

In Vitro Antibacterial and Antifungal Effects of a 30 kDa D-Galactoside-Specific Lectin from the Demosponge, *Halichondria okadai*

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Abstract—The present study has been taken to explore the screening of *in vitro* antimicrobial activities of D-galactose-binding sponge lectin (HOL-30). HOL-30 was purified from the marine demosponge *Halichondria okadai* by affinity chromatography. The molecular mass of the lectin was determined to be 30 kDa with a single polypeptide by SDS-PAGE under non-reducing and reducing conditions. HOL-30 agglutinated trypsinized and glutaraldehyde-fixed rabbit and human erythrocytes with preference for type O erythrocytes. The lectin was subjected to evaluation for inhibition of microbial growth by the disc diffusion method against eleven human pathogenic gram-positive and gram-negative bacteria. The lectin exhibited strong antibacterial activity against gram-positive bacteria, such as *Bacillus megaterium* and *Bacillus subtilis*. However, it did not affect against gram-negative bacteria such as *Salmonella typhi* and *Escherichia coli*. The largest zone of inhibition was recorded of *Bacillus megaterium* (12 in diameter) and *Bacillus subtilis* (10 mm in diameter) at a concentration of the lectin (250 µg/disc). On the other hand, the antifungal activity of the lectin was investigated against six phytopathogenic fungi based on food poisoning technique. The lectin has shown maximum inhibition (22.83%) of mycelial growth of *Botrydipodia theobromae* at a concentration of 100 µg/mL media. These findings indicate that the lectin may be of importance to clinical microbiology and have therapeutic applications.

Keywords—Antibacterial, *Halichondria okadai*, Inhibition zone, Lectin.

I. INTRODUCTION

LECTINS are ubiquitous proteins in the hemolymph and cells of many invertebrates and vertebrates with the activity based in the carbohydrate-binding protein that produces the agglutination of several types of cells, such as erythrocytes, bacteria and special cancerous cells through the interaction with specific oligosaccharides [1]. Such oligosaccharides and glycans are known to occur on the surface and the capsule of bacteria. In invertebrates lectins have been suggested to participate in the innate immune

response by inducing bacterial agglutination or by acting as opsonins to enhance phagocytosis by coelomycetes [2]. Besides role in cell recognition and host defense, lectins have been used as probes to determine sugar composition of glycan and glycoconjugates like bacterial lipopolysaccharide, cell surface glycoproteins and glycolipids for long time [3]. Cell lysates and cell-free plasma of several invertebrates also expressed antibacterial activity, though the activity of the latter may be possible due to small antimicrobial proteins [4]. These results strongly support the contention that invertebrates possess ‘immune-like’ defense mechanisms. Moreover, marine invertebrate lectins are also involved in other endogenous biological events such as biomineralization [5], embryonic development [6] and mediated the interaction between symbiont and host [7]. Despite their ubiquity, their functions in nature are not fully clear. Marine invertebrates rely solely on innate immunity, which includes both humoral and cellular responses, as they lack an adaptive immune system. Various methods employed to counteract infectious agents include, hemolymph coagulation, melanization, cell agglutination, encapsulation, nodule formation and phagocytosis [8]. The microbial load in natural marine habitat can number up to 10^6 bacteria and 10^9 virus ml^{-1} of seawater [9]. It is therefore imperative that animals develop a robust innate immune system for survival. Sponges, the evolutionarily oldest metazoan phylum (Porifera), share one common ancestor with the other metazoan phylum, the *Urmetazoa* [10]. Most sponges are marine sessile filter feeders that filtrate huge amounts of water (0.002-0.84 ml/s/cm^3 of sponge tissue) through their aquiferous canal system to extract edible material [11]. Hence, these animals are exposed to large amounts of bacteria and also viruses that are present in seawater [12]. Sponges have developed several strategies as they possess an efficient chemical defense system to inhibit bacterial growth [13]. Sponges are provided with efficient humoral and cellular defense/immune mechanisms that share, at the sequence level, surprisingly high similarity with the immune molecules found in humans [14]. Sponges are the most primitive invertebrates and their water-extractable lectins have been localized within the spherulous cells, which are assumed to participate in the formation of the sponging fibers [15]. A 27 kDa lectin was purified from demosponge *Suberites domuncula* and displayed antibacterial activity

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against the gram-positive bacteria *Staphylococcus aureus* and the gram-negative bacteria *Escherichia coli* [16].

Halichondria okadae (class Demospongiae) is a toxic marine black sponge and potent toxins, okadaic acid and halichondrins [17] were isolated from this sponge. Also, three lectins, HOL-I, HOL-II [18] and HOL-30 [19] have been purified from this sponge using affinity column chromatography. HOL-I and HOL-II had 21 kDa and 42 kDa polypeptides, respectively and they specifically recognized *N*-acetyl hexose (GlcNAc or GalNAc) and type 2 *N*-acetyllactosamine (Gal β 1-4GlcNAc), respectively. On the other hand, HOL-30 had the 30 kDa polypeptide and it recognized both branched complex type oligosaccharides with type-2 *N*-acetyllactosamine (LacNAc; Gal β 1-4GlcNAc) and type 1 (Gal β 1-3GlcNAc) by the frontal affinity chromatography technology (FACT) [19]. However, there is no evidence on the antimicrobial activity yet, the intent of the present investigation was taken to evaluate the antibacterial and antifungal activity of the HOL-30 purified from the demosponge against some human and phytopathogens.

II. MATERIALS AND METHODS

A. Chemicals

Lactosyl-agarose was purchased from Seikagaku Kogyo Co. Ltd., Japan. A standard protein marker mixture (Daiichi-III) for SDS-PAGE was purchased from Daiichi Pure Chem. Co. Ltd., Japan. BCA kit was purchased from Pierce Co. Ltd., USA. Agar, dextrose, peptone, beef extract were purchased from Merck Ltd., India and BDH Ltd., Bangladesh.

B. Purification of a Sponge Lectin, HOL-30

HOL-30 was purified from the marine demosponge *Halichondria okadae* as previously reported (Kawsar *et al.*, 2008). In brief, sponges were collected from intertidal zone and were immediately cut into small pieces and frozen at -80°C . For general preparation, 200 g (wet weight) of frozen sponge was dice homogenized in a commercial blender (Waring, USA) with 10 volumes (w/v) of Tris-buffered saline (TBS) (10 mM Tris(hydroxymethyl)aminomethane-HCl, pH 7.4, containing 150 mM NaCl) containing 10 mM of a protease inhibitor mixture. The homogenates were roughly filtered via filter bag in a sink to remove debris and the filtrates were centrifuged at 14,720 *g* in 500 mL centrifuge bottles for 1 h at 4°C with a Suprema 21 centrifuge equipped with an NA-18HS rotor. The supernatant was centrifuged again at 27,500 *g* for 1 h at 4°C and the supernatant was applied to affinity column of lactosyl-agarose (5 mL) being fitted with a Sephadex G-75 pre-column (5 mL). After applying the extracts, the lactosyl-agarose column was extensively washed with TBS. The lectin was eluted with 50 mM lactose in TBS and each 1 mL of elution was collected in tubes with a fraction collector and the eluted fractions as identified by UV spectrophotometer at 280 nm. Each chromatography step during washing and elution was monitored using a UV monitor by the absorbance at 280 nm.

C. Protein Determination

Protein concentrations were determined using BCA protein assay kit [20, 21] with bovine serum albumin as the standard by using measuring absorbance at 562 nm with spectrophotometer ND-1000 (Nano Drop Tech. Inc., USA).

D. SDS-Polyacrylamide Gel Electrophoresis

The molecular mass of the polypeptide was determined by SDS-PAGE. Purified lectin was mixed with an equal amount of sample buffer (20 mM Tris-HCl, pH 6.8; 0.2% SDS, and 20% glycerol) and then heated at 70°C for 15 min. Aliquots of 30 μL were applied to the well of a mini-slab gel (gel size: 80 mm \times 100 mm with 1 mm thickness; 12% and 5% polyacrylamide were used in separation and upper gels, respectively, constant current at 30 mA for 1 h) according to a previous report [22]. The following polypeptides were used as molecular mass markers; phosphorylase b (M_r 94 kDa), bovine serum albumin (M_r 66 kDa), ovalbumin (M_r 42 kDa), carbonic anhydrase (M_r 30 kDa), trypsin inhibitor (M_r 20 kDa), and lysozyme (M_r 14 kDa). After SDS-PAGE, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue (CBB) R-250 in 40% (v/v) and 10% acetic acid (v/v) followed by discoloration by excessive staining with 40% methanol and 10% acetic acid.

E. Hemagglutinating Assay

The lectin hemagglutination assay was performed using 1% (w/v) trypsinized and 0.25% glutaraldehyde-fixed rabbit and human erythrocytes as described previously [23]. Erythrocytes were suspended at a concentration of 1% (w/v) with TBS. In the general assay, 20 μL each of TBS, TBS containing 1% Triton X-100, and erythrocytes were added with 20 μL of the serially-diluted lectin with TBS in 96 well V-shape titer plates for 1 h. The hemagglutinating activity of the lectin was expressed as the titer defined as the reciprocal of the highest dilution giving positive hemagglutination.

F. Molecular Weight Determination by GPC

The purified lectin was dissolved in 2.5% glycerol and subjected to gel permeation chromatography (GPC) utilizing a Superdex 75 column (1.0 \times 65 cm) connected to an FPLC system (GE Healthcare, USA) in the presence of 50 mM galactose containing TBS. The elution time of the lectin from the column was detected by UV at an absorbance of 280 nm. Bovine serum albumin, ovalbumin, carbonic anhydrase and myoglobin were used as standard marker.

G. Strains of Tested Organisms

The bacterial and fungal strains used in this study were obtained from the Microbiology Laboratory, Department of Microbiology, University of Chittagong, Bangladesh. Gram-positive bacterial strains were *Bacillus subtilis* BTCC 17, *Bacillus cereus* BTCC 19, *Bacillus megaterium* BTCC 18 and *Staphylococcus aureus* ATCC 6538 and Gram-negative bacterial strains were *Salmonella typhi* AE 14612, *Salmonella paratyphi* AE 146313, *Shigella dysenteriae* AE 14396, *Shigella sonnei* CRL (ICDDR,B), *Escherichia coli* ATCC 25922, *Vibrio cholerae* (CRL (ICDDR,B) and *Pseudomonas*

sp. CRL (ICDDR,B). The fungal pathogens were *Alternaria alternata* (Fr.) Kedissler, *Botryodiplodia theobromae* Pat., *Curvularia lunata* (Wakker) Boedijin, *Colletotrichum corcori* Ikata (Yoshida), *Fusarium equiseti* (Corda) Sacc., and *Macrophomina phaseolina* (Tassi) Goid.

H. Medium and Culture

Standard NA medium was used for growing bacterial strains throughout the work. A 20 g of agar powder, 5 g of peptone, 3 g of beef extract and 0.5 g of NaCl was added slowly to 1000 mL water and the solution was mixed thoroughly with a glass rod. After 10 minutes of boiling, the medium was transferred into 500 mL conical flask and flask was closed with a cotton plug. The medium was autoclaved for 15 minutes at 121°C and 15 psi and ready to use bacterial culture. Older cultures were transferred to freshly prepared NA slants separately for each species via sterilized bacterial loop. In such a way, four test tubes were freshly prepared for each bacterial pathogen. These test tubes of inoculated slants were incubated at 35±2°C in incubator for 18-24 hours and each culture was used throughout for antibacterial screening studies. For preservation of the stock culture, one set of culture slants were kept in polythene bag, properly tied and preserved at 10°C.

I. Antibacterial Growth Inhibition Assay

The *in vitro* sensitivity of the bacteria to the test purified lectin was done by disc diffusion method [24]. In this method sterilized paper discs of 4 mm in diameter and petridishes of 150 mm in diameter were used throughout the experiment. The autoclaved Mueller-Hinton agar medium, cooled to 45°C, was poured into sterilized petridishes to a depth of 3 to 4 mm and after solidification of the agar medium; the plates were transferred to an incubator at 37°C for 15 to 20 minutes to dry off the moisture that develops on the agar surface. The plates were inoculated with the standard bacterial suspensions (as of McFarland 0.5 standard) by help of sterilized glass and allowed to dry for three to five minutes. Dried and sterilized filter paper discs were treated separately with 20µL (250 µg/disc) from 5% PBS solution of lectin using a micropipette, dried in air under aseptic condition and were placed at equidistance in a circle on the seeded plate. A control plate was also maintained in each case without any test material. These plates were kept for 4-6 hours at low temperature and the test materials diffused from disc to the surrounding medium by this time. The plates were then incubated at 35±2°C for 24 hours to allow maximum growth of the organisms. The antibacterial activity of the test agent was determined by measuring the mean diameter of zone of inhibitions in millimeter. Each experiment was repeated thrice. Lactose was used as negative control. All the results were compared with the standard antibacterial antibiotic ampicillin (20 µg/disc), (BEXIMCO Pharma Bangladesh Ltd.).

J. Antifungal Mycelial Growth Inhibition Assay

The *in vitro* antifungal activity of the purified lectin was determined by the poisoned food technique [25, 26]. PDA medium was used for the culture of fungi. A required amount of PDA was taken in conical flasks separately and was sterilized by autoclave (121°C, 15 psi) for 15 minutes. Purified lectin (in PBS solution) was mixed with sterilized melted PDA medium to have 100 µg/mL PDA and this was poured (about 20 mL/plate) in sterilized petridishes. At the center of each plate, 5 days old fungal mycelial block (4 mm in diameter) was inoculated and incubated at 27°C. A control set was also maintained in each experiment. Linear mycelial growth of fungus was measured after 3-5 days of incubation in triplicate. The average of two measurements was taken as mycelial colony diameter of the fungus in mm. All the antifungal results were compared with the standard antifungal antibiotic Nystatin (100 µg/mL PDA). Lactose was used as negative control. The percentage inhibition of radial mycelial growth of the test fungus was calculated as follows:

$$\% \text{ Inhibition} = (C-T/C) \times 100$$

Where, C = diameter of the fungal colony in the control petridish and T = diameter of the fungal colony in the treated petridish.

III. RESULTS AND DISCUSSION

HOL-30 which was purified from the marine demosponge *H. okadai* by lactosyl-agarose column (Fig. 1). About nine milligrams of *H. okadai* lectin was successfully purified from 200 g of marine sponge (wet mass) and it was concentrated 472 times by affinity purification (Table 1). It was confirmed to have the strong hemagglutinating activity against rabbit and human erythrocytes in the absence of any divalent cations (Table 2). The activity of the lectin was specifically cancelled by the co-presence of galactose and galactoside. It has shown to be a single 30 kDa band under both reducing and non-reducing conditions by SDS-PAGE (Fig. 2, lane L1 & L2). However, crude extract contained various proteins by SDS-PAGE (Fig. 2, lane C). The relative molecular mass of native HOL-30 as estimated by GPC on a Superdex 75 column connected to an FPLC system using TBS containing 50 mM lactose was shown to be 60 kDa (Fig. 3) suggesting that the protein is a non-covalently bound dimer. Since the target peak also appeared at the same position, when the lectin eluted from the column equilibrated with TBS (data not shown), it was suggesting that the native lectin does not interact with the column medium. The lectin also eluted at the same position by GPC in 20 mM CaCl₂ containing TBS. This property had showed the similarity with other demospongia lectins purified from *Geodia cydonium* [27] and *Suberites domuncula* [28].

A considerable number of lectins, including those isolated from sponges, were reported to react with D-galactose [29] as same as HOL-30 and the carbohydrate specificity is suggested to have important roles in modulating cellular responses in marine animals [30]. It has also been reported that several lectins from marine organisms can inhibit the growth of

TABLE I
PURIFICATION STEPS OF GALACTOSIDE-BINDING LECTIN FROM
H. OKADAI

Fraction	Titer (HU)	Total activity ^a	Specific activity ^b	Purification ratio (Fold)
Crude extract	256	51,200	0.51	1
Affinity purification	2048	15,360	241	472

^aTotal activity is shown by Titer × volume.

^bSpecific activity was shown by titer/mg of protein.

TABLE II
AGGLUTINATION OF DIFFERENT ERYTHROCYTES BY THE LECTIN

Erythrocytes sources*	Titer (HU)
Human	
Type A	1024
Type B	1024
Type O	2048
Rabbit	1024

*Trypsinized and glutaraldehyde fixed erythrocytes were used.

TABLE III
ANTIBACTERIAL ACTIVITY OF THE PURIFIED LECTIN (HOL-30)
AGAINST GRAM-POSITIVE BACTERIA

Name of bacteria	Diameter zone of inhibition in mm	
	Lectin (250 µg/disc)	Ampicillin* (20 µg/disc)
<i>Bacillus megaterium</i>	12±1	19±1
<i>Bacillus subtilis</i>	10±1	16±1
<i>Bacillus cereus</i>	4±1	18±1
<i>Staphylococcus aureus</i>	4±1	17±1

*Standard antibacterial antibiotic, Statistical analysis (RBD) at 1% level, organisms significant (F value 183.5), replica significant (F value 6.95).

pathogenic bacteria and fungi [16].

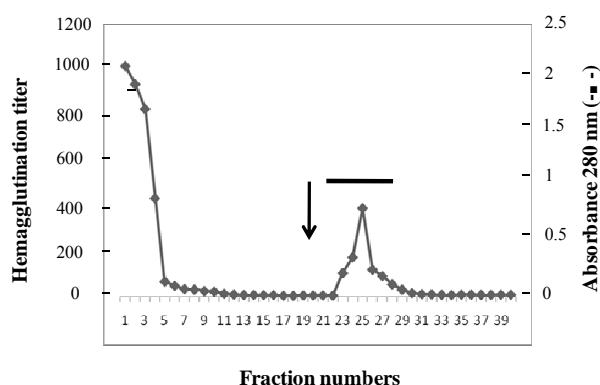


Fig. 1 Affinity purification of HOL-30. Crude extract of *H. okadai* was applied to a lactosyl-agarose column equilibrated with TBS. The column was washed with TBS and eluted with TBS containing 50 mM lactose (arrow). Closed circles show the absorbance at 280 nm. The column bound fractions shown by the bar were collected and designated as purified lectin after dialysis against TBS.

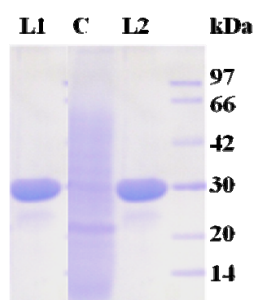


Fig. 2 SDS-polyacrylamide gel electrophoresis of HOL-30. Purified lectin (L) and crude extract (C) were subjected to SDS-PAGE under non-reducing (L1) and reducing (L2) conditions. Standard marker proteins (M) were used as follows, phosphorylase b (97 kDa); bovine serum albumin (66 kDa); ovalbumin (42 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20 kDa) & lysozyme (14 kDa).

In vitro antibacterial susceptibility by HOL-30 was assayed against eleven pathogenic bacteria and compared to that of antibacterial antibiotic, ampicillin. The results of the sensitivity test are presented in Table 3 and 4.

HOL-30 (250 µg/disc) exhibited a strong antibacterial effect on the gram-positive bacteria, as *Bacillus megaterium* and *Bacillus subtilis*. The diameter of zone inhibition by the addition of HOL-30 was significant effective for *Bacillus megaterium* and *Bacillus subtilis* to be 12 and 10 mm, respectively (Table 3). However, the lectin has inhibited less effect for *Bacillus cereus* and *Staphylococcus aureus*. On the other hand, HOL-30 did not inhibit well against gram-negative bacteria. Only *Shigella sonnei* and *Shigella dysenteriae* exhibited little sensitivity by the lectin (Table 4), though the control antibiotic, Ampicillin inhibited the growth against all gram-negative bacteria. Amongst the gram-positive and gram-negative bacteria, gram-positive bacterial strains

were more susceptible to the lectin as compared to gram-negative bacteria.

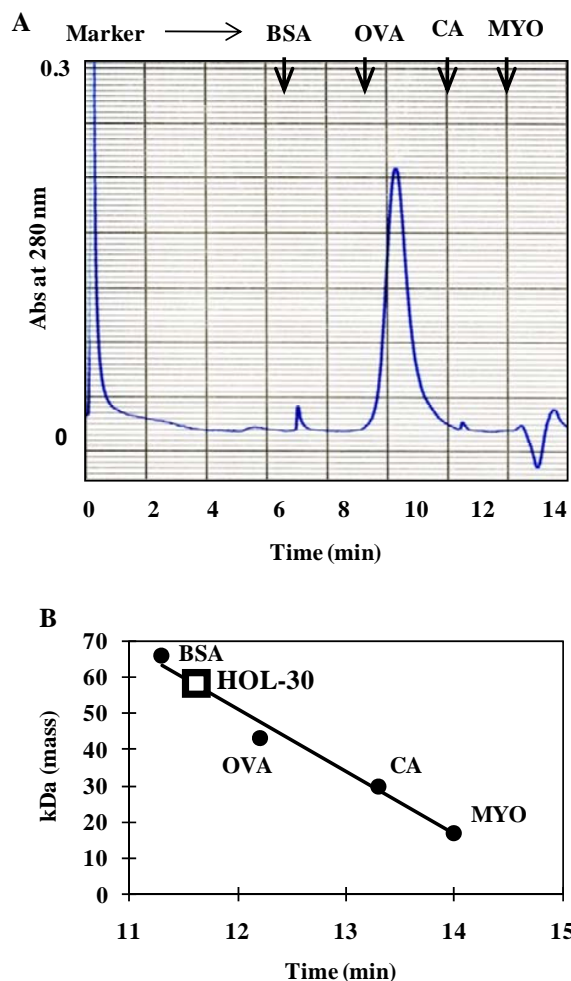


Fig. 3 GPC of HOL-30 and calibration curve for the determination of molecular weight. HOL-30 (20 μ g) was separated on Superdex 75 column using FPLC system at a flow of 0.5 mL/min (chart speed is 0.5 cm/mL). Calibration line (B) for the determination of molecular weight of HOL-30 was determined using standard molecular mass marker proteins as bovine serum albumin (BSA; 66 kDa), ovalbumin (OVA; 42 kDa), carbonic anhydrase (CA; 30 kDa) and myoglobin (MYO; 17 kDa).

This result suggest that the surface-exposed carbohydrates of bacteria were different even if they are belong same genus such as *Bacillus*. The glycome study approach to determine the structure of surface glycans of bacteria will give us much useful clinical information to prevent the disease using lectins. The glycan binding profile of HOL-30 has been analyzed by FACT and it specifically recognizes branched complex type oligosaccharides having lactosamine (Gal β 1-4GlcNAc) or sialyllactosamine (Sia α 2-3 Gal β 1-4GlcNAc) chains which were seen in fetuin and tyroglobulin [19]. Recently, a lectin purified from seeds of *Eugenia uniflora* had shown the similar

antibacterial activity with HOL-30, as it effectively inhibited the growth against gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* [31]. *Eugenia* lectin strongly bound to glycoproteins as fetuin and tyroglobulin.

In vitro antifungal susceptibility by HOL-30 was determined against six phytopathogenic fungi with antifungal antibiotic Nystatin as positive control. Generally, the lectin had less inhibited the growth of fungal than the case of bacteria. HOL-30 (100 μ g/mL in PDA medium) showed significant inhibition (22.83%) of mycelial growth against *Botryodiplodia theobromae* among all tested fungi (Table 5). On the other hand, the growth of *Alternaria alternata*, *Curvularia lunata*, *Macrophomina phaseolina* and *Colletotrichum corchori* (11.53-9.38%) was moderately inhibited by the lectin. However, the growth of *Fusarium*

TABLE IV
ANTIBACTERIAL ACTIVITY OF THE PURIFIED LECTIN (HOL-30)
AGAINST GRAM-NEGATIVE BACTERIA

Name of bacteria	Diameter zone of inhibition in mm	
	Lectin (250 μ g/disc)	Ampicillin* (20 μ g/disc)
<i>Shigella sonnei</i>	9 \pm 1	18 \pm 1
<i>Shigella dysenteriae</i>	7 \pm 1	16 \pm 1
<i>Salmonella paratyphi</i>	5 \pm 1	19 \pm 1
<i>Pseudomonas sp</i>	4 \pm 1	21 \pm 1
<i>Vibrio cholerae</i>	4 \pm 1	16 \pm 1
<i>Salmonella typhi</i>	0	15 \pm 1
<i>Escherichia coli</i>	0	15 \pm 1

*Standard antibacterial antibiotic, Statistical analysis (RBD) at 1% level, organisms significant (F value 183.5), replica significant (F value 6.95).

TABLE V
ANTIFUNGAL ACTIVITY OF THE PURIFIED LECTIN (HOL-30)
AGAINST PHYTOPATHOGENIC FUNGI

Name of bacteria	Percentage inhibition of fungal mycelial growth	
	Lectin (100 μ g/mL)	Nystatin* (100 μ g/mL)
<i>Botryodiplodia theobromae</i>	22.83 \pm 1	48.51 \pm 1
<i>Alternaria alternata</i>	11.53 \pm 1	45.52 \pm 1
<i>Macrophomina phaseolina</i>	10.98 \pm 1	40.62 \pm 1
<i>Curvularia lunata</i>	10.12 \pm 1	38.82 \pm 1
<i>Colletotrichum corchori</i>	09.38 \pm 1	40.78 \pm 1
<i>Fusarium equiseti</i>	03.55 \pm 1	50.55 \pm 1

*Standard antifungal antibiotic, growth measured-radial growth in cm.

equiseti was never inhibited by HOL-30, though the growth of all the six fungi was totally inhibited by antifungal antibiotic Nystatin (100 µg/mL PDA). Although the growth inhibition effect by HOL-30 against fungi was not strong, some other galactose-, mannose- or fucose-binding lectins have reported and showed strong antifungal activity [32]. During the course of evolution, marine invertebrates have developed defense strategies against various pathogens, firstly bacteria living in the surrounding water [33]. Reactions against these living pathogens constitutes immune defense. Sessile invertebrates, such as sponges and ascidians are well known to produce an astonishing variety of antimicrobial compounds [34], which help to control bacterial surface colonization [35].

The assigning of "ecological roles" to marine natural products is complicated by the nature of the symbiotic relationships observed in many marine invertebrates, including sponges. Symbiotic organisms associated with sponges have been identified as the sources of bioactive secondary metabolites as okadaic acid which is the toxic inhibitor for phosphatase produced by microorganisms in *Halichondria okadai* [17]. In the living system for sponge, the production of antimicrobial compounds is essential to maintain the ecological balance with symbiotic organisms. If the lectin functions for the purpose to regulate the eco-system of sponge, it was reasonable that the protein was produced as high yield in the animal.

IV. CONCLUSION

The results of the present study have shown that HOL-30 possess the potent *in vitro* antimicrobial activities, particularly against gram-positive bacteria. *In vivo* evaluation of the antimicrobial activity, along with toxicity studies of HOL-30 in *H. okadai* are expectable for the future studies to know the potent activity of the lectin.

APPENDIX

Abbreviations used:

HOL-30, *Halichondria okadai* lectin with molecular mass 30 kDa; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BCA, Bicinchoninic acid; TBS, Tris buffered saline; PBS, Phosphate buffered saline; NA, Nutrient agar; PDA, Potato dextrose agar.

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