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# ISOLATION, CHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITIES OF CELL WALL POLYSACCHARIDES OF *Laurencia microcladia* (RHODOMELACEAE, CERAMIALES)

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## ABSTRACT

Cell wall polysaccharides of the red algae *Laurencia microcladia* were isolated by alkaline treatment (KOH 1M, 10 mg $NaBH_4$, room temperature), yielding two sub-fractions by neutralization (AcOH, pH=5.2) and EtOH precipitation, respectively **A** and **B**. Paper electrophoresis revealed the existence of only one polysaccharide in each fraction. FT-IR spectra of both polysaccharides showed signals for COH (OH: 3400-3300 $cm^-$, CO: 1260-1000 $cm^-$) and CHC (1300 – 1000 $cm^-$) typical of cell wall algal polymers. An N-glyco derivative (1640 – 1560 $cm^-$) and a low acidic content (0.34 mol %) were structural features found, while proteins were not detected. Antimitotic activity was observed on *Lytechinus variegatus* (green bur) larvae, with chronic values of 38.18 mg/mL and 267.99 mg/mL for polysaccharides **A** and **B**, respectively**.** No bradykinin or acetylcholine antagonistic effect was observed when polysaccharides were assayed *in vitro* in guinea-pig ileum. Preliminary results pointed to neurotoxic effect of these macromolecules (5mg/100 mL) since asymmetry of the neural tube in chick embryo was also found.

**Keywords**: *Laurencia microcladia*, cell wall polysaccharides, *Lytechinus variegatus,* angiogenesis, antimitotic activity.

## ISOLAMENTO, CARACTERIZAÇÃO E ATIVIDADE BIOLÓGICA DE POLISACARÍDEOS DA PAREDE CELULAR DE *LAURENCIA MICROCLADIA* (RHODOMELACEAE, CERAMIALES)

## RESUMO

Polissacarídeos de parede celular da alga vermelha Laurencia microcladia foram isolados por tratamento alcalino (KOH 1M, 10 mg $NaBH_4$, temperatura ambiente), produzindo duas sub-frações por neutralização (AcOH, pH=5.2) e precipitação com EtOH, respectivamente A e B. Eletroforese em papel revelou a existência de apenas um polissacarídeo por fração. O espectro FT-IR de ambos polissacarídeos mostraram indícios de COH (OH: 3400-3300 $cm^-$, CO: 1260-1000 $cm^-$) e de CHC (1300 – 1000 $cm^-$), típicos em polímeros de parede celular de algas. Um derivado N-glicosil (1640 – 1560 $cm^-$) e baixo conteúdo ácido (0.34 mol%) são traços estruturais encontrados, ao passo **que proteínas não foram encontradas. A atividade antimitótica foi observada**

em larvas de Lytechinus variegatus (ouriço verde), com valores crônicos de 38.18 mg/mL e 267.99 mg/mL para os polissacarídeos A e B, respectivamente. Não foram observados efeitos antagonistas para bradicinina e acetilcolina para os polissacarídeos em testes in vitro com íleo de porco-da-índia. Resultados preliminares apontaram efeito neurotóxico dessas macromoléculas (5mg/100 mL) além de assimetria do tubo neural em embriões de galinha.

**Palavras Chave**: Laurencia microcladia, polissacarídeos de parede celular, Lytechinus variegatus, angiogênese, atividade antimitótica.

## INTRODUCTION

Over the last years, an increasing number of studies have been performed showing the bioactive potential of compounds produced by marine organisms. As example, several algae species (*Fucus* spp., *Sargassum* spp., e.g.) are sources of secondary metabolites or macromolecules (i.e. polysaccharides, glycoproteins) with antitumoral, antiviral, or immune-stimulant activity. In some cases, the referred to compounds are currently under pre-clinical or clinical investigation. Further, taking into account both genetic and chemical variability found in marine organisms, one might bear in mind the potential of the marine ecosystem as source of new pharmaceuticals.

The present study was carried out focusing on biological activities and preliminary elucidation of the chemical structure of cell wall polysaccharides of the red algae *Laurencia microcladia.*

## MATERIAL AND METHODS

*Biomass collection:*

The biomass of the red algae *Laurencia microcladia* was originated from the Armação do Itapocoroy beach (Santa Catarina State, Brazil) and collected in January-February/1998. About 815 g (fresh weight) were pre-cleaned by removing residuals of distinct nature and washed under tap water. The excess of water was drained off and small fragments of plant and animal tissues were removed out. About 66,5 g (fresh weight) of the selected biomass were collected and washed with 3 volumes of distilled/deionized water, repeatedly, dried (65°C) till constant weight and stored at –20°C.

*Cell wall polysaccharides isolation:*

Algae biomass (66,5 g, dry weight) was sequentially treated with 3 volumes of toluene and EtOH/3 days each and the residue freeze-dried. The dry biomass was shaken in 3 volumes of an aqueous 1.5% SDS (sodium dodecyl sulfate) solution (pH=5.0)/12 h agitation/4°C centrifuged (10.000 rpm/15 min/4°C), and the cytoplasm release in the supernatant (e.g. nucleic acids and proteins) was monitored through spectrophotometry, at 260 and 280 nm, respectively. The residual cell wall designed as SDS-CW (cell wall after detergent washing) was washed with 3 vol. water, treated three times with 90% DMSO (dimethyl sulfoxide)/12 h agitation/25° C and centrifuged (10.000 rpm/15 min/25°C). The extraction of the cell wall polysaccharides was then carried out with 4M KOH/12 h/25° C in a rotatory shaker, with $NaBH_4$ (10mg) added to the medium (Selvendran et al., 1985). After the treatment the suspension was centrifuged (10.000 rpm/15 min/4° C), the supernatant collected, treated with AcOH till pH=5.2 and centrifuged. The polysaccharide **A** fraction was obtained as precipitate and the supernatant was treated with excess of EtOH (3 volumes), yielding the polysaccharide **B** fraction. Residual potassium acetate was washed off through dialysis against distilled, with further lyophilizing and storage of the polysaccharides at – 20°C till analysis.

*Paper electrophoresis and infra-red spectrophotometry:*

Electrophoresis of the polysaccharides was performed on a cellulose acetate paper (2,5 x 14cm - Cellogel® Chemetron, Milan) as follows: 0.1M zinc acetate buffer (pH 6.6), 200V, 1hour. The detection was carried out by staining with aqueous 0.5% Toluidine Blue solution (Takashi et al., 1989). Infrared spectra were recorded for a KBr pellet of a test sample (3 mg, accurately weighed) with a BOMEM Michelson infrared spectrophotometer, operating with a laser frequency at 15799.7 $cm^{-1}$. The recorded data were analyzed using Win-Bomem (v. 3.01C) software.

*Biological activities assay:*

*Antimitotic activity*: The antimitotic assays were performed monitoring the larval development of previously *in vitro* fertilized eggs of *Lytechinus variegatus* (green bur), as previously described (Pessatti et al., 1998; CETESB, 1992). Briefly, 100 ml of a solution containing the fertilized eggs was added to 10 ml of polysaccharides **A** (6,725; 13,5; 27 and 54 mg/ml) or **B** (47,38; 94,75; 189,5 and 379 mg/ml) solutions, incubated for 36 h and treated with formaldehyde (4%). For each treatment, 6 replications were used and data of chronic toxicity, observed and non-observed concentration effects were recorded, by monitoring larval development through optical microscopy (Nippon Labphot, Japan). For purpose of control, sterile sea-water was used. Statistical analysis considered a previous data normalization, followed by Dunnetts ANOVA.

*Anti-BK and Ach* : Ileal strips (10-20mm long) were obtained from guinea-pigs (300-500g) of either sex and taken from the portion situated 10 to 30 cm proximal to the ileo-caecal junction. Preparations were set up for recording of isotonic contractions in 5 ml jacket organ baths containing Krebs Henseleit solution at 37°C continuously bubbled with air under 1g of tension (Calixto et al., 1984). After an equilibration period of 60 min, cumulative concentration-response curves for each agonist were obtained at 30 min intervals. The curves to bradykinin (BK) and acetylcholine (*Ach*) were obtained in the presence or absence of atropine (2.5 uM) or enalapril (kinase II inhibitor - 1uM). After obtaining concentration-response curve, different concentration (50 and 100 ug/ml) of the polysaccharides (**A-**LP4A and **B-**LP4B) were added to the bathing solution, 20 min before constructing new curves to the agonist. The mean maximal response obtained from the first two cumulative concentration-response curves was taken as 100% response value. In separate sets of experiments, in order to correct for spontaneous and/or vehicle-induced desensitization, control experiments for Bk and *Ach* were performed in the presence of the corresponding concentration of absolute ethanol.

*Chick embryo development*: The effects of the polysaccharides on the chick embryo development was performed by using fertilized eggs of *Gallus domesticus* kindly furnished by Macedo & Koerich Co. The eggs were incubated for 4 days as previously described (Mendes, 1994), and elapsed 24h from the beginning of the incubation, 10ml or 20ml* of a polysaccharide **A** or **B** solution (5g%-p/v) were injected into: area pellucida (**IAP test**), by mean of an opening in the egg shell ($\varnothing \cong$ 15mm); yolk sac (**IYS test**)* through an small opening ($\varnothing \cong$ 1 mm) on the apices of the yellow egg; or air chamber (**IAC test**). Manipulation was controlled incubating intact, or injected eggs with equal volume of sterile *Milli Q* water. After the injections, the openings were closed with adhesive tape and completed the incubation. In each experiment, 6 embryos were analyzed in respect to external morphology [morphogenesis level] (Eyal-Giladi, 1991; Bowden et al., 1983; Christ & Ordahl, 1995), angiogenesis level, total length and weight of the embryos and egg weight. Statistical analysis of the recorded data was performed

by calculating one-way ANOVA and Tukey test, using Prism Graph software (v 2.0).

## RESULTS AND DISCUSSION

The paper electrophoresis revealed the existence of only one polysaccharide in each fraction. FT-IR spectra of both polysaccharides showed signals for COH (OH: 3400-3300 $cm^{-}$, CO: 1260-1000 $cm^{-}$) and CHC (1300-1000 $cm^{-}$) typical of that macromolecules. An N-glyco derivative (1640-1560 $cm^{-}$) and a low acidic content (0.34 mol %) were found as structural features, while proteins were not detected. Antimitotic activity was observed on *L. variegatus* larvae, with chronic values of 38.18 mg/ml and 267.99 mg/ml for polysaccharides **A** and **B**, respectively (Fig. 1). No bradykinin or acetylcholine antagonistic effect was observed when polysaccharides were *in vitro* assayed in guinea-pig ileum.

Preliminary results pointed to neurotoxic effect of these macromolecules characterized for asymmetry of the neural tube in chick embryo. The polysaccharide **A**, administrated into area pellucida (**IAP** test) or air chamber (**IAC** test – Fig. 2), caused a 50% reduction in the embryo length. Concomitantly, the polysaccharide **B** induced an alteration of three orders of magnitude (**IAP** test) in the morphogenetic level of the embryos. Further, these macromolecules negatively affected blood vessel development (angiogenesis), regardless of the way of injection. The highest values were found for **IAP** test with alteration of the vascular network about 200% (polysaccharide **A**) and 250% (polysaccharide **B**). In fact, the data have shown a potential toxic effect on chick embryo development, mostly as compared to fucans, extracted from *Fucus vesiculosus* and commercially available for human emaciation regimen, or from *Ecklonia kurome* that have been found to be potent anticoagulant and cytotoxic macromolecules against several tumor cell lines and viruses (Nishino et al., 1989; Nishino & Nagumo, 1992). These findings prompted us to perform further analysis of the chemical structure of the polysaccharides and the results will be published elsewhere.

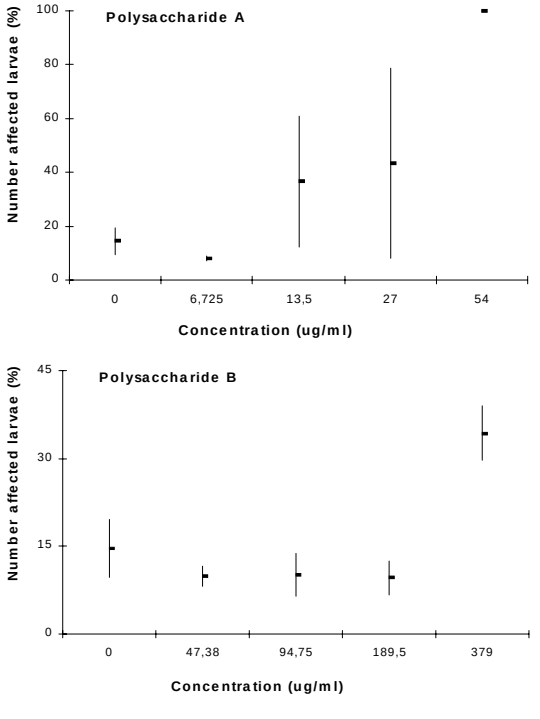


Figure 1. Effect of the *Laurencia microcladia* cell wall polysaccharides **A** and **B** on the larvae development of green bur (*L. variegatus*). The data were recorded after a 36h-incubation-time for the treatments and indicate chronic values of the toxic effect (mean ± standard deviation, $p < 0.05$) of the polysaccharides on the mitotic cycle along the embryo development.

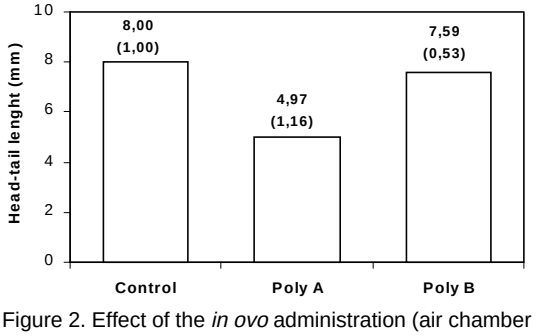


Figure 2. Effect of the *in ovo* administration (air chamber – IAC test) of *L. microcladia* cell wall polysaccharides **A** (Poly-A) and **B** (Poly-B), both at 5mg/100 mL, on the embryo's head-tail length [mean (standard deviation)], after 96 hour incubation.

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