.

The *G. firma* lectin was also found to exhibit significant larvicidal activity (53.33 ± 13.33 % average mortality) against mosquito (*Aedes aegypti)* larvae at a lectin concentration of 700 μg mL^{-1}. No studies have so far been done to investigate the larvicidal activity of algal lectins using mosquito larvae. However, Leite et al. (2005) reported that the incorporation of the *G. ornata* lectin in artificially prepared cowpea seeds affected the development of the *Callosobruchus maculatus* larvae which is an insect pest infesting cowpea seeds. This study gave some insights on the possibility of using the *G. ornata* lectin in biotechnological strategies for pest management of stored cowpea seeds. On the other hand, a weak larvicidal activity against mosquito larvae (8 % average mortality at 500 mg mL^{-1} lectin concentration) was reported by Mojica and Merca (2005) using the lectin from the internal organs of a black sea cucumber *Holothuria scabra* Jaeger. The observed larvicidal activity of the *G. firma* lectin suggests its potential application as an insecticide or mosquito repellant.

Antimicrobial assays were also performed on the *G. firma* lectin. In the filter paper disc and agar well assays that used 130 μg mL^{-1} lectin solutions, small zones of inhibition, approximately 1-2 mm from the edge of the disc or agar well, were observed using the gram-negative *Escherichia coli* and the gram-positive *Staphylococcus aureus*. However, no antimicrobial activity was observed using the cylinder cup method and lectin solutions at 500 and 1000 μg mL^{-1}. There are very few reports on the antimicrobial properties of algal lectins. Liao et al. (2003) reported the antimicrobial properties of lectins from the marine algal species *Eucheuma serra* and *Galaxaura marginata*. Lectins from these algae were able to inhibit the growth of *Vibrio vulnificus* which is a known pathogen in marine organisms and can be acquired by humans upon eating undercooked infected seafoods, getting punctures from stingrays, or even entering through open wounds when swimming in infected waters.

CONCLUSIONS AND RECOMMENDATIONS

This study was able to show that the red seaweed *Gracilaria firma* Chang & Xia is a potential source of a lectin that exhibits biological activities such as inhibition of seed germination and cell division, larvicidal activity and toxicity effects that have pharmaceutical and agricultural applications. Thus, an additional economic value of the seaweed may be possible aside from its being a valuable source of agar. The isolation process included commonly used and less expensive biochemical techniques such as buffer extraction, ammonium sulfate precipitation and isoelectric precipitation. However, the use of more sensitive techniques such as affinity chromatography may be recommended in order to improve its yield.

It is recommended that the antimicrobial activity of the isolated lectin be tested using other bacteria and more sensitive techniques. The isolated lectin may also be subjected to other biological tests to further uncover its potential for other useful applications. For instance, the cell line assay may be done to determine its potency as an anti-cancer agent, a biological function that was already established for lectins isolated from other sources.

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STATEMENT OF AUTHORSHIP

The first author prepared the conceptual framework of the study in consultation with and with guidance from the second author, conducted the literature search, performed all the experiments and prepared the manuscript. The second author provided the expertise in addressing the problems encountered during the whole duration of the study, and in the processing and interpretation of the results. The final editing of the manuscript was done by the first two authors. The third and fourth authors made important suggestions in interpreting the results of the biological assays and also contributed in editing the manuscript.

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