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Artigo

Chemical Composition and Anticariogenic Activity of *Tambja stegosauriformis* Nudibranch

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Composição Química e Atividade Anticariogênica do Nudibrânquio *Tambja stegosauriformis*

Resumo: Extrato do nudibrânquio da espécie *Tambja stegosauriformis* foi obtido por imersão em metanol, concentrados em um rotaevaporador e secos por liofilização. A identificação dos metabólitos secundários presentes nos extratos foi realizada por cromatografia líquida de alta eficiência com detector DAD, acoplada à espectrometria de massa (HPLC-DAD-MSn). Foram identificados oito alcaloides pirrólicos no extrato de *T. stegosauriformis*. Todos os alcaloides derivam da estrutura base com dois anéis pirrólicos interligados e presença de bromo ou não, as quais foram reportadas previamente na literatura. A atividade antibacteriana do extrato foi testada em amostras padrão de *Strepcococcus mutans* ATCC ("American Type Culture Collection") 25175 e *Lactobacillus casei* ATCC 393, determinando a Concentração Inibitória Mínima (CIM) e Concentração Bactericida Mínima (CBM) do extrato frente a essas bactérias. A atividade citotóxica das concentrações do extrato de *T. stegosauriformis* com atividade antibacteriana foi verificada em *Artemia salina*. O extrato de *T. stegosauriformis* apresentou atividade bacteriostática frente às espécies *S. mutans* (CIM = 1,175mg / mL) e *L. casei* (CIM = 0,5875 mg / mL). Os resultados indicam um potencial efeito anticariogênico de substâncias presentes no extrato do nudibrânquio *T. stegosauriformis*

Palavras-chave: Nudibrânquios; produtos naturais marinhos; alcaloides pirrólicos; atividade antimicrobiana

Abstract

Extract from *Tambja stegosauriformis* nudibranch were obtained by immersion in methanol, concentrated in a rotary evaporator and dried by lyophilization. The identification of the secondary metabolites present in the extracts was performed by high performance liquid chromatography with DAD detector, coupled to mass spectrometry (HPLC-DAD-MSn). Eight pyrrole alkaloids were identified in the *T. stegosauriformis* extract. All alkaloids identified and reported previously in the literature are derived from the base structure of two interconnected pyrrole rings, some compounds are substituted with bromine. The antibacterial activity of the extracts was tested on *Streptococcus mutans* ATCC (American Type Culture Collection) 25175 and *Lactobacillus casei* ATCC 393 samples, determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract against these bacteria. The cytotoxic activity against *Artemia salina* was detected in the *T. stegosauriformis* extract with antibacterial activity. The *T. stegosauriformis* extract presented bacteriostatic activity against *S. mutans* (MIC = 1.175mg / mL) and *L. casei* (MIC = 0.5875 mg / mL). The results indicate a potential anticariogenic effect of substances present in the *T. stegosauriformis* extract.

Keywords: Nudibranchs; marine natural products; pyrrolic alkaloids; antimicrobial activity.

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Chemical Composition and Anticariogenic Activity of *Tambja stegosauriformis* Nudibranch

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1. Introduction

The marine environment is an important source of chemical and biological diversity. In recent years, many compounds with pharmacological potential have been developed from marine invertebrates. Extracts of several marine species were evaluated revealing biological activities attributed to substances isolated from these organisms.[1,2] Several biomolecules isolated directly from marine animals or from microbes associated to them present antimicrobial activity.[3,4]

Gastropod mollusks, called nudibranchs and commonly known as sea slugs, produce toxic compounds for defense against predators. These compounds are produced by the nudibranchs themselves or obtained from

their preys, hydrozoans and corals.[5] Among the compounds isolated from several species of nudibranchs are alkaloids, indole and pyrrolic derivatives, and sesquiterpenes. Many compounds isolated from nudibranchs have been proven to present antitumor, antimalarial, antifungal, anti-inflammatory and antibacterial properties.[4-6]

Streptococcus and *Lactobacillus* are important saccharolytic oral bacteria that degrade carbohydrates into organic acids via the Embden-Meyerhof-Parnas pathway, resulting in caries.[7] In efforts to prevent caries, several natural products have been analyzed against cariogenic bacteria.[8-10]

Nudibranchs present an extensive chemical diversity with terpenes been the major metabolites isolated, however there are many nudibranchs yet to be studied.[11] The Brazilian coast has been poorly explored, with only 200 species reported.[12] Most of the new Brazilian species described were found in the southeast region of the country, in the states of Rio de Janeiro and São Paulo.[13,14] The chemical characterization and evaluation of the biological activity of secondary metabolites, isolated from Brazilian nudibranch species is still scarce.

Therefore, the chemical characterization and *in vitro* pharmacological activity evaluation of Brazilian nudibranchs species are fundamental approaches for advancement of knowledge in this area. The nudibranch *Tambja stegosauriformis*, known only from Brazil, was first described in 2005 and the first chemical study was carried out in 2012.[15,16] The aim of this study is to evaluate the chemical composition and anticariogenic activity of the extract from *T. stegosauriformis* nudibranch, collected from the Brazilian coastline.

2. Experimental Section

2.1. Sample collection

Four specimens of *T. stegosauriformis* were collected by SCUBA at depths of 10-20 meters, off Papagaio Island in the city of Cabo Frio, Rio de Janeiro in November 2017. The specimens were preserved in methanol and transported to the laboratory. The authors have the register (SISGEN A85E6DF) for research involving genetic patrimony and the appropriate collection authorizations. The identification of the specimens was carried out according to specialized literature and with the assistance of a zoologist.[15]

2.2. Extraction of biological material

The extract was produced by the following procedure. The methanol preservation was evaporated in a Rotavapor at 40 ºC and the remaining material was sequentially extracted three times with methanol (3 x 50 mL). The resulting extract solution was then filtered and concentrated under reduced pressure in a Rotavapor and freeze-dried. The nudibranchs collected yielded 317.19 mg of MeOH extract.

2.3. Chemical characterization

Extract was solubilized in methanol (1 $mg.mL^{-1}$) and analyses were performed using HPLC-DAD-MSn with a PDA detector and an ion trap mass spectrometer (positive ion mode); X-Bridge C18 column (2.1 × 150 mm) with a 10 µL injection. The elution was carried out using water (0.1 % formic acid) and acetonitrile gradient. MS parameters: ion source ESI voltage 4 kV; nebulization with nitrogen at 30 psi at a gas flow rate 9L/min. Ion source temperature at 310 ° C, skimmer 1: -10 V. The spectra were scanned in the range of 50-3000 m/z.

2.4. Bacterial inoculum preparations

The cariogenic bacterial samples of *Streptococcus mutans* (ATCC 25175) and *Lactobacillus casei* (ATCC 393) were used to

prepare two different inoculum. Bacteria were kept at –20°C in Brain Heart Infusion (BHI) medium (Becton, Dickinson and Company, Sparks, MD, USA) with 20 % glycerol and activated when transferred into BHI agar (Becton, Dickinson and Company), and incubated at 36°C for 24 h, with 5 % CO_2. Initially, the bacterial samples were evaluated to verify the degree of purity. Then, isolated bacterial colonies were selected and transferred to 0.85 % saline solution until an Optical Density (O.D.) of 0.15 and 0.20 at 520 nm (Libra S2 Colorimeter, Biochrom, Cambridge, England) for *S. mutans* and *L. casei*, respectively, corresponding to approximately 10^8 colony forming units per milliliter (CFU/mL) for each bacteria. Afterward, each bacterial sample received a decimal dilution to obtain both inoculum at the concentration approximately of 10^7 CFU/mL.

2.5. Determination of antibacterial activity

The antibacterial activity of *T. stegosauriformis* extract was determined by Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[17] with modifications proposed by da Cunha *et al*. (2013).[18] The MIC was performed in 96-well microplates, adding 5 µL of inoculum per well in 100 µL of BHI (Becton, Dickinson and Company) medium (5 x 10^5 CFU/mL as final concentration) and concentrations of *T. stegosauriformis* extract ranged from 2.35 to 0.0011 mg/mL. MIC was also performed for DMSO that was used as the extract's diluent (concentrations ranged from 0.012 to 12.5 % - v/v). The positive control was wells with inoculum and 0.05 % chlorhexidine digluconate, and the sterility control was comprised of wells with extract and medium without the inoculum. The inoculated BHI (Becton, Dickinson and Company) medium without test compounds comprised the negative controls. Microplates were incubated at 36°C for 24 h, with 5 % CO_2 and MIC was defined as the lowest concentration of extract or DMSO that allowed no visible bacterial growth. Which was confirmed by the reduction of resazurin adding 10 µL of this salt (Sigma-Aldrich, St. Louis, Missouri, USA) (100 µg/mL) per well. MBC was determined by subculturing 50 µL aliquots of dilutions equal to and greater than MIC on BHI agar (Becton, Dickinson and Company). Plates were incubated at 36°C for 48 h, with 5 % CO_2 and MBC was defined as the lowest concentration of extracts or DMSO that allowed no bacterial growth. The purity analysis was performed by streaking on BHI agar (Becton, Dickinson and Company) 10 µL of the wells with the highest concentration of *T. stegosauriformis* extract and at the same time visible growth after incubation. Two separate experiments were conducted in triplicate.

2.6. Cytotoxicity bioassays

Two bioassays with *Artemia salina* (brine shrimp) were used to evaluate the cytotoxic potential of the plant extract, the brine shrimp hatchability and brine shrimp lethality assay.

The brine shrimp hatchability assay was performed according to Carballo *et al*. (2002)[19], with modifications. Artificial sea water was prepared by adding 34 g of salt to 1 L of distilled water and pH was adjusted to 8.5 with sodium bicarbonate. The assay was performed in 96-well microplates, 175 µL of the cyst solution containing 15-30 cysts in artificial sea water was added to each well and 25 µL of TS extract (MICs final concentrations) for the test group or DMSO (final concentration 6.25 % and 3.125 %) for the control group. The plates were incubated at room temperature with constant light and the number of brine shrimp larvae (nauplii) and unhatched cysts were evaluated after 24 h.

For the brine shrimp lethality test, Rahman *et al*. (2018) method was applied with modifications.[20] *A. salina* cysts were placed in

the hatching chamber containing artificial sea water and kept at room temperature under constant aeration and light for 24 h. The newly hatched phototropic nauplii were concentrated by placing an artificial light at one end of the hatching chamber. From ten to fifteen of these nauplii (175 µL solution) were counted against a lighted back-ground and transferred to each well of a 96-well microplate to which were added 25 µL of TS extract (MICs final concentrations) for the test group or DMSO (final con-centration 6.25 % and 3.125 %) for the control group. The plates were incubated at room temperature and under constant light for 24 h.

Both experiments were conducted in triplicate. Hatching inhibition and lethality percentage were calculated using the following formula proposed by Aftab, Zechel and Sajid (2015)[21], with modifications:

$$A = [(B/C)-(D/E)] \times 100$$

Where, A = Hatching inhibition or lethality percentage; B = Number of live or free nauplii, after 24 h in control group; C = Initial number of larvae or cysts in control group; D = Number of live larvae or free nauplii, after 24 h in test group; E = Initial number of larvae or cysts in test group. This formula was not applied when there were no live larvae or free nauplii.

3. Results and Discussion

3.1. Chemical analysis

The analyzes of the methanolic extract led to the detection of eight pyrrolic alkaloids derivatives (Figure 1) being: Tambjamycin A $[M+H]^+$ =190, B $[M+H]^+$ = 267 and 269], C $[M+H]^+$ = 246, D $[M+H]^+$ = 324 and 326, G $[M+H]^+$ = 297, J $[M+H]^+$ = 339, K $[M+H]^+$ = 260 and 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde [M+H] = 191 were previously identified and reported in the literature as tambjamycin or tambjamines. All alkaloids derived from the base structure of two interconnected pyrrole rings, some compounds are substituted with bromine.[16,22]

The analysis of mass spectra obtained by fragmentation (HPLC-UV-MS) of the *T. stegosauriformis* indicated structures that follow a pattern of fragmentation according proposal for the structural characteristics of tambajamycins with a bipyrrolic system. It was observed the presence of compounds with a bromine atom due to the presence of two ions such as $[M+H]^+$ in *m/z* 267 and 269 (tambjamycin B), $[M+H]^+$ in *m/z* 324 and 326 (tambjamycin D) and $[M+H]^+$ in *m/z* 339 and 337 (tambjamycin J) with approximately the same intensity. Other compound as tambjamycin K (*m/z* 260) can loss the group C_5H_{10} (70Da) affording the peak *m/z* 190. Some tambanycins have groups that are eliminated in their neutral and non-nitrogenous form such as tambjamycins G, J and K.

Previous chemical investigation of the nudibranch *T. stegosauriformis* led to the identification of alkaloids known as tambjamycin or tambjnamine. A chemical study was carried out to verify if the mollusks captured and accumulated substances from their prey, bryozoans and sponges. The study revealed the composition of alkaloids in *T. stegosauriformis* (predator) and *B. dentata* (prey) presented variations, being that the bryozoan (prey) presents a greater variety of alkaloids than the mollusk (predator).[16] In fact, the sequestering of metabolites from prey species is seemingly the most common source of nudibranch natural products.[11]

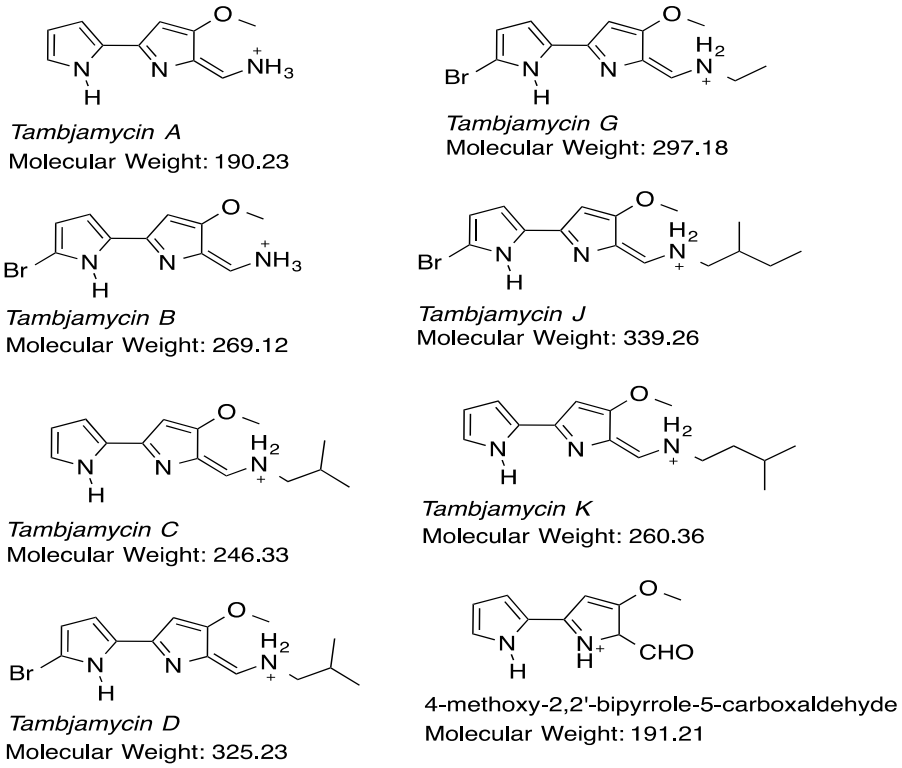

Figure 1. Pyrrolic alkaloid derivatives detected in the methanolic extract of *Tambja stegosauriformis* by HPLC-DAD-MSn

3.2. Bacteriostatic and bactericidal activities of *T. stegosauriformis* extract

The *T. stegosauriformis* extract presented bacteriostatic activity against *S. mutans* and *L. casei,* MIC values were 1.175 mg/mL and 0.5875 mg/mL, respectively. The DMSO MIC for both bacteria (12.5 %) was lower than to those present in the MIC values. No bactericidal activities were found for the concentration of *T. stegosauriformis* extract tested, so MBC values were over the highest test concentration (2.35 mg/mL) (Table 1).

The 0.05 % chlorhexidine digluconate (positive control) presented bacteriostatic and bactericidal action against both bacteria, as expected. Sterility and negative control wells showed absence and presence of bacterial growth, respectively.

The marine environment harbors great biodiversity making marine biotechnology an extremely promising field for scientific research and the chemical synthesis of new drugs. Secondary metabolites produced by various marine species have unique and unusual chemical structures and present antimicrobial activity.[3,4]

Organic acids such as lactic, acetic, formic and propionic acids, produced by cariogenic microorganisms as products of carbohydrate metabolism are able to enamel hydroxyapatite demineralization and proteolytic breakdown of tooth hard tissues.[23] *Streptococcus* and *Lactobacillus* are oral bacterial genus that degrade carbohydrates into organic acids, resulting in caries. According to Antonio *et al*. (2011) the most important anticariogenic properties of a product are inhibition of growth of the cariogenic bacteria and inhibition of the enamel demineralization process, one of these properties was detected in the present study.[8]

Table 1. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of *T. stegosauriformis* extract (mg/mL) against *S. mutant* and *L. casei*, in two separate experiments

Bacteria	1st experiment		2nd experiment		Mode	
	MIC	MBC	MIC	MBC	MIC	MBC
S. mutans ATCC 25175	1.175	> 2.35	1.175	> 2.35	1.175	> 2.35
L. casei ATCC 393	0.5875	> 2.35	0.5875	> 2.35	0.5875	> 2.35

The present study revealed that *T. stegosauriformis* extract presented bacteriostatic activity against *S. mutans* and *L. casei*. The DMSO MIC for both bacteria was 12.5 %, less than ones presented as solvent in the MIC values, so it was not responsible for the absence of bacterial growth in both MICs. To our knowledge, this is the first report that evaluates the anticariogenic proprieties of this gastropod's extracts. These results suggest that the *T. stegosauriformis* extract possesses potential pharmacological action and is a suitable candidate for further investigation in order to seek new strategies for the treatment of dental caries.

3.3. Cytotoxicity activity of *T. stegosauriformis* extract

The cytotoxicity analyses were performed for two *T. stegosauriformis* extract concentrations, the MIC values against *S. mutans* (1.175 mg/mL) and *L. casei* (0.5875 mg/mL), using brine shrimp hatchability and lethality assay. No free or live nauplii were observed in both assays for the highest concentration analyzed (1.175 mg/mL). The 0.5875 mg/mL of *T. stegosauriformis* extract presented 71.1 % ± 8.5 brine shrimp lethality, while the same concentration showed 36.8 % ± 5.9 brine shrimp hatching inhibition (Table 2).

Table 2. Percentage of hatching inhibition or lethality of brine shrimp at two concentrations of *T. stegosauriformis* extract

Assay / concentration	Hatch inhibition			Lethality		
	1st well	2nd well	3rd well	1st well	2nd well	3rd well
1.175 mg/mL	100 %	100 %	100 %	100 %	100 %	100 %
0.5875 mg/mL	31 %	43 %	37 %	63 %	80 %	70 %

The brine shrimp cytotoxicity assays are fast, simple and inexpensive methods[24] and are frequently used to assess an extract´s potential cytotoxic activity.[19-21] The *T. stegosauriformis* extract concentrations analyzed in this study showed cytotoxic response against *A. salina.* According to Carballo *et al*. (2002) the brine shrimp hatchability and the lethality test when used together increase the sensibility in screening for natural marine cytotoxic products.[19]

Although the extracts analyzed presented cytotoxic properties it is possible that other types of extraction methods or fractions can produce different responses in the brine shrimp assay.[20] Additionally, our results might reflect the presence of potent antineoplastic compounds in *T. stegosauriformis* extract[21] requiring further investigation into this property.

Tambjamines have also been isolated from the nudibranchs *Tambja eliora*, *Tambja abdere* and *Roboastra tigris* and shown to have antibacterial, antifungal activity, antimitotic effects and cytotoxic activity. It is known that the genus Tambja is a group with great diversity of species already described in the literature which brings great potential to find new substances with potential biological activity. [14,25,26]

Thus, the discovery of new prototypes with biological activities can bring new marine products with potential for the treatment of several diseases, as well as contributing to the planning of drug prototypes and the synthesis of new compounds that are more potent and less toxic.

4. Conclusions

The study of the extract of *T. stegosauriformis* nudibranch led to the identification of eight pyrrolic alkaloids derivatives that were previously reported in the literature as tambjamycin or tambjamines. The extract presented antibacterial effect and cytotoxic activity. This is the first research focused on the anticariogenic effect of nudibranch extract from the Brazilian coast. The results indicate the importance in the discovery of secondary metabolites that can be useful to future investigations of a potential anticariogenic product**.**

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