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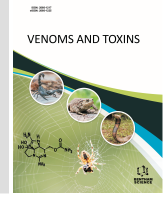
Venoms and Toxins, 2023, *3*, e070523216622

RESEARCH ARTICLE



Jellyfish Tissue Extract as Inhibition Effect of Jellyfish *Olindias sambaquiensis* Müller (1861) Sting

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ARTICLE HISTORY

Received: December 06, 2022
Revised: March 24, 2023
Accepted: March 28, 2023

DOI:
10.2174/2666121703666230507184946

Abstract: ***Background***: *Olindias sambaquiensis*, the most abundant species of jellyfish along the southern coast of Brazil, frequently stings bathers during the summer months, when the occurrence of this species usually reaches a peak.

Objective: As jellyfish are rich in protein and carbohydrates, and as these biomolecules could provide a natural defense against stings, this study investigates whether any of the components present in the umbrella of jellyfish species occurring in the south of Brazil can inhibit the nematocyst discharge of the tentacles of *O. sambaquiensis*.

Methods: Sting tests were conducted in humans, with live tentacles of *O. sambaquiensis*, to evaluate different lyophilized extracts of different exumbrellar jellyfish tissues obtained at different times of the year to determine their capacity to reduce pain and alter skin color.

Results: Of all the species of jellyfish used in this study (*O. sambaquiensis*, *Chiropsalmus quadrumanus* and *Tamoya haplonema*), only the lyophilized extract of the cubozoa *C. quadrumanus* umbrella showed the capacity to inhibit the pain associated with nematocyst stings.

Conclusion: Tests on a lyophilized extract obtained from organisms caught in summer and winter suggested that the biomolecule responsible for the biological activity is carbohydrate since this biomolecule would signal the recognition of the species. Jellyfish are rich in protein and carbohydrates, and as these biomolecules could provide a natural defense against stings. This study investigates whether any of the components present in the umbrella of jellyfish species occurring in the south of Brazil can inhibit the nematocyst discharge. Of all the species of jellyfish used in this study, only the lyophilized extract of the cubozoa *C. quadrumanus* umbrella showed the capacity to inhibit the pain associated with nematocyst stings of the *O. sambaquiensis.* It is suggested that the biomolecule responsible for the biological activity is carbohydrates.

Keywords: Cnidarians, nematocyst discharge inhibition, exumbrellar jellyfish tissue, pain and skin tests, public health, *Olindias sambaquiensis.*

1. INTRODUCTION

Olindias sambaquiensis (*Limnomedusa*, Olindiidae) is a *Hydromedusa* species endemic to the Atlantic Ocean, occurring in the southeast of Brazil up to the coast of Uruguay and northern Argentina. It can occur in high densities in the warmer months, between late spring and early autumn [1, 2].

Although little is known about the biology of this species in studies on the distribution of its size classes along the south coast of Brazil, *O. sambaquiensis* has two well-defined cohorts during the year; one in spring/summer and the other in autumn/winter [3]. The spring/summer cohort has high densities of organisms and coincides with the summer vacation period in the southern hemisphere when it becomes a serious public health problem due to the stings suffered by bathers in Brazil.

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Various studies have been conducted seeking to determine the extent of the outbreaks [1], the associations between these outbreaks, climatic conditions [4, 5] and medical records [6, 7]. Other studies have focused on identifying and characterizing the toxins of the species [8, 9]. The *O. sambaquiensis* toxin presents a new class of cytolytic peptides with myonecrosis activity [9], later confirmed by Knittel *et al.* [10] by the presence of serine proteinase and phospholipase. Bueno *et al.* [11] also highlights that the toxin from this hydrozoa significantly interferes with noradrenergic neurotransmission in smooth muscle.

In the last decade, there have been efforts to understand the processes associated with the nematocyst discharge of jellyfish, which have led to advances in our knowledge of the subject. Several studies have drawn attention to the fact that the process of nematocyst discharge is not solely a mechanical reaction but is principally chemical and is related to Ca^{2+} efflux and the osmotic balance of the internal fluid of

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nematocysts [12, 13]. These discoveries have prompted the development of formulations and lotions with compounds that inhibit nematocyst discharge when in contact with human skin [14, 15]. Originally conceived based on the natural defenses of the clownfish (*Amphiprion*), these formulations have the following characteristics: (a) they are highly hydrophilic, (b) they are based on glycosaminoglycans (mucopolysaccharides) that mimic those in the umbrellas of jellyfish, (c) they contain competitive antagonists bound to amino acids and sugars secreted by the skin, which inhibit the activation of non-selective chemoreceptors, and (d) they contain Ca^{2+} antagonists [14] such as Mg^{2+}, Co^{2+} and La^{3+} [16].

In the case of stings by *O. sambaquiensis*, Mianzan *et al.* [17] demonstrated the capacity of vinegar to prevent the discharge of nematocysts adhered to human skin after contact. However, its application is limited to water when the tentacles remain adhered to some region of the victim's body. In this context and based on the fact that the proteins and carbohydrates in jellyfish can provide a natural defense against stings [3], lyophilized extracts of the umbrella of jellyfish that normally occur along the southern coast of Brazil were investigated in relation to the inhibition of the discharge of nematocysts of *O. sambaquiensis* live tentacles. Moreover, an attempt was made to identify the bioactive ingredient in the umbrella extract in experiments on humans.

2. MATERIALS AND METHODS

2.1. Obtaining Biological Material

The jellyfish capture was carried out by bottom trawls used in artisanal shrimp fishing, being the jellyfish sampled once a month between 2012 to 2015 in the region near the mouth of the river Itajaí-açu (south of Brazil). The identification of the organisms was aided by the work of Morandini *et al.* [18]. After capture, the organisms were carefully packed in seawater coolers and transported to the laboratory for subsequent analysis. In this study, we used only the species with the highest organic content in the umbrellas, according to De Barba *et al.* [3], with the hydrozoa *O. sambaquiensis* and cubozoans *Chiropsalmus quadrumanus* (F. Müller 1859) and *Tamoya haplonema* (F. Müller 1859) being tested.

In the laboratory, the organisms were washed in fresh water, and the umbrellas (exumbrellar tissue) were frozen at -20°C and lyophilized in a LioTop® bench lyophilizer (Model L101, 5 kg capacity) and then triturated. The lyophilized extracts, consisting of 5 to 10 organisms per extract, were subjected to biochemical analyses and tests on humans. Some specimens of *O. sambaquiensis* were treated differently after collection for use in the experiments on humans (experimental sting model).

2.2. Biochemical Analyses

Biochemical analyses were performed only for umbrellas of *C. quadrumanus* because these showed a positive response to the inhibition of nematocyst discharges of *O. sambaquiensis*. The protein content was determined by the Kjeldahl method [19]. The method used to determine the lipid content was extraction in petroleum ether. The dosage of carbohydrates, by total hexose, followed the method proposed by DuBois *et al.* [20]. Ash was determined by burning the dry material in a muffle furnace at 550°C for 2 h. All the analyses were performed in duplicate (carbohydrates) or quadruplicate (protein, lipid and ash contents).

The lyophilized samples of umbrellas of *C. quadrumanus*, obtained at various times of the year, were mixed in equal amounts and homogenized for an average value of their biological activity. Lyophilized extracts of *C. quadrumanus* from summer and winter samples were also prepared, attempting to relate the variation in biochemical composition to the capacity to inhibit nematocyst discharges.

2.3. Experimental Model in Animals and Humans

The species selected for the sting tests was the hydromedusa *O. sambaquiensis* because it contains toxins in its tentacles and is associated with occurrences of stings in the region. The methodology used was adapted from Kimball *et al.* [15] and Tønseth *et al.* [21]. The specimens of *O. sambaquiensis* collected in the trawls were carefully transported to the laboratory in a container with cooled seawater collected from the sampling site to prevent activation of the nematocysts and inactivation of the toxins. The tentacles were removed from the organisms with scissors, stored in 50 mL Falcon-type test tubes with filtered sea water, and kept at 4°C until their use within a period of not more than 24 hours. At the time of the tests, the live tentacles were transferred to Petri dishes containing 0.2 g of tentacles (standardized mass) to be used in the treatment applied to animals and humans.

2.3.1. Animals

Initially, tests were carried out on animals per the guidelines for ethical conduct in the care and use of animals (Committee on Animal Research and Ethics - CARE) of the University of Vale do Itajaí (UNIVALI) under process 012/13. Ten Swiss mice were used (25 to 35 g, 2 to 3 months of age, 50-50% male and female), kept under controlled conditions (25 ± 2°C, light/dark cycle of 12 h, 55 ± 5% air humidity), with water and food *ad libitum*.

The sting model was defined as a segment of 2.0 cm x 2.5 cm from the back skin of animals that had been dehaired 24 h before the beginning of the experiment. The sting was induced, and the animals were anaesthetized with ether to induce envenomation. A sample (0.2 g) of live tentacles, whose viability had been previously tested on humans to confirm their activity, was then applied to the shaved area and left in contact with the skin for 4 min. At the end of this time, the tentacles were carefully removed, and the area was observed for up to 10 min after application. In this assay, we evaluated the feasibility of nematocyst stings by confirming erythema's presence for subsequent test formulations. However, this test was excluded from the study due to a lack of visual response to injury in the skin of the animals (n = 10), obtaining negative results for the presence of skin lesions.

2.3.2. Humans

For testing in humans, the research project was approved by the Research Ethics Committee of UNIVALI under process 684.398-CAAE 31300614.0.0000.0120. All volunteers were informed about the details of the test procedure by an Informed Consent Form (ICF). For the use of humans in the

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trials, the inclusion criteria adopted were: healthy individuals, at least 18 years of age, and people in the community who wished to contribute to the research. Exclusion criteria included sick individuals with a history of bleeding, a local wound, active peptic ulcer with local burning less than 24 h previously, a history of allergic responses and/or previous experience of jellyfish stings (in the environment or through having previously participated in the test), and under 18 and over 60 years old. During the experiments, a doctor was present for possible intervention due to an allergic reaction. However, as noted by Haddad Jr. *et al*. [7], *O. sambaquiensis* stings are not associated with severe effects, and local effects are more common than systemic effects.

The assays were performed on the anterior surface of the forearm of each of the volunteers, in a standardized area of 2.5 x 2.5 cm, for the tests with the different formulations. The treatments were applied 4 min prior to stings with live tentacles of *O. sambaquiensis*. After treatment, 0.2 g of tentacles was applied directly onto the skin in each pre-delimited site, where they remained for 4 min. The tentacles were removed, and the area was observed for 10 minutes. The skin manifestations were recorded as the presence of erythema (change of color or skin color test) classified as (-) without reaction; (+) mild erythema; and (++) erythema and papules, as shown in Fig. (**1**) (modified from Brockow *et al*.) [22]. The pain perceived by the volunteers was also recorded, using the pain scale for erythema with scores ranging from 0 to 10 (0 to 2 for mild pain, 3 to 7 for moderate pain and 8 to 10 for intense pain). In all of the assays, a positive control (without treatment) was included to ensure that the tentacles used maintained their nematocyst sting capacity on the forearms of the volunteers. A control was also conducted with neutral cream to compare the treatments and ensure that the vehicle used in the preparation of the formulations was not interfering with the action of the compounds tested.

2.4. Formulations and Treatments

The assays were grouped into a) lyophilized umbrella samples from different species of jellyfish, b) lyophilized umbrella from *C. quadrumanus* obtained in summer and winter samples, and c) Ca^{2+} antagonists. These compounds were previously incorporated, separately or in combination, into a neutral base of the pharmaceutical cream (neutral cream) of the lipophilic and hydrophilic semi-solid emulsion (details below). The application to specific demarcated sites on the forearms of the volunteers was previously standardized in a total volume of 0.1 mL (160 mg) using a displacement pipette.

2.4.1. Assay 1 - Determination of the Species with Inhibitory Activity against Stings by O. sambaquiensis

As previously mentioned, for the assay, exploratory experiments were carried out to determine whether any jellyfish species had constituents in the umbrella that showed inhibitory activity against the nematocyst sting, following the criteria of higher organic content and abundance, according to De Barba *et al*. [3]. For this purpose, lyophilized extracts of the umbrella of the *Hydromedusa* species *O. sambaquiensis* and the cubomedusae species *C. quadrumanus* and *Tamoya haplonema* were added to the neutral cream at a concentration of 5% (w:w). According to the previously described model, the creams were then used in the assays on humans to test their ability to inhibit stings by nematocysts of *O. sambaquiensis* live tentacles. Positive controls were included, *i.e*., tests without treatment (PC) and with the neutral cream (NC) were also conducted. Eight volunteers participated in these tests, with at least two repetitions for each treatment. Based on the results obtained, the other tests were performed only with the cubozoan *C. quadrumanus* extract.

2.4.2. Assay 2 – Seasonal Variation of the Biochemical Composition of C. quadrumanus Umbrellas

Assays were conducted with lyophilized extract (5% m/m) of *C. quadrumanus* in a neutral cream to observe the inhibition of stings by nematocysts, which could be associated with the seasonal variation of the biochemical composition of the umbrellas.

For this purpose, fifteen volunteers were used, demarcating 4 sting sites, with the following treatments: (a) PC: positive control (without treatment); b) NC: neutral cream; c) Winter: lyophilized extract of *C. quadrumanus* obtained in winter samples; and d) Summer: lyophilized extract of *C. quadrumanus* obtained in summer samples.

Fig. (1). Patterns of skin color. (**a**) no reaction (-); (**b**) weak erythema (+) and (**c**) erythema and papules (++). Modified from Brockow *et al*. (2002). (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

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2.4.3. Assay 3 - Antagonists of the Ca^{2+}

Creams were tested with a calcium channel blocker (verapamil at 5% m/m) [23], with EDTA at 0.35% m/m [24, 25] and the metals lanthanum, cobalt and magnesium in combination (0.35% each, m/m) [16], to determine the possibility of increasing the inhibition of stings by nematocysts of *O. sambaquiensis*, alone or in conjunction with the lyophilized extract of *C. quadrumanus* at 5%. For each treatment, seven volunteers participated, with at least four replicates per treatment.

The treatments were: a) PC: positive control (without treatment); b) NC: neutral cream; c) Verapamil; d) EDTA; e) Verapamil, EDTA and extract: verapamil, EDTA and lyophilized extract of *C. quadrumanus*; and f) Metals and extract: verapamil, EDTA, lyophilized extract of *C. quadrumanus* and metals (lanthanum, cobalt and magnesium).

2.5. Statistical Analysis

For each assay, the mean and standard deviation were estimated for the reported pain (based on the Pain Scale) associated with the controls (PC and NC) and the treatments applied. Analysis of variance (ANOVA) and the a posteriori Tukey test for a 95% significance level ($P < 0.05$) were performed for comparison between the biochemical analyses and between the controls and the treatments for the pain indices. For the classification of skin color, comparisons were made between the treatments and the PC and NC in relation to the occurrence of classes of color manifestation.

3. RESULTS

3.1. Assay 1 – Determining the Species with Biological Activity

In the tests using the lyophilized extracts of jellyfish, the cubozoan *C. quadrumanus* demonstrated the highest capacity to reduce the pain associated with stings and to prevent the reaction of a change in skin color in the volunteers (Fig. **2**). The other cubozoan, *T. haplonema*, showed a slight reduction in pain and change in skin color, but there was no significant difference in relation to the controls. The species responsible for stings, *O. sambaquiensis*, did not demonstrate the capacity to auto-neutralize the stings of its tentacles.

3.2. Seasonal Biochemical Composition of *C. quadrumanus* Umbrellas

The analysis of the umbrellas of *C. quadrumanus* indicated a seasonal variation in the biochemical composition, with higher carbohydrate contents in the summer, while in winter, they presented higher values of proteins (Table **1**). The contents of lipids and ashes did not show significant variations between the analyzed periods.

3.2.1. Assay 2 - Extracts of C. quadrumanus

Both the winter and summer lyophilized extract of *C. quadrumanus* presented lower scores for associated pain, compared with the positive controls and the neutral cream, and for the change of skin color (Fig. **3**). On the other hand, the summer extract showed a higher efficiency in blocking pain and reducing the change in skin color, being significantly different in relation to the positive control. This result indicates that seasonal variations in the composition of an umbrella of *C. quadrumanos* influence the ability to inhibit the discharge of *O. sambaquiensis* nematocysts.

3.2.2. Assay 3 - Antagonists of the Ca^{2+}

In relation to the Ca^{2+} antagonists, only the treatment with the lyophilized extract of *C. quadrumanus* combined with verapamil and EDTA resulted in a reduction in pain and lessened the change in skin color (Fig. **4**). This reduction in pain was greater than that observed in assays 1 and 2 described above. Likewise, a trend (though not significant) was observed toward a reduction in pain and a skin color change for the treatment with EDTA. On the other hand, for the treatment with metals, the results suggest an antagonistic effect against the action of the extract of *C. quadrumanus*.

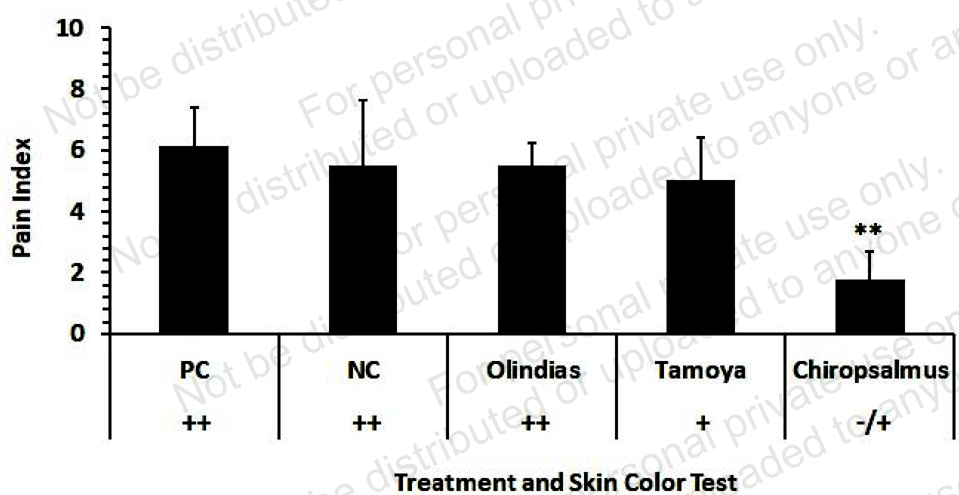

Fig. (2). Average (columns) and standard deviation (lines) for pain and classes of skin color for assay on stings by tentacles of *O. sambaquiensis* on human skin. PC: positive control (without treatment); NC: neutral cream control (vehicle); Olindias: lyophilized extract of *O. sambaquiensis*; Tamoya: lyophilized extract of *T. haplonema*; Chiropsalmus: lyophilized extract of *C. quadrumanus*. ** Indicates significant difference ($P < 0.05$) in relation to PC and NC.

4. DISCUSSION

O. sambaquiensis is the most abundant species of *Hydromedusa* on the south coast of Brazil, and it presents a public health problem since it is the main organism responsible for stings suffered by bathers and fishermen [4, 5] and possibly related to oceanographic conditions [26]. This sting is caused by the discharge of nematocysts, which contain a mixture of toxins whose principal action is to activate or block the ion channels [8]. According to Haddad *et al.* [7], stings by *O. sambaquiensis* result in local manifestations of pain, erythema and edema, with oval and round patches on the skin and sometimes marks left by small tentacles less than 20 cm long.

Table 1. Average percentages (%) and standard deviation of lipids, carbohydrates, proteins and ash of the lyophilized extract of *C. quadrumanus* of the organisms obtained in summer and in winter.

-	Summer	Winter	Sig.
Lipids	2.8 ± 0.007	2.7 ± 0.005	-
Carbohydrates	7.2 ± 0.4	4.7 ± 0.2	*
Proteins	18.5 ± 0.01	23.6 ± 0.02	*
Ash	51.9 ± 7.1	63.1 ± 1.02	-

Note: *Represents statistical differences between season according ANOVA.

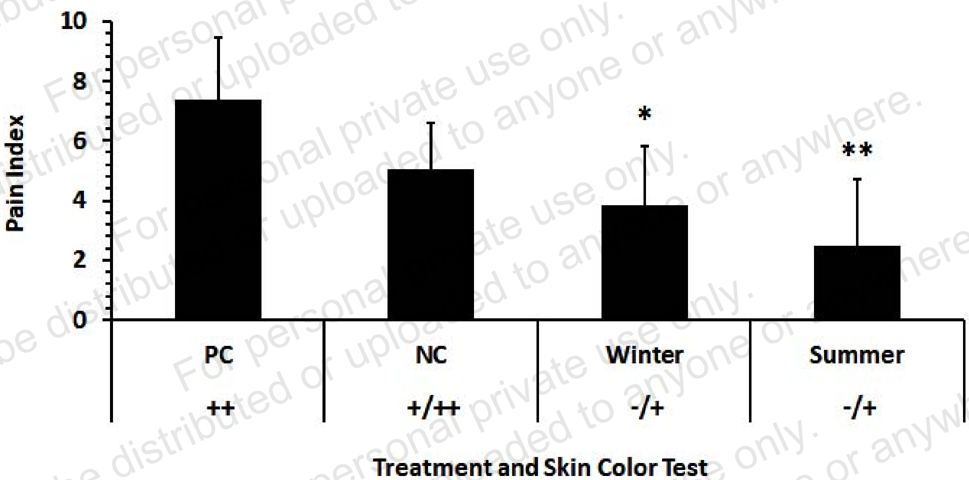

Fig. (3). Average (columns) and standard deviation (lines) of the pain and classes of skin color for the assay on stings by tentacles of *O. sambaquiensis* on human skin. PC: positive control (without treatment); NC: neutral cream control (vehicle); Winter: lyophilized extract of *C. quadrumanus* of the winter; Summer: lyophilized extract of *C. quadrumanus* of the summer. * Indicates a significant difference ($P < 0.05$) in relation to PC and ** indicates significant difference ($P < 0.05$) in relation to PC and NC.

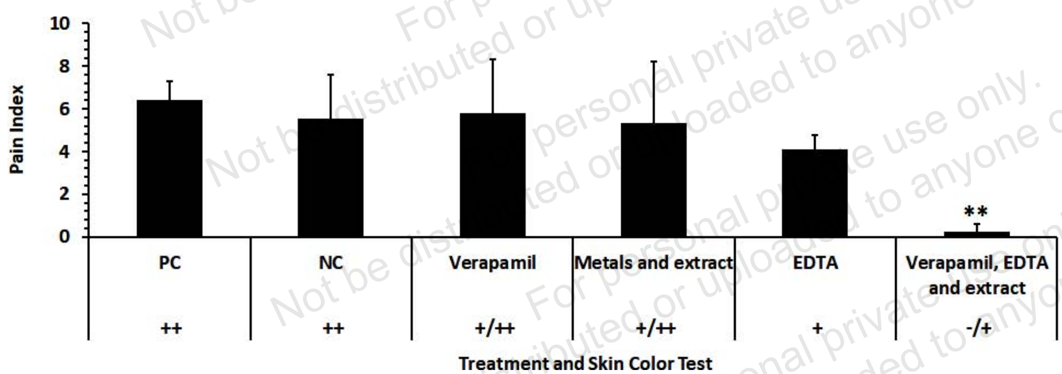

Fig. (4). Average values (columns) and standard deviation (lines) of pain and classes of skin color for the assay on stings by tentacles of *O. sambaquiensis* on human skin. PC: positive control (without treatment); NC: control of neutral cream (vehicle); Verapamil: neutral cream with Verapamil; Metals and extract: neutral cream with verapamil, EDTA, lyophilized extract of C. quadrumanus and metals (lanthanum, cobalt and magnesium); EDTA: neutral cream with EDTA; and Verapamil, EDTA and extract: neutral cream with verapamil, EDTA and lyophilized extract of *C. quadrumanus*. ** Indicates significant difference ($P < 0.05$) in relation to PC and NC.

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Although the hydrozoan *O. sambaquiensis* and the cubozoans *C. quadrumanus* and *T. haplonema* belong to the group of jellyfish with a higher organic content comprised of carbohydrates and proteins [3], only the umbrella of *C. quadrumanus* showed biological activity. It is known that the cubozoans differ from other cnidarians in terms of body structure, habitat, behavior, nervous system [27], and sensory organs [28]. They are active swimmers and predators, unlike *Hydromedusae* and *Scyphomedusae*, which demonstrate a more planktonic lifestyle [29]. However, the absence of biological activity of the extract of *T. haplonema*, also a cubozoa, may indicate specific differences within the group and in the ecological niche and responses to environmental stimuli.

The effect of seasonality observed for the constituents of the organic matter of *C. quadrumanus* indicated higher protein content in winter and carbohydrates in summer. Lucas [30] points out that protein is used as an energy reserve and is more abundant in the gonads than in the umbrella of the cnidarians. Furthermore, the higher content of carbohydrates in the summer could be interpreted as increased food availability since protein is used for reproduction in the summer. According to De Barba *et al.* [3], *Rhacostoma atlanticum* (Hydrozoa) presents seasonality in the biochemical composition in the same study area and is also associated with the reproductive cycle of the species.

In any case, the relationship between the higher carbohydrate content of the umbrella and the greater efficiency in inhibiting the process of discharging *O. sambaquiensis* nematocysts has already been highlighted by Boulware [14] and Kimball *et al.* [15] for other jellyfish. According to these authors, glycosaminoglycans (mucopolysaccharides) could mimic the umbrellas of jellyfish since this biomolecule would be used as a signal of recognition of the species itself in the hunting process.

Since the work of Lubbock [31], carbohydrates have been highlighted as a biomolecule that would not present a stimulus to the discharge of nematocysts. Later, Lubbock [32] confirmed that clown fish mucus (which naturally inhibits the release of nematocysts by jellyfish) is rich in polysaccharides, while Throrington and Hessinger [33] suggested that one of the nematocysts discharge chemoreceptors may be associated with N-acetylated sugar. In jellyfish, polysaccharides are associated with proteins and can retain water, giving consistency to these gelatinous organisms. For this reason, jellyfish are often cited as an important source of hyaluronic acid used in the treatment of arthritis [34].

Tests using formulations with Ca^{2+} antagonists were carried out to assess whether their addition to the formulation of the *C. quadrumanus* extract could promote an increase in the effect of inhibiting stings caused by nematocyst discharge. A significant increase in the effect was observed with the mixture containing verapamil, EDTA and the extract of *C. quadrumanus* in relation to a reduction in pain and skin color change. This increase may be associated with EDTA and its action on the Ca^{2+}, which may inhibit the formation of the pressure gradient inside the nematocysts since the efflux of Ca^{2+} is the chemical discharge mechanism of the nematocysts [12]. The latter authors also demonstrated verapamil's selective efficiency in inhibiting nematocyst discharge in studies with sea anemones. The result of the action of EDTA in the mixture indirectly confirmed the effect of the extract on the action of the ion channels and, consequently, on the stimulation of nematocyst discharge.

The same action of EDTA may also have been responsible for the failure to inhibit pain observed for the mixture containing metals (lanthanum, cobalt and magnesium) and the lyophilized extract of *C. quadrumanus*, together with verapamil. Several authors highlighted that these metals could inhibit nematocyst discharge [14-16, 35] but may be chelated by EDTA [36]. However, the inability to reduce pain observed in the treatment using metals associated with the extract of *C. quadrumanus* indicates an antagonistic effect of the mixture, including inhibition of the action of the cubozoa extract. It could be that the complexes formed to act on the molecule responsible for the inhibitory action indicate that this mixture would not be suitable for potentiating the effect of the extract.

Previous studies on stings by *O. sambaquiensis* [4, 5] have confirmed the effectiveness of using vinegar and cold compresses for relieving symptoms resulting from stings. However, Mianzan *et al.* [17] argues that vinegar prevents the discharge of intact nematocysts adhered to the victim's skin after contact with the water but does not promote pain relief after stings, in contrast to the results obtained by Fenner [37] for other species of jellyfish. On the other hand, Haddad Jr. and Barreiros [38] attribute pain relief from the stings to using cold compresses and the anesthetic effect of lowering the temperature. According to Cegolon *et al.* [39], there are many proposals for treating stings by jellyfish, but so far, the evidence is weak, highlighting the need for further studies on this subject.

CONCLUSION

Tests on a lyophilized extract obtained from *C. quadrumanus* caught in summer and winter suggested that the biomolecule responsible for the biological activity in inhibiting stings caused by nematocyst discharge of the *O. sambaquiensis* is a carbohydrate since this biomolecule would be used as a signal of recognition of the species.

AUTHOR'S CONTRIBUTIONS

Resgalla Jr C – Study concept or design

De Barba FM – Data collection

Bazi CC – Data collection

Pessatti ML – Data analysis or interpretation

LIST OF ABBREVIATIONS

ICF	=	Informed Consent Form
NC	=	Neutral Cream
PC	=	Positive Controls

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

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The research project was approved by the Research Ethics Committee of UNIVALI under process 684.398-CAAE 31300614.0.0000.0120.

HUMAN AND ANIMAL RIGHTS

Tests were carried out on animals per the guidelines for ethical conduct in the care and use of animals (Committee on Animal Research and Ethics - CARE) of the University of Vale do Itajaí (UNIVALI) under process 012/13.

All procedures performed in studies involving human participants were per the ethical standards of institutional and/or research committees and with the 1975 Declaration of Helsinki, as revised in 2013.

CONSENT FOR PUBLICATION

All volunteers were informed about the details of the test procedure by an Informed Consent Form (ICF).

AVAILABILITY OF DATA AND MATERIAL

The data and supportive information are available within the article.

FUNDING

This study was funded by National Council for Scientific and Technological Development (CNPq) (Grant Nos. 484467/2012-3 and 406583/2012-9).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors like to thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for the research grant and the National Council for Scientific and Technological Development (CNPq) for resources granted to the research project.

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