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CELLULAR AND MOLECULAR BIOLOGY

Proteomic characterization and biological activities of the mucus produced by the zoanthid *Palythoa caribaeorum* (Duchassaing & Michelotti, 1860)

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Abstract: Mucus, produced by *Palythoa caribaeorum* has been popularly reported due to healing, anti-inflammatory, and analgesic effects. However, biochemical and pharmacological properties of this mucus remains unexplored. Therefore, the present study aimed to study its proteome profile by 2DE electrophoresis and MALDI-TOF. Furthermore, it was evaluated the cytotoxic, antibacterial, and antioxidant activities of the mucus and from its protein extract (PE). Proteomics study identified14 proteins including proteins involved in the process of tissue regeneration and death of tumor cells. The PE exhibited cell viability below 50% in the MCF-7 and S-180 strains. It showed IC50 of 6.9 µg/mL for the J774 lineage, and also, favored the cellular growth of fibroblasts. Furthermore, PE revealed activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* (MIC of 250 µg/mL). These findings revealed the mucus produced by *Palythoa caribaeorum* with biological activities, offering alternative therapies for the treatment of cancer and as a potential antibacterial agent.

Key words: Antibacterial, cytotoxicity, proteomic, Palythoa.

INTRODUCTION

In recent decades, there have been increasing discoveries of new substances isolated from marine organisms (Quintana et al. 2015, Nalini et al. 2018). Indeed, biological properties may have various therapeutic applications for health (Blunt et al. 2015, Kim et al. 2012). One of these bioactive compounds, the protein molecules, are promising alternatives for the development of therapeutic compounds. Proteomic characterization is an important strategy for detecting proteins with numerous beneficial effects on health (Beaulieu et al. 2015). Well-defined biological properties of proteins include antioxidants (Robinson et al. 2017), antibacterial (Sila et al. 2014, Perumal et al. 2015), antiproliferative (Gue et al. 2006, Burgos-Hernandez 2012) and antihypertensive (Patricia et al. 2016).

The zoanthid *Palythoa caribaeorum*, a phylum Cnidaria, is an organism abundant on the coast of Pernambuco, Brazil. In this region exists a high degree of endemism and competitiveness, which makes the environment more dynamic. The development of the zoanthid *Palythoa caribaeorum* is vegetative and displays high rates of regeneration. During low tide, the polyps of this zoanthid release mucus known as ox drool, which serves mainly to protect the colony against desiccation and pathogens (Soares et al. 2006, Almeida et al. 2012).

The great diversity of biocomposites synthesized by marine organisms may represent therapeutic applications to human health (Vizetto-Duarte et al. 2016). According to what the population says, this mucus may contain healing, anti-inflammatory, and analgesic effects (Soares et al. 2006). Because of the lack of information about this mucus, the scientific community has been trying to better understand its biological properties. In addition, due to an increase of bacterial resistance and the search for new antitumor agents, it occurs a current demand for research new therapeutic agents (Padhi et al. 2014).

Therefore, the current study evaluated the antibacterial activity and cytotoxicity of the *P. caribaeorum* mucus and its protein extract. In addition, antioxidant activities of the mucus was performed. Finally, proteomic characterization of mucus was carried out.

MATERIALS AND METHODS

Collection of biological material

Samples of mucus produced by the zoanthide *Palythoa caribaeorum* were collected on the beach of "Porto de Galinhas" in the municipality of "Ipojuca", located in the south coast of "Pernambuco"/Brazil (8°30'24'' S, 34°59'52'' W). The mucus was collected directly from its colonies through digital stimulation, with the aid of gloves. The collected material was transported in a hermetically sealed thermal box and sealed at 4°C to the final storage site (-20°C) and, then, lyophilized. The Registration number of National System for the Management of Genetic Heritage and Associated Traditional Knowledge - SISGEN of the *Palythoa Caribaeorum* is A7F30E7.

Preparation of mucus

The lyophilized mucus (1g) was rehydrated with 15 mL of ultrapure water and taken to the ultrasonic bath at low temperature (≈ 4°C) for 40 min. The material was then centrifuged for 15 minutes at 112 xg in a microcentrifuge at 20°C. The supernatant was collected, lyophilized, and stored at -20°C.

Total protein dosage

The 400 mg of lyophilized mucus was diluted in 1mL of ultrapure water. The dosage of proteins was performed by the 2-D Quant Kit (GE Healthcare Corp., USA), following the manufacturer instructions. A standard curve was determined using BSA (bovine serum albumin).

Total protein extraction

After quantitation by the 2-D Quant Kit, the total proteins were precipitated using the 2-D Clean-Up Kit (GE Healthcare Corp., USA) following the manufacturer instructions. This kit works by quantitatively precipitating proteins while leaving behind in solution substances such as detergents, salts, lipids, phenolic and nucleic acids which can cause interference. The proteins were pelleted by centrifugation and the precipitate was washed to further remove non-protein contaminants. The mixture was centrifuged again and the precipitated proteins easily suspended in distilled water for biological analyzes.

Cells and culture conditions

Epitheloid cervix carcinoma cells (HeLa 0100), macrophages (J774.A1), human fibroblast (CCD1072Sk), breast adenocarcinoma (MCF-7) and Sarcoma (S-180), a heterogeneous lineage of tumor cells from mice of mesodermal origin were used (Debnath et al. 2017). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10%) and penicillin-streptomycin (1%) at 37°C and 5% CO_2. Cells were counted (10^4 -10^6 cells/ml) for the cell viability assay by the MTT method

(3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide).

Cytotoxicity assay

An MTT assay was used to determine the cellular viability of the enzyme (da Silva et al. 2019). The cells were inoculated into 96-well plates at a density of $1x10^4$-$1x10^6$ cells/ml and after 24h incubation. The protein extract was exposed for 48 hours at the final concentrations of 100, 50, 25, 12.5 and 6.25 µg/mL. The protein extract was solubilized in distilled water. After the treatment period, 25µL of MTT solution (5mg/mL) was added, and the plates were incubated for 3h. After incubation, the supernatant was removed and 200µl of DMSO (Dimethylsulfoxide) added. The absorbance was measured on a Microplate Reader (BioteK Elx808) in the length of 570nm. Cytotoxicity was expressed in cell viability (A^{570} of treated cell population X100/A^{570} of untreated cell population). DMSO was used as a positive control (Miller et al. 2018).

Antimicrobial activity

The antimicrobial activity was evaluated by the broth microdilution method according to the *Clinical and Laboratory Standards Institute* (CLSI 2015). The bacteria were maintained at -80°C in brain heart infusion broth (BHIB) containing 20% glycerol. Initially, Müeller-Hinton broth was evenly distributed in the well plates. Samples (mucus and proteins) were then added by serial dilution to obtaining concentrations ranged from 0.5 to 250 µg/mL. The bacterial suspensions *Escherichia coli* ATCC® 25922, *Klebsiella pneumoniae* ATCC® *700603, Staphylococcus aureus* ATCC® 29213 and *Staphylococcus epidermidis* clinical isolate were adjusted in the density of 0.5 McFarland standard, diluted and deposited into the wells to obtain a final concentration of 10^5 UFC/well. Then, the microplates were incubated at 35°C for 24 hours. The minimum inhibitory concentration (MIC) was determined as the lowest concentration capable of inhibiting microbial growth. This method used resazurin dye as an indicator of microbial growth. Minimum bactericidal concentration (MBC) was determined after the MIC results. An aliquot of 10 µL was aseptically removed from each well in which no visible bacterial growth was observed, it was seeded on Müeller-Hinton agar and the plates were incubated at 35°C for 24 hours. After this period, the MBC was determined as the lowest concentration containing no microbial growth.

Determination of total antioxidant activity

Lyophilized mucus was utilized to evaluate, the antioxidant activity by the method of Re et al. (1999). The ABTS + radical were dissolved in an appropriate volume of distilled water, then added to a solution of potassium persulfate and left at 27 °C for 16 hours in the dark. After that time, 1 mL of the solution was dissolved in ethanol until an absorbance of 0.70 (± 0.2) at 734 nm was obtained.

The effect of the mucus on the antioxidant activity was determined using a 30 µL aliquot of each mucus diluted (100 and 200 mg/mL) in the test tubes containing 3.0 mL of $ABTS^+$. Absorbance was measured at different periods (6, 15, 30, 45, 60, and 120 minutes). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard reference. The final value obtained corresponds to the percentage of antioxidant activity.

Proteomic profile of mucus

Two-dimensional electrophoresis

Two-dimensional electrophoresis (2-DE) was adopted to separate the proteins present in the mucus. Thus, in the first dimension 400 µg of total protein precipitated by the 2D clean up kit

was solubilized in 250 µL of hydration solution (7M urea, 2M thiourea, 4% CHAPS, 100 mM DTT, 0.002% bromophenol blue and 2% Pharmalite pH 3-10) and used for rehydration of the Immobiline DryStrips tapes for a period of 16 hours at 27°C, using IPGbox (GE Healthcare Corp., USA). The isoelectric focusing of the samples embedded in the tapes was performed in IPGPhor 3 apparatus (GE Healthcare Corp., USA). Subsequently, the tapes were equilibrated with buffer containing 6 M urea, 50 mM TRIS-HCl pH 6.8 buffer, 30% glycerol, 2% SDS and DTT (10 mg/mL) for 15 minutes. Then, the process was repeated using both the same buffer and incubation time, however, this time iodoacetamide (25 mg/mL) was used instead of DTT. The second dimension was performed by running on 12.5% polyacrylamide gel (SDS-PAGE). The gels were stained with a Coomassie Brilliant Blue solution and their images scanned with the aid of Image Scanner III (GE Healthcare Corp., USA). At last, ImageMaster 2D Platinum Software Version 7.0 (GE Healthcare Corp., USA) analyzed the spots.

Trypsinization of spots

The spots found were cut with the aid of a sterile scalpel and added to tubes containing 200µL of destaining solution (50% methanol, 5% acetic acid). After 12 hours, this solution was removed, the wash solution (25mM NH_4HCO_3:50% acetonitrile) added and vortexed for 10 minutes, then the procedure was repeated twice. Then, by the addition of 100% acetonitrile, spots were dehydrated and, the gels were dried in SpeedVac (Concentrator 5301, Eppendorf). Trypsin was prepared in 25 mM NH_4HCO_3 buffer according to the manufacturer's instructions (Invitrogen Inc, USA). The spots were kept immersed in the trypsin solution at 37°C for 20 hours. After the end of trypsin digestion, the enzyme was inactivated and the peptides were extracted organically by the addition of 30 µL of 5% TFA: 50% ACN solution. After incubation for 1 hour, the solution containing the peptide's extract was transferred to a new tube, and lastly, the solution was concentrated using a SpeedVac (Concentrator 5301, Eppendorf).

Mass spectrometry analysis

To obtain the spectra, a mass spectrometer of the MALDI-TOF (Matrix-assisted laser desorption/ ionization time-of-flight) type Autoflex III (Bruker Daltonics, Billerica, MA) was used, equipped with solid phase laser Nd: YAG (355 nm). The spectra acquisition was performed in a positive reflected mode, in the detection range m/z 700 - 5000, with an acceleration of the ions at 19 kV. An external calibration was performed with standard peptide mixtures (Bruker Daltonics) using the following standards: (monoisotopic mass): Angiotensin II ([M+H]+1046.5418), Angiotensin I ([M+H]+1296.688), Substance P ([M+H]+1347.7354), Bombesin ([M+H]+ 1619.8223), ACTH clip 1-17 ([M+H]+2093.0862), ACTH clip 18-39 ([M+H]+2465.1983) and Somatostatin 28 ([M+H]+3147.4710). The spectra were analyzed using the software FlexAnalysis version 3.0 (Bruker Daltonics).

After extraction of proteins, the samples were diluted and homogenized in 10µL of 0.1% trifluoroacetic acid (TFA). Then, a 2µL aliquot was mixed containing the same volume of matrix (alpha-cyano-4-hydroxycinnamic acid, 10mg/ mL) and 1µL of that mixture was applied onto the MALDI plate. All samples were evaluated in triplicate.

The spectra obtained were analyzed using the Mascot algorithm (Matrix Biosciences) against the NCBI and SWissProt protein databases. The parameters used were: Taxonomy: Metazoa; Fixed modifications: cabamidomethyl (C), variable modifications: Oxidation (M).

Statistical analyses

Cytotoxicity results were reported as mean ± SD. One-way ANOVA followed by the Bonferroni test was used for multiple comparisons. For the antioxidant tests, ANOVA was followed by the Tukey test. Differences were considered statistically significant when $p < 0.05$. Graph Pad Prism program (version 5.00) was used to perform the statistical analyses.

RESULTS AND DISCUSSION

Cytotoxicity assay

In regard to cytotoxicity, it is noteworthy that a compound is considered a good candidate to inhibit cell growth when its cellular viability is below 50% according to Na et al. (2007).

In S-180 cells at concentrations of 100, 50, 25, 12.5 and 6.25µg/mL of mucus, the cell viability exhibited respective values of 79.05 ± 3.95; 91.01 ± 4.62; 88.26 ± 5.66; 87.6 ± 5.1; 96.54 ± 5.2% (Fig. 1a). In regard to the protein extract (PE), the values of cellular viability were 28.98 ± 2.49; 62.48 ± 3.63; 80.82 ± 4.70; 84.34 ± 3.0; 85.20 ± 4.84%. (PE) presented viability below 50% in the concentration of 100 µg/mL, which confirmed that the protein extract was as effective as DMSO (21.45%). At the concentration of 50 µg/mL, PE had a cytotoxic effect against sarcoma cells, as previously published by Cai et al. (2012).

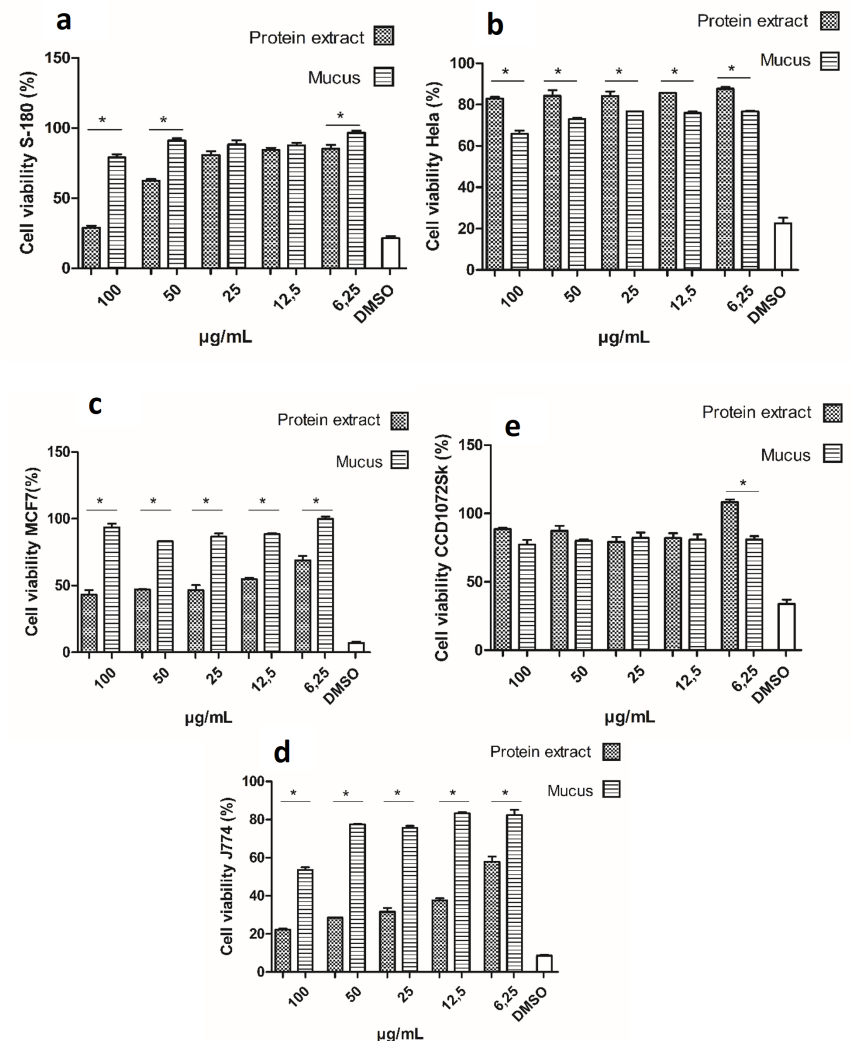

Figure 1. Cell viability of mucus and mucus protein extract against ascites S-180 (a) HeLa (b) MCF7 (c) J774 (d) and CCD1072 Sk (e). Protein extract; Mucus. Statistical differences were determined by ANOVA followed by the Bonferroni test. * P <0.05.

In the study of cytotoxicity against HeLa cells, the mucus presented cell viability between 60% and 80%. At the concentrations of 100, 50, 25, 12.5 and 6.25 µg/mL, the values were respectively 65.96 ± 1.44; 73.02 ± 1.37; 76.76 ± 0.005; 75.96 ± 1.64; 76.68 ± 0.96% (Fig. 1b). The protein extract presented the following viability values: 82.93 ± 1.2; 84.29 ± 5.1; 84.2 ± 5.34; 85.68 ± 1.04; 87.70 ± 3.73% (Fig. 1b).

As for MCF-7 cells, the cellular viability values for mucus were 93.58 ± 5.6; 83.12 ± 1.4; 86.58 ± 5.02; 88.38 ± 2.34; 99.93 ± 4.52%. The protein extract presented viability below 50% in the concentrations of 100, 50 and 25 µg/mL, presenting respective values of 43.1 ± 5.4; 46.87 ± 1.15; 46.45 ± 2.5% (Fig. 1c). These findings demonstrated that at low concentrations, this protein extract decreased viability this cell line.

When tested against MCF-7, total protein from other marine organisms, such as fish, showed viability above 70%, even at a high concentration (1000 µg/mL) (Gue et al. 2006). Protein molecules, such as natural polymers extracted from *Lentinus polychrous*, showed viability above 50% at concentrations of 100 and 50 µg/mL (Thetsrimuang et al. 2011).

The (PE) presented viability below 50% in the concentrations of 100, 50, 25 and 12.5 µg/mL in cells J774, according to the following values of 22.18 ± 1.08; 28.52 ± 0.24; 31.60 ± 3.29; 37.57 ± 1.88% respectively. The mucus did not present significant cytotoxicity in any of these concentrations, as described in fig. 1D. Macrophage-toxic molecules are important to combat intracellular infections. For instance, in the case of tuberculosis, the *Mycobacterium tuberculosis* (intracellular pathogen) invades macrophages and causes tuberculosis (Lira et al. 2009). Mucus (PE) can be a good source of bioactive molecules that can have a therapeutic use against tuberculosis.

The fibroblast viability (CCD1072Sk) observed after exposure to mucus was 77.32 ± 5.76; 80.09 ± 2.27; 82.23 ± 5.67; 80.85 ± 5.64; 80.89 ± 5.34% at the concentrations of 100, 50, 25, 12.5 and 6.25 µg/mL, respectively. The mucus PE presented viability of 88.58 ± 1.30; 87.3 ± 2.59; 79.24 ± 4.23; 82.0 ± 1.30; 108.32 ± 3.19%, respectively, according to Fig. 1e. The results showed that both the mucus and the protein extract did not present cytotoxicity. Interestingly, an increase in cell viability was observed at the concentration of 6.25 µg/mL of PE. Based in these results is possible to infer that PE can act on fibroblast growth. In addition, there are previous findings showing that molecules of protein origin, such as the LL37 peptide promoting endothelial cell proliferation (Ramos et al. 2011).

Concentrations that inhibit 50% of cell growth (IC_{50}) are shown in table I. Mucus presented IC_{50} values greater than 100 µg/mL in all cells tested as well as in the PE for HeLa and CCD1072Sk cells. These data suggest that mucus is not effective in inhibiting cell growth according to Akindele et al. (2015). The S-180, MCF7, and J774 strains raised two important conclusions: first, PE is toxic for S-180 and MCF-7; the second conclusion is that (PE) even more toxic for J774 cells presenting IC_{50} of 6.9 ± 2.24 µg/mL.

Table I. IC 50 values in S-180, Hela, MCF7, J774 and CCD1072Sk cells after 48 hours exposure to *P. caribaeorum* mucus and its protein extract.

IC_{50} µg/mL	Mucus	Protein extract
S-180	>100	51,67 ± 3,73*
HELA	>100	>100
MCF-7	> 100	54,94 ± 4,1*
J774	>100	6,9 ± 2,24*
CCD1072Sk	>100	>100

***P<0,05 Compared to mucus.**

There is a great demand for novel antitumor compounds that are efficient and specific. One of the most commonly used is synthetic Doxorubicin that exhibits IC50 of 0.38 ± 0.09 and 5.90 ± 0.44 µg/mL for MCF-7 and S-180 cells, respectively. However, chemotherapeutic agents e.g. doxorubicin, can have limitations. For instance, these chemotherapeutic agents are not selective or specific; both can affect tumor cells and normal cells, as well as present contraindications in the presence of generalized infections (Schmitt & Breinig 2006). The compounds of protein origin have a specificity of action, consequently are more effective and have reduced side effects. Another advantage of using these molecules as antitumor agents is that they are of natural origin, while Doxorubicin is synthetic (Chen et al. 2013).

In this way, the protein extract showed expressive results when compared with the mucus, mainly in the cells of S-180, MCF-7, and J77, significantly decreasing the cell viability. Additionally, protein extract has shown that it can be effective against tumor cells in minimal amounts. It also increased the viability of fibroblasts. Finally, it is possible that the proteins present in the mucus are involved in the healing activity.

Antimicrobial activity

Initially, the mucus of *Palythoa caribaeorum* was tested to evaluate the antimicrobial activity against *Escherichia coli*, *Klebsiella pneumoniae, Staphylococcus aureus*, and *Staphylococcus epidermidis* (Table II). The results demonstrated that this mucus did not present activity against the tested bacteria (MIC > 250 µg/mL), corroborating the study ensuing of Guarnieri et al. (2018) in which the mucus produced by *Palythoa caribaeorum* also did not present antimicrobial activity against *E. coli, K. pneumoniae, S. aureus*.

Regards the protein extract from the mucus of *Palythoa caribaeorum,* the MIC of this protein was 250 µg/mL for all the bacteria tested (Table II). Other authors highlighted the antibacterial activity of proteins extracted from corals. The corals *Phyllogorgia dilatata* presented a peptide denominated Pd-AMP1 which has antimicrobial activity, mainly against *K. pneumoniae* and *S. aureus* (Lima et al. 2013). He verified that the crude extract of *Palythoa caribaeorum* did not present significant results against the same bacteria. Therefore, the protein content of marine organisms consists of promising molecules with antibacterial activity.

Antioxidant activity

The mucus was tested at a concentration of 100 and 200 mg/mL at predetermined times. The ABTS radical inhibition values at the concentration 100 mg/mL were 0.0; 3.51; 19.8; 22.2; 24.3; 51.1% at the respective times of 6, 15, 30, 45, 60 and 120 minutes. At the concentration of 200 mg/mL, the inhibition values at those respective

Table II. Antimicrobial activity of mucus and its protein extract from *Palythoa*.

Microorganism	Gram	Mucus	Protein Extract
		MIC (µg/mL)	
Escherichia coli ATCC® 25922	-	> 250	250
Klebsiella pneumoniae ATCC® *700603*	-	> 250	250
Staphylococcus aureus ATCC® 29213	+	> 250	250
Staphylococcus epidermidis clinical isolate	+	> 250	250

MIC: Minimum inhibitory concentration; ATCC: American Type Culture Collection.

times were 0.0; 30.5; 20; 34.24; 33.37; 58.62% (fig. 2). Results were different ($p < 0,05$), except at 6 and 30 minutes. Mucus presented ABTS radical inhibition above 50% at 120 minutes. According to Lima et al. (2013) crude extract and alcoholic extracts of *Palythoa caribaeorum* showed antioxidant activity with inhibition above 50%. For the author, this percentage is significant.

The values represented in TEAC (Antioxidant Activity equivalent to trolox) of 100 mg/mL are 213; 746.3; 456.3; 799.6; 771.8; 1164.11, respectively. Whereas, the concentration of 200 mg /mL are 165.22; 327.44; 584.11; 615.22; 649.66; 1056.33, respectively.

The high concentrations that the mucus presented antioxidant action can be explained by the fact that it was tested in its entirety, without isolation of molecules. However, it has already been proven in cytotoxic and antimicrobial activity that the mucus protein isolate, in this work, presented results confirming its biological potential.

Proteomic identification of *P. Caribaeorum* mucus

Many studies have been conducted regarding the proteome of the marine organisms in an attempt to discover new compounds with important biological activities (Knigge 2015). In this context, the current work evaluated the protein contents in the mucus produced by *Palythoa caribaeorum*. For this purpose, 2-DE platform was adopted. Protein quantification showed a yield of 0.57%.

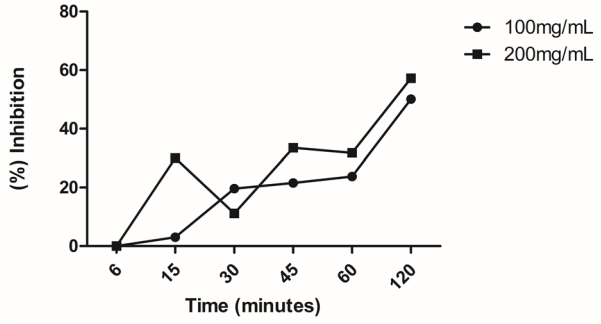

Figure 2. Activity of elimination of the ABTS radical + (%) of the mucus produced by *Palythoa caribaeorum*, at the concentrations of 100 and 200 mg / mL, in times of 6, 15, 30, 45, 60 and 120 min. Using trolox as standard. Statistical differences were determined by ANOVA followed by the Tukey test. * P <0.05.

In this study, the tool adopted to evaluate proteomics, the 2-DE, identified about 76 spots in the samples of mucus obtained from *P. caribaeorum*. There were 79% that behave as basic proteins (Fig. 3a). There was 24% of the identified spots that presented pI between 7-9. Whereas, 23% of the spots represented acidic proteins (pI 6) (Fig. 3b). The electrophoretic profile revealed that 34% of spots refer to proteins of molecular weight in the range of 10-20 kDa; 26% of the spots referred to proteins 30-40 kDa; 9% 40-60 and 8% referred to proteins 80-100 kDa (Fig. 3c).

A study in MALDI-TOF identified 14 proteins by the MASCOT database (Matrix Biosciences), as described in table III. The main functions of the identified proteins are also shown in Table III.

All proteins spots are shown in Fig. 4, the spot 8 was identified as glutamyl-tRNA synthetase. Recent finding demonstrates its involvement in the resistance mechanism to biocides by probiotic bacteria, such as *Lactobacillus pentosus* (Casado Muñoz et al. 2016). Therefore, the presence of glutamyl-tRNA synthetase in the mucus may be involved in the corals' ability to overcome the effects of oxidative stress. Corals have been experiencing these effects of oxidative stress due to the effects of climate change, such as rising temperatures in the seas (Voolstra et al. 2011).

An important protein identified as Zinc finger protein 654 corresponds to the spot 71. This protein seems to be involved in the destruction of tumor cells (Wang et al. 2005). It suggested that this protein of mucus participates in the process of cellular inhibition corroborating with

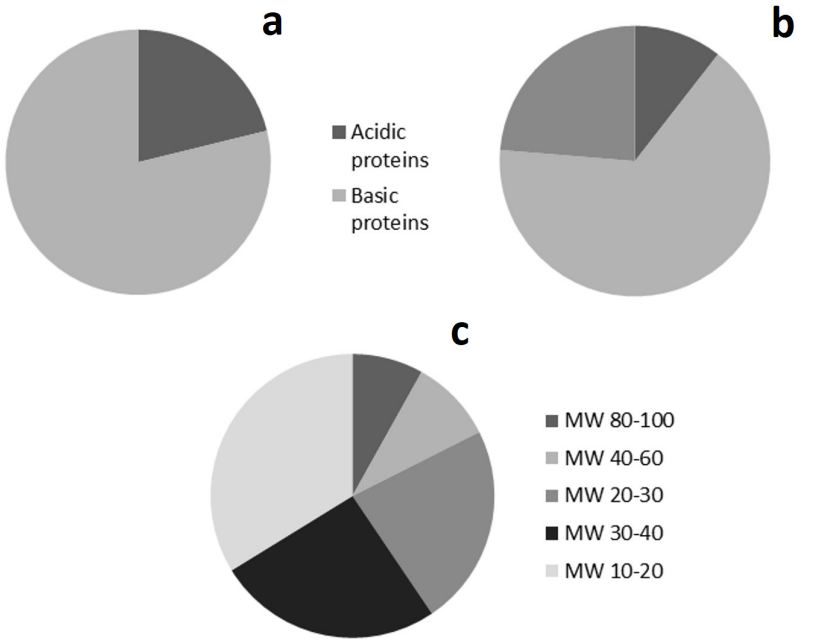

Figure 3. Data from SDS-PAGE gel spots (12.5%) identified from the ImageMaster 2D Platinum Software Version 7.0. (a) Classification of proteins; (b) Isoelectric point; (c) Molecular weight (KDa).

inhibition of the MCF-7, S-180, and J774 cells observed in this study.

Deleted in malignant brain tumors 1 protein-like is another protein identified (spot 56) and may also be involved in the death of tumor cells. This protein is involved in innate immune defense and is expressed in the lungs of preterm newborns. Its gene has been mentioned as a candidate tumor suppressor against brain, lung, and digestive tract cancer (Mollenhauer et al. 2001).

Hypothetical protein NECAME_18605 and Hypothetical protein PFICI_09699 correspond to spots 76 and 14, respectively. Both are hypothetical proteins (SPH), known as proteins with unknown structures (Tan et al. 2014). There are no records of the biological functions of these proteins in the current literature.

The proteasomes that correspond to spot 32, also identified in the mucus, play important and vital roles for living beings (Table III), so it is possible that these properties may become a potential therapeutic strategy.

Beta-2-microglobulin (B2M) corresponds to spot 61. The presence of this protein in the mucus has unknown function, but can be used in the induction of apoptosis of tumor cells (Yang et al. 2006). Another protein, Uroporphyrinogen-III synthase (URO-synthase III), that corresponds to spot 34, has no known function for *Palythoa*. However, there is a possibility that the protein has been involving in the maintenance of the normal functioning of the *Palythoa*, as well as in humans (Table III).

The identification of spot 33 corresponds to PAS domain S-box, which can be found in several species, including mammals, insects, plants, fungi, and cyanobacteria. For its function cited in table III, it should be associated with the protection of *Palythoa* against UV rays, which mucus together with zooxanthellae guarantees during low tide (Lesser et al. 1989).

The metalloproteinase-disintegrin, corresponding to spot 65, makes up the extracellular matrix and is involved in the process of tissue regeneration (Table III). Guarnieri et al. (2018) described the presence of proteolytic

Table III. List of proteins identified by MASCOT and expressed in the mucus produced by Palythoa caribaeorum, against the protein databases of NCBI and SWissProt. Species and functions of each protein are described below.

ID	Spot	Protein	Coverage	pI_{THE}	MW_{THE}	pI_{FO}	MW_{FO}	Species	Functions
319435166	8	glutamyl-tRNA synthetase	58%	5	55,23	5,25	56	*Dietzia cinnamea*	Catalyze a binding of an amino acid to the tRNA molecule (Farah et al. 2014)
630028390	14	Hypothetical protein PFICI_09699	45%	6,18	9,52	7,12	39	*Pestalotiopsis fici*	Unknown
697511537	32	Proteasome subunit aloha type-1	48%	6,08	30,353	6,05	31	*Fulmarus glacilis*	It participates in cell cycle regulation, differentiation, inflammatory responses, signal transduction pathways, apoptosis (Huang & Chen 2009)
464316105	33	PAS domain S-box protein, parcial	48%	4,9	30,069	5,68	29	*Leptospira interrogans serovar*	Works as sensors to light stimuli (Zhulin et al. 1997); Has function in signal detection and transduction (Pellequer et al. 1998)
768927828	34	Uroporphyrinogen-III sythase	47%	5,33	30,087	5,92	29	*Bacillus subtilis*	It is involved in the conversion of hydroxymethyl to bilane uroporphyrinogen III. Its deficiency causes Congenital Erythropoietic Porphyria (PEC)) (Bishop et al. 2010)
637279932	56	Deleted in malignant brain tumors 1 protein-like	81%	5,16	15,122	5,97	17	*Anolis carolinensis*	Tumor supressor (Mollenhauer et al. 2001)
LIN-51_TETNG	60	protein lin-52 homolog	35%	4,53	12,637	4,77	12	*tetraodon nigroviridis*	It participates in a large protein complex with important transcriptional regulators of cell proliferation and death (Bhaskar et al. 2012)
B2MG-CHICK	61	Beta-2-microglobulin	27%	5,84	13,148	4,96	13	*Gallus gallus*	It is associated with inflammatory and hematological diseases (Skare et al. 2014)
VM3B4-BOTSA	65	Zinc metalloproteinase-disintegrin-like bothrojarin-4 (fragment)	28%	5,36	11,436	5,65	10	*Bothrops jararaca*	Participates in fabric remodeling (Oliveira et al. 2013)
659870098	66	Killer protein	85%	6.73	10,8	5,83	10	*Caulobacter sp.*	Causes cell death by blocking calcium channels (Schmitt & Breinig 2006); Check resistance to fungal infections (Magliani et al. 2008)
543371659	70	mitochondrial import inner membrane translocase subunit Tim10	54%	5,9	10,638	6,97	10	*Pseudopodoces humilis*	It participates in the import and insertion of transmembrane proteins in the inner membrane of mitochondria. It acts as a chaperone-like protein that protects the hydrophobic precursors and guides them through the mitochondrial intermembrane space (Muehlenbein et al. 2004)
831563256	71	Zinc finger protein 654	13%	7,12	15,05	7,16	14	*Fundulus heteroditus*	Regulates programmed cell death (Wang et al. 2005)
657576201	73	deoxynucleoside triphosphate triphosphohydrolase SAMHD1-LIKE isoform X2	60%	7,71	14,995	8,27	13	*Stegastes partitus*	Blocks early replication of the virus. And it is still highly expressed in dendritic cells (Goldstone et al. 2011)
915245772	76	Hypothetical protein NECAME_18605	78%	9,91	11,658	8,37	11	*Necator americanos*	Unknown

THE= theoretical; FO= found; MW= molecular weight; pI= Isoelectric point.

enzymes as the matrix metalloprotease (MMPs) in the mucus produced by Palythoa caribaeorum. It may be associated with increased fibroblast viability (Fig. 1d).

The spot 70 was identified as Mitochondrial import inner membrane translocase subunit Tim10, however there are no reports of its function for *Palythoa*.

Deoxynucleoside triphosphate triphosphohydrolase SAMHD1-LIKE isoform X2 protein that corresponds to the spot 73, has also been identified. There are no reports of the function of this protein in the mucus. However, since it is involved in the HIV virus restriction factor (Franzolin et al. 2015), it has the potential for therapeutic use against HIV.

The Lin-52 and Killer Proteins that correspond to spot 60 and 66, respectively, have functions involved in cell death (Table III). Their presence in the mucus may be associated with *Playthoa* defense against pathogens. However, the discovery of these proteins proves their direct relationship with the protective function of mucus. In addition, these proteins may have caused the cell death of MCF-7, S-180, and J774 cells (Fig. 1a, c, d) by blocking calcium channels (Schmitt & Breinig 2006).

Despite the important results presented in this study, it is important to mention that future studies are necessary to isolate and characterize these biomolecules with the objective of applying them as a therapeutic option.

CONCLUSIONS

The extract protein from mucus produced by *Palythoa caribaeorum* showed antibacterial and antioxidant activities. Also, inhibited the growth of tumor cells. The proteomic profile revealed proteins that can justify this biological action. In addition, this tool was able to detect proteins involved in the process of tissue regeneration and death of tumor cells.

In view of the above, the mucus becomes an important source of biomolecules with attractive biological activities. Suggesting more in-depth studies that allow the use of these proteins.

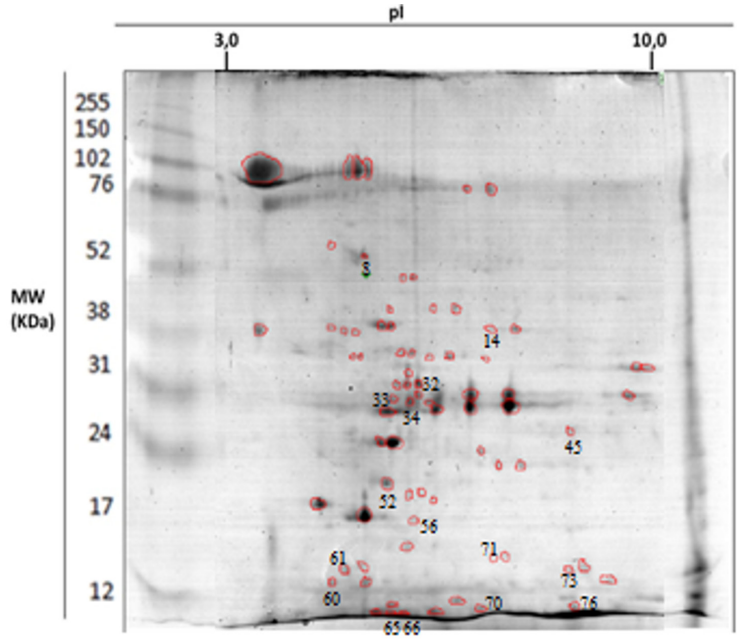

Figure 4. Proteomic profile of mucus produced by *Palythoa caribaeorum*, analyzed by Two-dimensional Electrophoresis SDS-PAGE (12.5%) stained with Comassie Blue. Strips of IPG (3-10) linear, 13cm, were used for isoelectric focusing.

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