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CELLULAR AND MOLECULAR BIOLOGY

New lectin isolated from the tropical sponge *Haliclona (Reniera) implexiformis* (Hechtel, 1965) shows antibiofilm effect

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Abstract: A lectin from the marine sponge *Haliclona* (Reniera) *implexiformis* (HiL) was isolated by affinity chromatography on Sepharose™ matrix. HiL showed specificity for galactose and its derivatives. The glycoproteins porcine stomach mucin (PSM) and bovine stomach mucin (BSM) were potent inhibitors. Hemagglutinating activity of the lectin was maximal between pH 5.0 and 9.0. The lectin remained active until 60°C. The presence of $CaCl_2$ and EDTA did not affect the hemagglutinating activity. In SDS-PAGE, HiL showed a single band of 20 kDa under reduced conditions, whereas in the non-reducing conditions, it showed a band of 20 kDa and one additional band of 36 kDa. The average molecular mass determined by Electrospray Ionization Mass Spectrometry (ESI-MS) was 35.874 ± 2 Da in native and non-reducing conditions, whereas carboxyamidomethylated-lectin showed 18,111 Da. These data indicated that HiL consists in a dimer formed by identical subunits linked by disulfide bonds. Partial amino acid sequence of HiL was determined by mass spectrometry, and revealed that it is a new type of lectin, which showed no similarity with any protein. Secondary structure consisted of 6% α-helice, 31% β-sheet, 18% β-turn and 45% random coil. HiL showed significant reduction in the number of viable cells of *Staphylococcus* biofilms.

Key words: Antibiofilm, circular dichroism, lectin, mass spectrometry, purification, sponge.

INTRODUCTION

Marine sponges are multicellular, sessile and filtering organisms, belonging to the phylum Porifera, the oldest of the Metazoans. More than 8,500 species found in marine and freshwater ecosystems have been described (van Soest et al. 2012).

These organisms have demonstrated great biotechnological potential; they are promising sources of bioactive natural products, such as nucleosides, sterols, alkaloids, fatty acids, glycolipids, amino acid derivatives, peptides and lectins (Carroll et al. 2019, Laport et al. 2009).

Lectins are proteins or glycoproteins of non-immune origin capable of reversibly binding carbohydrates without altering their structures (Vasta et al. 2004). Lectins isolated from marine sponges have shown many interesting biological activities, including mitogenic activities, pro-inflammatory effects, microorganism association, as well as antimicrobial, cytotoxic, and anticancer activities (Garderes et al. 2015).

Haliclona is a genus of marine sponges with the largest number of lectins ever isolated. To date, six lectins have been purified: HL from *Haliclona* sp., HCL from *Haliclona cratera*, H1, H2 and H-3 from *Haliclona caerulea*, and HMA from *Haliclona manglaris* (Carneiro et al. 2013a, b, 2015, Pajic et al. 2002, Mebs et al. 1985). Among these lectins, several biological properties have been described, such as β-carotene protection, cytotoxic effects against *Artemia* nauplii, HeLa and FemX cells and the induction of apoptosis and autophagy in MCF7 cells.

Beyond *Haliclona* lectins, some lectins isolated from marine sponges have demonstrated bacterial antibiofilm activity. For instance, ALL from *Aplysina lactuca*, AFL from *Aplysina fulva* and CCL from *Chondrilla caribensis* were able to significantly reduce biofilm biomass produced by *Staphylococcus* ssp. and *Escherichia coli* (Carneiro et al. 2017a, 2019, Marques et al. 2018).

A bacterial biofilm is a community of cells attached to a biotic or abiotic substrate, surrounded by a produced extracellular polymeric matrix. It acts as a physical barrier that increases bacterial resistance to antimicrobial agents, and it is one of the main problems in nosocomial infections (O'Toole et al. 2000, Song et al. 2018). Therefore, biomolecules with antibiofilm activity may be useful tools in the treatment of bacterial infections.

In the present work, we report the isolation, biochemical properties, carbohydrate-binding specificity and antibiofilm effect of a new lectin denominated HiL (*Haliclona (Reniera) implexiformis* Lectin).

MATERIALS AND METHODS

Animal collection

Specimens of the marine sponge *Haliclona (Reniera) implexiformis* (Heitchel, 1865) were collected in the intertidal zone of Pacheco Beach, Ceará, Brazil. Fresh sponges were transported to the laboratory and stored at –20°C until use. All collections were authorized through our registration with SISBIO (Sistema de Autorização e Informação em Biodiversidade, ID: 33913-8). The species was identified, and a voucher was deposited (ID: UFPEPOR2691) at the Zoology Department of the Federal University of Pernambuco, Pernambuco, Brazil.

Extraction and purification of the lectins

Sponge genetic access was authorized through our registration with SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado, ID: A1792FE).

After collection, the sponges were cut into small pieces, ground in a mortar, and homogenized in 50 mM Tris-HCl, pH 7.6, containing 150 mM NaCl and 20mM $CaCl_2$ (TBS-Ca^{2+}) at 1:2 (w/v). The mixture was strained through nylon tissue and centrifuged for 20 min at 9000 ×*g* at 4°C. The supernatant (crude extract) was collected and assayed for hemagglutinating activity and protein concentration (Bradford 1976).

The crude extract was loaded into an HCl-activated Sepharose™ column (1.5 × 6.0 cm) previously equilibrated with TBS. Unbound proteins were washed with the same buffer, and the retained fraction was recovered through elution with 0.3 M galactose in TBS. Fractions eluted with galactose in TBS were pooled, dialyzed against distillated water, freeze-dried and stored until use.

Hemagglutination and inhibition assay

Hemagglutinating activity and inhibition assays were performed in microtiter plates with V-bottom wells using the two-fold serial dilution method according to Sampaio et al. (1998). Erythrocytes from human (A, B and O) were

used in their native and protease (trypsin and papain)-treated forms. One hemagglutinating unit (HU) was defined as the amount of lectin able to agglutinate and, hence, precipitate erythrocytes in a suspension after 1 h.

The following sugars and glycoproteins were used in the inhibition assay: D-xylose, D-ribose, L-fucose, L-arabinose, L-rhamnose, D-galactose (Gal), D-mannose, D-glucose (Glc), D-glucosamine, D-galactosamine, *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), D-galacturonic acid, D-fructose, D-sucrose, D-melibiose, α-D-lactose, D-lactulose, D-maltose, D-rafinose, methyl-α-D-glucoside, methyl-α-D-galactoside, methyl-β-D-galactoside, methyl-β-D-thiogalactose, phenyl-β-D-galactoside, 4-nitrophenyl-α-d-galactoside, 4-nitrophenyl-β-D-galactoside, 2-nitrophenyl-β-D-galactoside, bovine submaxillary mucin (BSM), and porcine stomach mucin (PSM) type 2 (mucins were purchased from Sigma Aldrich, lots M3895 and M1778, respectively).

The effect of temperature, pH and divalent ions on the hemagglutinating activity of the lectin was evaluated according to pre-established methods (Sampaio et al. 1998). In brief, effect of temperature on stability lectin was evaluated by incubation of HiL in several temperatures (25, 30, 40, 50, 60, and 70°C) for 1h, followed by hemagglutinating activity assay.

The effect of the pH on lectin stability was evaluated by serial dilution of HiL in distinct buffers (20 mM sodium acetate, pH 4 and 5; 20 mM sodium phosphate, pH 6; 20 mM tris-HCl, pH 7 and pH 8; 50 mM glycine buffer, pH 9 and 10) and incubation for 1h. Then, erythrocytes suspension was added and hemaglutinating activity was evaluated after 30 m.

Finally, the effect of EDTA and Ca^{2+} was evaluated by incubation of HiL in 20 mM tris-HCl, pH 7.6, containing NaCl 150 mM and 5 mM $CaCl_2$ and 5 mM EDTA. After incubation hemagglutinating assay was evaluated.

Molecular mass determination

The molecular mass of HiL under denaturing condition was estimated by SDS- PAGE in 15% gel in the presence and absence of β-mercaptoethanol, followed by staining with Coomassie Brilliant Blue (Laemmli 1970).

Native molecular mass was evaluated by size exclusion chromatography (SEC), as described by (Chaves et al. 2018), using a BEH HR SEC column (0.78 × 30 cm, 1.7 µm particle size, Waters Corp.) coupled to an H-Class UPLC system (Waters Corp, MA, USA).

Average molecular mass and quantification of sulfhydryl groups were determined by Electrospray Ionization-Mass Spectrometry (ESI-MS), as described by (Carneiro et al. 2017a).

Amino acid sequencing

Automated Edman degradations were performed in the Shimadzu model PPSQ-31A protein sequencer (Shimadzu Corp., Japan). PTH-amino acids from the N-terminus sequence were separated on a 2.0 × 250 mm Wakosil ODS column (Wako Pure Chemical Corp., Osaka, Japan) connected to a model LC-20AT pump. The absorbance was detected at 269 nm with a UV–Vis SPD-20A detector.

A MS/MS approach was employed to determine internal sequences. First, an SDS-PAGE was conducted, as described above. Then, protein spots were sliced, discolored, reduced and carboxyamidomethylated, as described by Shevchenko et al. (2006). After that, protein spots were digested with trypsin (Promega, WI, USA) and chymotrypsin (Roche, Switzerland) at ratio of 1:50 (enzyme:substrate) for 16 h at 37°C. Peptides were extracted and concentrated, as described by Shevchenko et al. (2006).

The peptides were separated on a reverse phase C18 nanocolumn (0.075 x 100 mm) coupled to a nanoAcquity system. The eluates were directly infused in a hybrid mass spectrometer (ESI-Q-ToF) (Synapt HDMS, Waters Corp, MA, USA). The instrument parameters were adjusted, as described by Carneiro et al. (2013a). MS/MS spectra were interpreted manually, and sequenced peptides were searched online against NCBI and UniProt databanks.

Circular dichroism analysis

Circular Dichroism (CD) spectroscopic measurement was performed on a Jasco J-815 spectropolarimeter (Jasco International Co., Tokyo, Japan) connected to a peltier with controlled temperature conforming to Carneiro et al. (2017b). The DICHROWEB web server was used to perform analyses of secondary structure prediction (Whitmore & Wallace 2004).

The lectin was submitted to temperatures ranging from 70°C to 105°C with a ramp rate of 3°C.min^{-1} and sampling at each 1°C. Melting temperatures (Tm) of HiL in the presence and absence of galactose (10 mM) were calculated by monitoring the changes in ellipticity at 205 nm as a function of temperature (Greenfield 2006a, b).

Evaluation of antibacterial and antibiofilm activity

Strains and culture conditions

For the present study, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *Escherichia coli* ATCC 11303 strains were used. The strains were inoculated in Tryptic Soy Agar (TSA; Himedia, India) for 24 h at 37°C. Isolated colonies were inoculated into 5 mL of Tryptic Soy Broth (TSB; Himedia, India) for an additional 24 h at 37°C. Cells were harvested by centrifugation (5 min at 9000 x *g*), and the bacterial suspension was adjusted to a final concentration of 1 x 10^6 colony forming units (CFU/mL) by turbidimetry (620 nm) and calibration curves previously determined for each strain.

Effect of HiL on planktonic cells

Antibacterial activity was carried out according to the microdilution test in broth medium using 96-well plates in accordance CLSI with some modifications, as described Vasconcelos et al. (2014). HiL was used at concentrations from 7.8 to 500 µg.mL^{-1}. The lowest concentration of the compounds that inhibit a visible growth based on turbidity was considered as a MIC.

Biofilm assays

-Biomass quantification

The quantification of biofilm biomass was determined using the crystal violet (CV) staining method according to Stepanović et al. (2000) with some modifications. The biofilms were developed in the presence and absence of different lectin concentrations (7.8 to 500 µg.mL^{-1}) for 24 h. After 24 hours of incubation, the plates were washed with sterile distilled water three times for removal of weakly adherent cells. Subsequently, the wells were filled with 200 µL of 95% methanol for 5 minutes. Then, 200 µL of 1% violet crystal were added to the wells for 5 minutes at 25°C. Excess dye was removed and the plates washed with distilled water. The remaining dye was removed with 33% acetic acid elution. The eluted stain from each well was quantified by measuring the optical density at 590 nm (OD_{590}) using a microplate reader (SpectraMax i3).

-Quantification of biofilm-entrapped cells

After formation of the biofilms, the culture medium was removed, and the plates were

washed twice with sterile distilled water to remove weakly adherent cells. Subsequently, 200 µL of sterile distilled water were added to each well and submitted to ultrasonic bath (Cristófoli/EQM-CF) for 8 minutes to detach the cells embedded in the biofilms. Serial decimal dilution of the bacterial suspension was plated in TSA medium and incubated at 37°C for 24 hours. The number of Colony Forming Units was determined and the results expressed in terms of Log_{10} CFU/mL.

RESULTS

Lectin isolation

The *H. implexiformis* crude extract showed lectin activity against native and enzyme-treated human and rabbit erythrocytes. In affinity chromatography, the lectin was adsorbed in the Sepharose™ matrix, and it was recovered by elution with 0.3 M lactose (Fig. 1). Lectin eluted from the column represented 78% of total hemagglutinating activity present in the extract. This procedure increased 17-fold specific lectin activity compared to the crude extract (Table I).

Hemagglutination assays and inhibition by sugars and glycoproteins

The carbohydrate-binding specificity of the lectin was determined by the inhibition of hemagglutinating activity by sugars and glycoproteins. Several galactose-related carbohydrates showed inhibitory activity at distinct levels. 2-nitrophenyl-β-D-galactoside was the most potent inhibitor with minimum inhibitory concentration (MIC) of 0.19 mM, followed by phenyl-β-D-galactoside and 4-nitrophenyl-β-D-galactoside with MIC of 0.39 mM, whereas the glycoprotein PSM type 2 presented MIC at 0.03 µg.mL^{-1} (Table II).

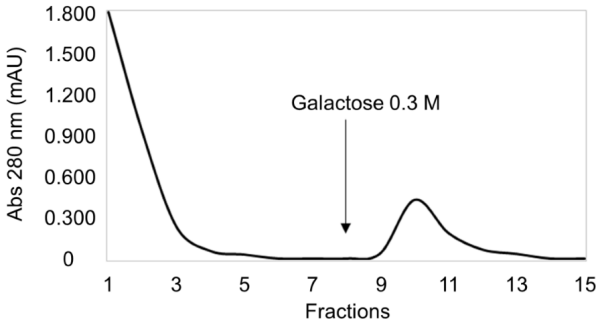

Figure 1. Affinity Chromatography of HiL. Unbound proteins were eluted by washing with TBS, and the retained fractions were eluted by 0.3 M galactose.

Effects of pH, temperature and divalent cation on hemagglutinating activity

Hemagglutinating activity of the lectin was maximal between pH 5.0 and 9.0, but outside these values, hemagglutinating capacity was reduced at pH 4.0 and 10.0. The lectin was stable up to 60°C with total loss of activity after heating at 90°C. The presence of $CaCl_2$ and EDTA did not affect the hemagglutinating activity (Fig. 2).

Molecular mass

SDS-PAGE of the purified lectin revealed one single band of approximately 20 kDa in the presence of reducing agents. The same band of 20 kDa and a broad additional band of 36 kDa were observed in the absence of 2-ME (Fig. 3a).

Table I. Purification procedure of the lectin from *Haliclona* sp.

Fraction	Volume (mL)	Titer (HU.mL^{-1})	Protein total (mg)	Hemagglutinating activity total (HU)	Specific activity (HU.mg^{-1})	Purification (fold)	Yield (%)
Crude extract	41	512	61	20,992	346	1	100
Affinity fraction	8	2048	2.8	16,384	5851	17	78

Table II. Hemagglutinating activity inhibition by sugars and glycoproteins.

Sugar	MIC[a]
2-nitrophenyl-β-D-galactoside	0.19 mM
Phenyl-β-D-galactoside	0.39 mM
4-nitrophenyl-β-D-galactoside	0.39 mM
D-galactose	0.78 mM
α-**D**-lactose (Galβ(1➔4)α-Glc)	0.78 mM
D-lactulose (Galβ(1➔4)Fru)	0.78 mM
methyl-β-D-galactoside	0.78 mM
methyl-β-D-thiogalactose	0.78 mM
4-nitrophenyl-α-**d**-galactoside	0.78 mM
D-galactosamine	1.56 mM
N-acetyl-D-galactosamine	1.56 mM
D-melibiose (Galα(1➔6)Glc)	1.56 mM
D-raffinose (Galα(1➔6)Glcα(1➔4)Fru)	1.56 mM
methyl-α-D-galactoside	1.56 mM
L- arabinose	25 mM
L-rhamnose	50 mM
Glycoproteins	
PSM type 2	0.03 $\mu g.mL^{-1}$
BSM	25 $\mu g.mL^{-1}$
[a] Minimal inhibition concentration.	

In native form (i.e., SEC), the lectin presented molecular mass estimated at 18.8 kDa, indicating an uncommon behavior on SEC (Fig 3b).

The lectin was submitted to ESI-MS in distinct forms: native, non-reduced and carboxyamidomethylated (CAM). Both non-reduced and native lectin showed average molecular mass of 35,876±2 Da, indicating the absence of free cysteines.

On the other hand, CAM-lectin showed 18,111 Da. This value represents an increment of 171 Da in relation to expected reduced monomer (35,876 Da / 2 = 17,938 Da). Since each acetamide linked to a thiol group produces an increment of 57 Da, this result suggests the presence of three cysteines (3 x 57 Da = 171 Da), all involved in disulfide bonds (Fig. 4).

Amino acid sequencing

The first seventeen amino acids of the lectin NH_2-terminal were determined: [1][A/G]XPGVTF[L/Y]YVGRILTSM, which showed no similarity with any known protein. Microheterogeneities were found in position 1 (A/G) and 8 (L/Y). In position 2, an unidentified amino acid was found.

Tryptic and chymotryptic digestions produced seven and three identified peptides, respectively (Table III). In some cases, overlapping between distinct peptides was observed. For instance, C-terminal of the peptide T5 (Val-Met-Asp-Arg) overlapped with NH_2-terminal of the peptide Q3, which, in turn, overlaps its C-terminal sequence (Gly-Glu-Asn-Ile-Leu) with the peptide T2. The C-terminal sequence of T2 (Leu-Ala-Tyr-Pro-Arg) overlapped with Q1. Thus, a continuous sequence of 44 amino acids (MW = 5090.46) was obtained. However, no similarity was found with any protein.

Some peptides were considered similar. For examples, peptides T1 and T5 have amino acid sequences very similar, with modification: Ser for Val. Peptides T2 and T2' are distinct due oxidized-Met found in T2'.

All sequences, including NH_2-terminal, totaled 66% of the amino acid sequence of HiL.

Circular dichroism

The theoretical secondary structure of HiL consisted of 6% α-helix, 31% β-sheet, 18% β-turn and 45% random coil. Melting temperatures (Tm) of the lectin and complex lectin-ligand (galactose) were 64.9 and 90.7°C, respectively (Fig. 5).

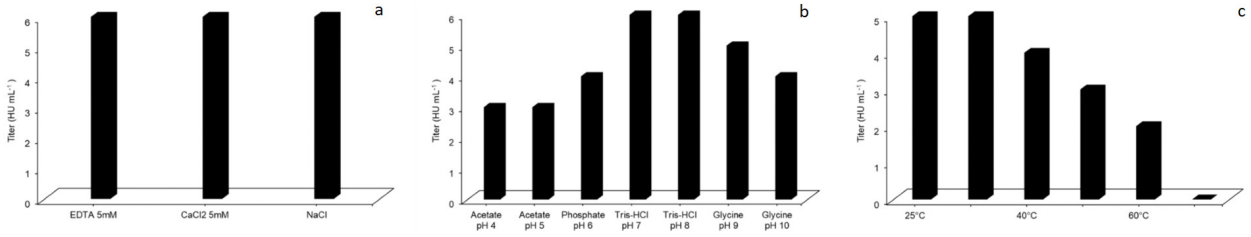

Figure 2. **Properties of the hemagglutinating activity of HiL. Effect of calcium ion and EDTA (a), pH (b) and temperature (c) on the hemagglutinating activity of HiL. Hemagglutinating activity was expressed in logarithm scale as units of titter.**

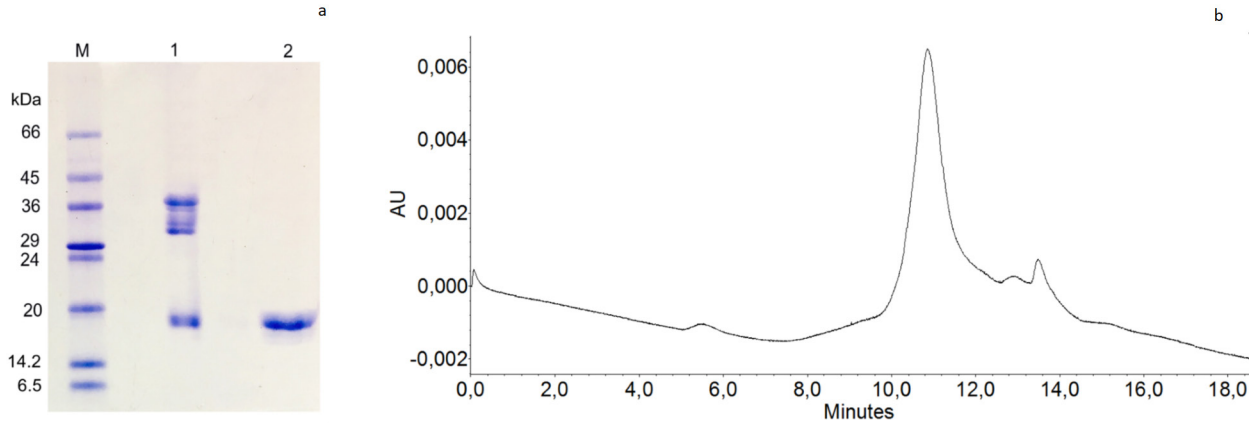

Figure 3. **(a) SDS–PAGE (15%) of purified HiL. (M) Molecular marker; (1) 20 µg of HiL in the absence of reducing agents; (2) 20µg of lectin in the presence of β-mercaptoethanol. (b) Size exclusion chromatography of HiL. BEH HR SEC column SEC column (0.78 x 30 cm, 5 um particle size, Waters Corp.) was equilibrated and eluted with Tris-HCl, 50 mM, pH 7.6, containing NaCl 500 mM. Approximately 20 µg of HiL were loaded. UPLC operated at flow of 0.3 mL.min^{-1}.**

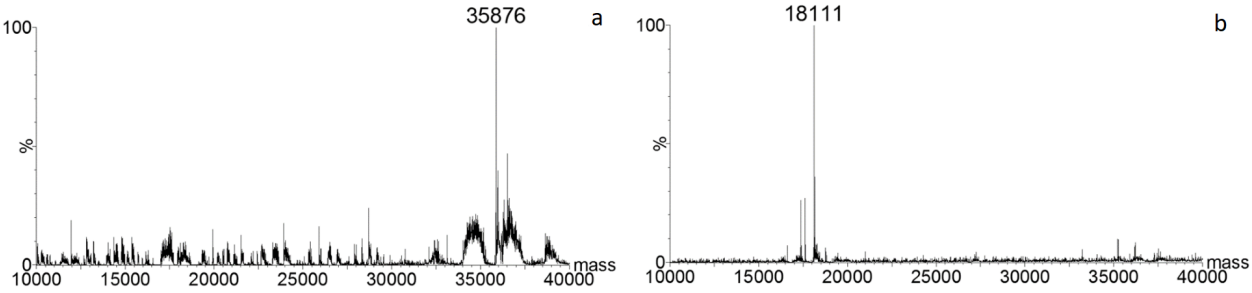

Figure 4. **Molecular mass determination of HiL by ESI–MS. (a) Deconvoluted ESI mass spectra of lectin. The lectin (10 pmol µl^{-1}) was dissolved in a solution of ACN 50% containing 0.2% FA and infused into the NanoESI source coupled to an ESI-Q-ToF mass spectrometer. (b) Deconvoluted mass spectra of CAM-lectin.**

Antibacterial and antibiofilm activity

HiL had no effect against planktonic cells; thus, the MIC values could not be determined. HiL was able to significantly reduce the biomass of *Staphylococcus* biofilms in the highest concentrations tested (Fig. 6a-b). On the other hand, the lectin did not cause biomass reduction of *E. coli* biofilms (Fig. 6c).

In agreement with biofilm biomass data, HiL showed significant reduction in the number of viable cells of *Staphylococcus* biofilms. As shown in Fig. 5, a reduction of about 2.0 logs for *S. aureus* strains and approximately 1.5 logs

Table III. Peptide sequences of HiL determined by MS/MS.

Peptide	*m/z*	Sequence	Mass		Δ (Da)
			Observed	Calculated	
T1	580.16	DSQPLVMDVR	1158.30	1158.57	0.27
T2	854.70	WNDM*EGNLLLAYPR	1706.37	1706.84	0.47
T2'	846.69	WNDMEGNLLLAYPR	1690.38	1690.81	0.43
T3	799.73	SSDKTTGVLATYDLK	1597.45	1597.78	0.33
T3'	591.25	TTGVLATYDLK	1180.50	11863	0.13
T4	982.75	STTLPDCFWLLSQLQR	1963.50	1963.98	0.48
T5	576.67	PDYSENPSQDDVQPLVMDVR	2302.67	2303.03	0.36
Q1	578.60	LAYPRQQAEVDLALF	1732.79	1732.91	0.12
Q2	571.59	GVFDNPPQLQPGVGCK	1711.77	1711.83	0.06
Q3	862.35	VM*DVRWNDM*EGNIL	1722.69	1722.83	0.04

***oxidized Methionine.**

for *S. epidermidis* (Fig. 6d,e) was observed. The same effect was not observed for *E. coli*, as the same number of viable cells in the control of non-HiL-treated bacteria was observed at all concentrations tested (Fig. 6f).

DISCUSSION

In this work, a new lectin extracted from the marine sponge *H. implexiformis* (HiL) was isolated and characterized. The lectin was purified by affinity chromatography on HCl-activated Sepharose™ matrix, an efficient method to isolate galactose-binding lectins (Carneiro et al. 2017a, 2019, Marques et al. 2018, Moura et al. 2006).

HiL proved to be most active at a basic pH. Similar behavior is observed in *Haliclona manglaris* agglutinin, *Haliclona cratera* lectin, H-1 from *H. caerulea* and in most lectins isolated from marine sponges, except for H-2 from *H. caerulea* which showed strong activity at acidic pH values (4-5). As observed in H-2, HiL remained active when exposed up to 80°C for 60 min (Carneiro et al. 2013a, b, 2015, Pajic et al. 2002).

Studies of the effects of EDTA and requirement for divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) of several sponge lectins, including *Haliclona caerulea* lectins (H-1, H-2, H-3) and *H. manglaris agglutinin,* showed that the hemagglutinating activity was not affected by the presence of these ions (Carneiro et al. 2013a, b, 2015). Similar results were observed in this work.

Thus, HiL showed several biochemical characteristics common to marine sponge lectins, such as non-specificity for blood types, hemagglutinating activity independent of divalent ions, thermostability and resistance to pH variations (Carneiro et al. 2013b, 2017b, 2019, Pajic et al. 2002, Marques et al. 2018, Kawsar et al. 2008).

Such as HiL, several lectins from marine sponges recognize some derivates of galactose (Carneiro et al. 2017a, 2019, Marques et al. 2018, Moura et al. 2006, Miarons & Fresno 2000, Medeiros et al. 2010). Nevertheless, some

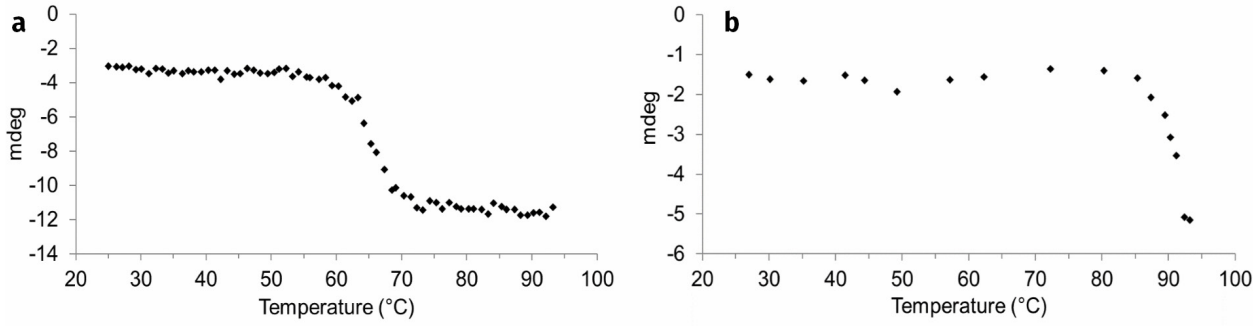

Figure 5. Determination of HiL Tm from changes in CD as a function of temperature. The HiL was solubilized in PBS, pH 7, in the absence (a) and presence (b) of galactose.

important aspects of inhibition profile of HiL deserve more attention.

HiL showed specificity for galactose and non-reducing terminal carbohydrate containing galactose. The preference for β-galactosides observed in the inhibition assay suggests the involvement of C1-substituent groups in the interaction of lectin with sugar. For example, 4-nitrophenyl-β-D-galactoside is twice as effective as 4-nitrophenyl-α-D-galactoside. The same is true for methyl-β-D-galactoside and methyl-α-D-galactoside. Like some marine algal lectins, HiL seems to possess a hydrophobic region near its carbohydrate-binding site since the inhibition shown by nitrophenyl-D-galactosides was stronger than that of methyl-D-galactosides (Sampaio et al. 1998).

The nitro ($-NO_2$) radical displacement from ortho-director on 4-nitrophenyl-α-D-galactoside to para-director on 2-nitrophenyl-α-D-galactoside increased lectin inhibitory efficiency two-fold and generated greater binding stability among all sugars, ensuring that it would have greater inhibitory power with MIC of 0.19 mM.

The nature of the C1-substituent group, such as methyl, phenyl and nitrophenyl, may increase affinity, but does not define specificity. Substitution of the hydroxyl by other C2 groups decreases affinity; for example, inhibition of D-galactose falls twice when amino or acetyl radicals are attached at that carbon.

Although C1 appears to be an important point of recognition, a similar degree of inhibition of β-D-lactulose (Galβ1➔Fru) and raffinose (Galα1➔6Glc α1➔4Fru) indicates that C1 cannot be considered as a key point for lectin recognition.

The configuration at C-4 of the pyranose ring is important since neither glucose nor glucose derivatives showed any inhibitory activity against HiL. The difference in structure between galactose and glucose can be seen when comparing the symmetrical ring structures where the hydroxyl group at C-4, in Haworth projection, in glucose is below the plane of the ring (equatorial), whereas with galactose, it is above the plane of the ring (axial). Owing to their axial position in C4, L-arabinose and L-rhamnose were able to inhibit, but only in high concentrations. In addition, the C-6 position in the binding site is important since D-galacturonic acid and D-melibiose failed to inhibit the lectin.

Some *O*-linked glycoprotein that exhibited GalNAc and D-galactose as terminal in their structures, such as PSM and BSM, were also able to inhibit HiL. Affinity for BSM and PSM were also observed in the lectins isolated from *Haliclona caerulea*, *Craniella australiensis*, *Aplysina*

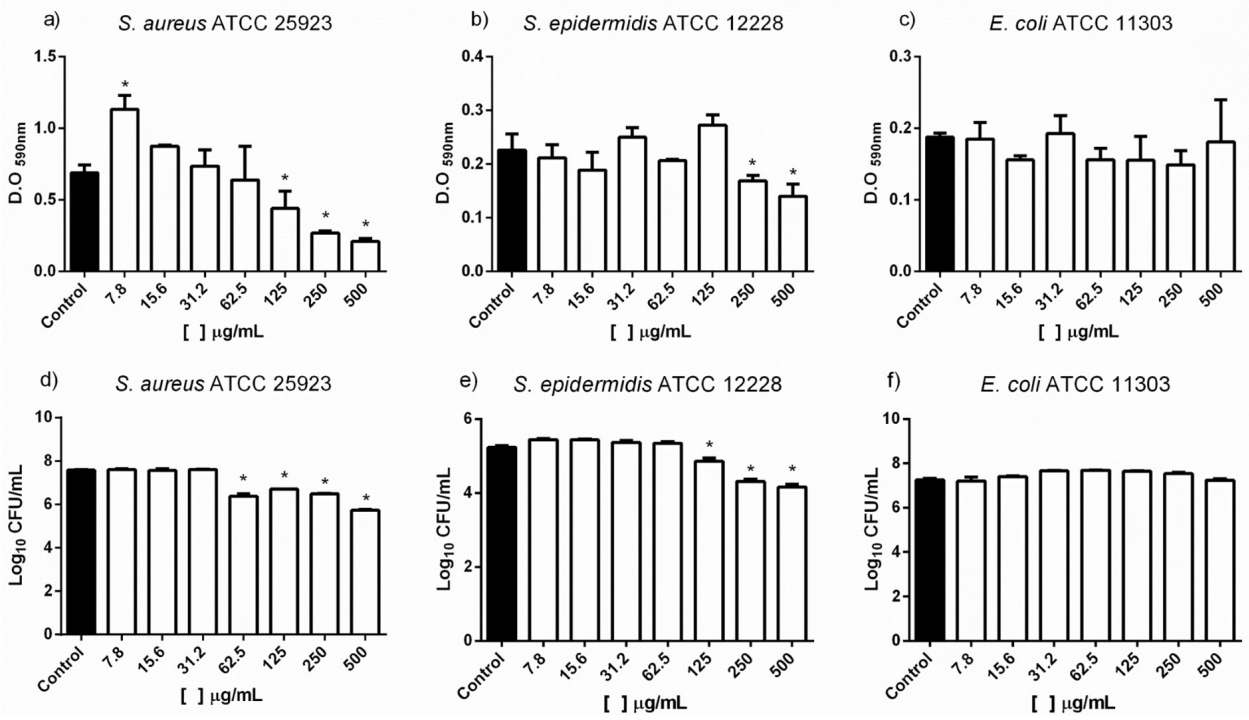

Figure 6. Activity of HiL against biofilm formation of Gram-positive and Gram-negative bacteria. Total biomass quantification measuring the intensity of crystal violet stain for *S. aureus* (a), *S. epidermidis* (b) and *E. coli* (c) biofilms; colony forming units per mL for *S. aureus* (d), *S. epidermidis* (e) and *E. coli* (f) biofilms. *Significantly different ($p < 0.05$) compared to the control group.

lactuca and *A. fulva* (Carneiro et al. 2013a, 2017a, 2019, Xiong et al. 2006).

Structurally, HiL also showed properties similar to those of other sponge lectins. For instance, the presence of disulfide bonds, predominance of β-conformation, and unique amino acid sequence are features found in lectins from *Cliona varians*, *A. fulva* and *Chondrilla caribensis*, respectively (Marques et al. 2018, Carneiro et al. 2017a, Moura et al. 2006).

However, an uncommon aspect observed in HiL was its quaternary structure. Despite SEC showed inconclusive results for determination of quaternary structure of HiL, SDS-PAGE and MS analysis make clear the predominance of HiL as a dimer linked by disulfide bonds. This is an uncommon quaternary organization for sponge lectins. CvL and lectins from *Aplysina lacunosa* and *A. archeri* are tetramer linked by disulfide bonds, while *Haliclona caerulea* lectin H-2 and *Haliclona cratera* lectin are dimer maintained by weak interactions (Carneiro et al. 2013b, Pajic et al. 2002, Moura et al. 2006, Miarons & Fresno 2000).

In recent years, some lectins from marine sponges have been isolated and their amino acid sequences partially determined (Carneiro et al. 2017a, 2019, Marques et al. 2018, Garderes et al. 2016). However, only a few lectins from these organisms have had their primary structures fully determined. In fact, the last sponge lectins to have its primary structure deposited were H-3 from *Haliclona carulea* and CCL from Chondrilla caribensis (Carneiro et al. 2013a, Sousa et al. 2021).

Attempts to determine amino acid sequence of sponge lectins fail for many reasons, such as the small amount of protein for animal biomass, presence of isoforms and glycoforms, and

possible differential expression levels in certain seasons (Carneiro et al. 2013a, b).

In this study, in particular, the difficulty of determining the full amino acid sequence arose because of (1) low occurrence of *H. implexiformis* along our coastline and (2) absence of similar proteins in current databanks, which makes sequencing by direct techniques (MS/MS and Edman degradation) very complicated. Therefore, new experiments will be conducted to determine primary structure of HiL and classify it in some animal lectin family.

HiL exhibited no effect on plankton cell growth since, at the tested concentrations; it was not possible to determine the MIC. However, this lectin was able to reduce the total biomass and viable cell number of *S. aureus* and *S. epidermidis* biofilms. Previous studies using lectins from marine sponges also showed results similar to ours (Carneiro et al. 2017a, 2019, Marques et al. 2018). Carneiro et al. 2019 evaluated antibacterial activity of AFL, which did not inhibit planktonic growth of Gram-positive and Gram-negative bacteria tested. However, the lectin significantly reduced the biomass biofilm of the bacteria *S. aureus*, *S. epidermidis*, and *E. coli*.

Another lectin isolated from the marine sponge *Chondrilla caribensis* (CCL) by Marques et al. 2018 was able to agglutinate the same strains evaluated in our study, but showed no inhibition of planktonic growth of these bacteria; even so, it showed a significant reduction in biofilm biomass.

Antibiofilm activity related to HiL results from increased antimicrobial resistance. Biofilm formation represents an important mechanism of virulence of many bacterial pathogens and a strategy used by these same bacteria to survive in natural environments or in colonized tissues of some hosts (Raafat et al. 2019, Costerton et al. 1999). According to our findings, HiL was able to reduce by 50% the total biomass and up to 2.0 logs the number of viable *S. aureus* cells. In fact, *S. aureus* can be considered the most problematic pathogen of the genus *Staphylococcus*, as it is known to cause numerous acute and chronic infections (Gordon & Lowy 2008).

Similar results were observed in *S. epidermidis* biofilm. However, *E. coli* showed greater resistance to lectin activity. This could be attributed to the inherent resistance of Gram-negative bacteria, intrinsic to their metabolic activity, and/or presence of an outer membrane (MacGowan & Macnaughton 2017).

According Coelho et al. (2009), the recognition of glycans in the bacterial membrane by lectins may cause an inhibitory effect in the biofilm formation. Moreover, some studies have shown that galactose-binding lectins may recognize glycans in bacterial surface and distinguish bacteria Gram-positive from Gram-negative bacteria, as ADEL, a lectin from *Aplysia dactylomela*. In addition, ADEL cause agglutination and inhibit biofilm formation of *S. aureus,* but not of *E. coli* (Carneiro et al. 2017b).

The mechanism of activity of lectins on biofilm formation is not yet fully determined, but some mechanisms are suggested, as bacterial adhesion inhibition, reduction of expression of genes associated with biofilm formation, down-regulation of *quorum-sensing* factors, recognition of carbohydrates in the SPE and disruption of the biofilm matrix (Islam et al. 2009, Cavalcante et al. 2013).

In conclusion, we have isolated a new lectin with unique amino acid sequence and antibiofilm activity potential to prevent infections caused by bacterial biofilms, mainly those caused by *Staphylococcus* spp.

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