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BIOLOGY

Inhibiting effect of cholesterol isolated from marine red seaweed *Plocamium brasiliense* in the Eastern Amazon Region, Brazil

Rainiomar Raimundo Fonseca[1] Carlos Jose Brito Ramos[2] Antônio Pedro Souza Filho[3] Valeria Laneuville Teixeira[2,4*]

[1]Universidade do Estado do Amazonas (UEA), Parintins, AM, Brasil.
[2]Universidade Federal Fluminense (UFF), Niterói, RJ, Brasil.
[3]EMBRAPA, Amazônia Oriental/CPATU, Belém, PA, Brasil.
[4]Universidade Federal do Estado do Rio de Janeiro (UNIRIO), 22290-240, Rio de Janeiro, RJ, Brasil. E-mail: valerialaneuville@gmail.com. *Corresponding author.

ABSTRACT: In general, allelopathy can be defined as a biological process by which an organism produces one or more metabolites that can change the germination, growth, survival, and reproduction of other organisms, and thus influencing the stability of agroecosystems. Cholesterol is a very abundant sterol isolated from the marine red alga *Plocamium brasiliense* that has been studied regarding its potential inhibitory effects on seed germination, radicle elongation, and hypocotyl development of the weeds *Mimosa pudica* (malicia) and *Senna obtusifolia* (mata-pasto). Cholesterol was isolated from a hexane extract by chromatographic methods. Over a 15-day period, germination bioassays were performed at 25 ºC with a 12-h photoperiod. Radicle elongation and hypocotyl development were assayed at 25 ºC with a 24-h photoperiod. Later, Petri dishes 9.0 cm in diameter were coated with filter paper, and 25 seeds were placed in a germination chamber. Six pre-germinated seeds were placed in the Petri dish for 2-3 days. After 10 days, radicle and hypocotyl extensions were measured, and the inhibitory potential of cholesterol was assessed at 5, 10, 15, and 20 ppm. In both herbs, M. pudica and *S. obtusifolia*, they were significantly affected by the action of cholesterol, achieving higher percentages of inhibition in seed germination (50% and 33%, respectively), radical germination (68% and 60%, respectively), and hypocotyl development (66% and 55%, respectively). The inhibition effects were dose-dependent in all experiments, having more pronounced allelopathic effects at 20ppm.
Key words: phytotoxins, *Mimosa pudica, Senna obtusiloba,* marine sterol.

Efeito inibidor do colesterol isolado da alga vermelha marinha *Plocamium brasiliense* na Amazônia Oriental, Brasil

RESUMO: Em geral, alelopatia pode ser definido como o processo biológico no qual um organismo produz metabolitos que alteram a germinação, o crescimento, a sobrevivência e a reprodução de outros organismos, influenciando a estabilidade de agroecossistemas. O colesterol, o mais abundante esterol isolado da alga vermelha marinha *Plocamium brasiliense*, foi estudado quanto ao seu potencial efeito inibitório na germinação de sementes, alongamento das radículas e desenvolvimento do hipocótilo das ervas daninhas *Mimosa pudica* (malicia) e *Senna obtusifolia* (mata-pasto). Colesterol foi isolado do extrato em Hexano por métodos cromatográficos. Durante 15 dias, os bioensaios de germinação foram realizados a 25 ºC e fotoperíodo de 12 horas, enquanto os bioensaio de alongamento da radícula e do hipocótilo foram realizados a 25 ºC e fotoperíodo de 24 horas. Posteriormente, placas de Petri de 9,0 cm de diâmetro foram revestidas de papelfiltro, e 25 sementes foram mantidas em câmaras de germinação, enquanto seis sementes pré-germinadas foram postas em placas de Petri por 2-3 dias. Após dez dias, a extensão da radícula e do hipocótilo foi medida. O potencial inibitório do colesterol foi avaliado a 5, 10, 15 e 20 ppm. Em ambas as ervas *M. pudica* e *S. obtusifolia,* foram afetadas significativamente pela ação do colesterol alcançando maiores percentuais de inibição na germinação das sementes (50% e 33%, respectivamente), alongamento da radícula (68% e 60%, respectivamente) e desenvolvimento do hipocótilo (66% e 55%, respectivamente). Os efeitos inibitórios foram dose-dependentes em todos os experimentos, tendo efeitos alelopaticos mais acentuados a 20 ppm.
Palavras-chave: fitotoxinas, *Mimosa pudica, Senna obtusiloba,* esteróis marinhos.

INTRODUCTION

Brazil has a coast of more than 7300 km^2 with great biodiversity and potential industrial use of its chemical components especially the so-called natural products or secondary or special metabolites. However, much more needs to be studied regarding natural products of marine organisms on the Brazilian coast.

Here, we studied natural products from seaweed with various biological activities, e.g., antiviral, anti-leismanioses, etc. (AMAYA-GARCÍA et al., 2021). We have particularly studied the inhibitory potential of extracts, fractions, and isolated products of seaweed on pasture weeds (FONSECA et al., 2012; RAMOS et al., 2019).

We previously showed that the acetone extract has fractions of different polarities, and a mixture of two diterpenes pachydictyol A and

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isopachydictyol A was obtained from the brown alga *Dictyota menstrualis* (Hoyt) Schnetter, Hörning & Weber-Peukert (FONSECA et al., 2013). These fractions were evaluated on their inhibitory effects on seed germination and on the elongation of the radicle and hypocotyl of pasture weeds *Mimosa pudica* and *Senna obtusiloba* in the Brazilian Amazonia.

In a second study, two lipophilic extracts and an isolated natural product (the meroditerpenoid atomaric acid) were obtained from the marine brown alga *Stypopodium zonale* (J.V. Lamouroux) Papenfuss to identify and characterize their potential inhibitory effects on seed germination and on the elongation of the radicle and hypocotyl of the pasture weeds *Mimosa pudica* and *Senna obtusiloba*. The extracts were active, and the atomaric acid showed particular potential inhibitory with mean values of 59% for *M. pudica* and 49% for *S. obtusifolia* (RAMOS et al., 2019).

In another study, the authors demonstrated the chemical components of the extracts of the red seaweed *Plocamium brasiliense* (Greville) M. Howe & W.R. Taylor (FERREIRA et al., 2010; VASCONCELOS et al., 2010). The authors suggested that the inhibitory effects could be due to the presence of halogenated monoterpenes. These inhibitory effects included reduced seed germination and reduced elongation of the radicle and hypocotyl of the pasture weeds *M. pudica* and *S. obtusiloba*. Here, we continue these studies of benthic seaweed including an evaluation of the inhibitory effects of cholesterol isolated from the red algae *Plocamium brasiliense* and its impact on pasture weed development.

MATERIALS AND METHODS

Plant material

Specimens of the macroalgae *P. brasiliense* were collected during a low tide period (0.1 m) in the sublittoral of Enseada do Forno beach in the municipality of Armação de Búzios on the blue coast located north of the state of Rio de Janeiro (22045'S and 41052' W) using a free diving technique at depths of 50 cm to 3.5 m. After collection, the algae were washed with seawater and screened for the removal of sediment and associated organisms. Samples were then transported to the Laboratory of Natural Products of Marine Algae (ALGAMAR) in the Department of Marine Biology, Universidade Federal Fluminense - UFF /RJ.

Isolation and chemical identification

The air-dried material alga (292,5 g) extracted successively with n-hexane (3 x 1.5l) at room temperature (± 30 ºC), for 24 h. The three crude extracts *P. brasiliense* were combined and the solvents were evaporated under pressure in a rotatory evaporator, with a water bath below 40 ºC, yielding a red residue (EBPBHEX; 1.37 g). Preliminary studies of the extract obtained from *P. brasiliense* were carried out by thin layer chromatography (CCD) using commercial chromatographic plates of aluminum foil with silica gel 60 F254 (Merck). After inspection in a darkroom under white and ultraviolet light at wavelengths of 254 and 365 nm, the plates were revealed by spraying inan exhaust chamber with a ceric sulfate solution [$Ce(SO_4)_2.5H_2O$] at 2% in sulfuric acid (H_2SO_4); the samples were subsequently heated but not above 100 ºC.

Chromatography was used to isolate the chemical constituents in the n-hexane extract (liquid-solid type (adsorption)). Merck silica gel (70 - 230 mesh) was used as a solid phase for EBPBHEX in a glass column (70 cm long and 4 cm in diameter with 29 cm of adsorbent height). About 500 mg of EBPBHEX was absorbed into the silica gel and applied to the top of the column. In this first fractionation, 153 fractions of 10 ml were obtained up to F97, and 20 ml from F98-F153. In these analyses, it was possible to confirm the presence of several metabolites including cholesterol (fractions F112 to F117). Cholesterol (Figure 1) was identified by spectroscopic analyses of 1H and ^{13}C NMR and compared with data from the literature. The chemical structure of cholesterol isolated from *P. brasiliense* (IUPAC: 3β-cholest-5-en3-ol) was confirmed by its molecular weight (386.65 g/mol). NMR used a Varian VNMRS 500 MHz – Magneto - 11.75 Tesla (500 MHz for the 1 H frequency) with an opening of 54 mm. The scanner was ultra- shielded and equipped with an anti-vibration system (NMR from LAReMN from IQ-UFF).

Allelopathic Bioassay

An allelopathic bioassay was developed in a germination chamber with a constant temperature

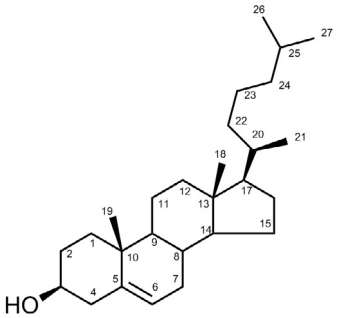

Figure 1 - Compounds of secondary metabolism called cholesterol obtained from the marine macroalgae *P. brasiliense*.

of 25 ºC and a photoperiod of 12 h. Seed germination was monitored for 15 days with daily counts and elimination of germinated seeds. The germinated seeds were defined as those with root extension equal to or greater than 2.00 mm (SOUZA FILHO et al., 2010). The 9.0 cm diameter Petri dishes were lined with qualitative filter paper. Each received 25 seeds constituting an experimental plot.

The plant development occurred under constant temperature conditions of 25 ºC with a 24 h photoperiod of continuous light in a germination chamber with cold white fluorescent lamps and a luminous flux of 10 $\mu mol.m^{-2}.s^{-1}$.

Six pre-germinated seeds were added to each and monitored for approximately three days. The length of the radicle and hypocotyl was measured after ten days of growth. All Petri dishes received 3.0 mL of solution containing cholesterol (test solution). After evaporation of the ethyl ether solvent, an equivalent volume of distilled water was added. In the control, the metabolite was added only once (at the beginning of the experiment) and from then on, only distilled water was added whenever necessary. Both germination and radicle and hypocotyl developments were made and evaluated in triplicate according to the literature (SOUZA FILHO et al., 2010; FONSECA et al., 2012). The solutions containing cholesterol were tested at concentrations of 5, 10, 15, and 20 ppm.

Mimosa pudica (malicia) and *Senna obtusiloba* (mata-pasto) are common weeds and were used to study allelopathic effects. The seeds of these two species were collected in cultivated pasture areas in the municipality of Terra Alta, State of Pará and were cleaned and treated to break the dormancy. They were then immersed in sulfuric acid for 20 min as specified by SOUZA FILHO et al. (1998).

To calculate the LD_{50}, the empirical points of the inhibition/concentration relationship were considered, where they were adjusted by the linear equation Inhibition = A, Concentration = B, considering the value when 50% of the individuals tested in each period suffer inhibition.

The experimental design and statistical analysis for all bioassays were distilled in a hierarchical model with two factors. The data were transformed into a completely randomized type with four replicates using arcsine water $\sqrt{x}$ for a normal distribution. The values were then submitted to analysis of variance via a F test. The means were compared by regression analysis when the treatment effects showed a significant difference ($P < 0.01$). SAS software was used for analysis (SAS, 1998).

RESULTS AND DISCUSSION

We obtained 292.5 g of dry mass of seaweed, which generated 31.35 g of extract mass in hexane. From the crude extract, we used 500 mg for fractionation, generating a mass of 143.4 mg or 28.68% of the fraction of the extract. Unfortunately, we were unable to isolate halogenated monoterpenes in sufficient quantity for the tests. They are natural products in large numbers, but in amounts less than 5 µg. The inhibition potential of the isolated metabolite (3β-cholest-5-en3-ol) of *P. brasiliense* was evaluated on seed germination, radicle development, and the hypocotyl of two weeds in the Amazon region. The fractionation of the n-hexane extract, EBPBHEX, occurred in chromatographic column in the fractions F113-F120. Those provided cholesterol (Figure 1), a white, yellow solid substance with a pleasant taste and odor. The chemical isolation of cholesterol obtained from hexane extract was identified via ^{1}H and ^{13}C NMR.

The F113 fraction showed a cholesterol signature: a broad duplet at 5.34 ppm (J = 4.9 Hz) corresponding to the unsaturated system (H-6), another signal at 3.54 ppm related to carbinolic hydrogen, and signals from 0.6 to 2.4 ppm referring to the five existing methyl groups. This information-together with the 13C experiments, HSQC, and the literature-suggest that the isolated metabolite is 3β-cholest-5-en3-ol. Knowing the effects of allelopathy and its inter- and intra-specific interactions are important to any ecosystem. According to REZENDE et al. (2003), invasive plants can damage pasture and thus livestock.

The novel cholesterol retarded seed germination of invasive plant species in a dose-dependent manner. Development of the radicle and hypocotyl of the seeds was also impaired with stronger effects at 20 ppm.

Regardless of the receptor species, the allelopathic effects of cholesterol were more intense on the development of the radicle of the seeds with inhibition higher than 50% at 20 ppm: These were 68% for *M. pudica* radicle and 60% for the development of *S. obtusifolia* radicle (Figure 2). Variations in the intensity of allelopathic effects on germination of the receptor species were observed indicating that the germination sensitivity varied: *S. obtusiloba* < *M. pudica*. The phytotoxicity of this allelochemical was at least 50% inhibition, which makes it useful in practical applications (DUDAI et al., 1999). *M. pudica* (malice) was the most sensitive (F113) (Figures 2A-C). INOUVE et al. (2010) also showed inhibition using two sterols with a cholesterol-like skeleton. The experiments with F113 showed higher percentages of

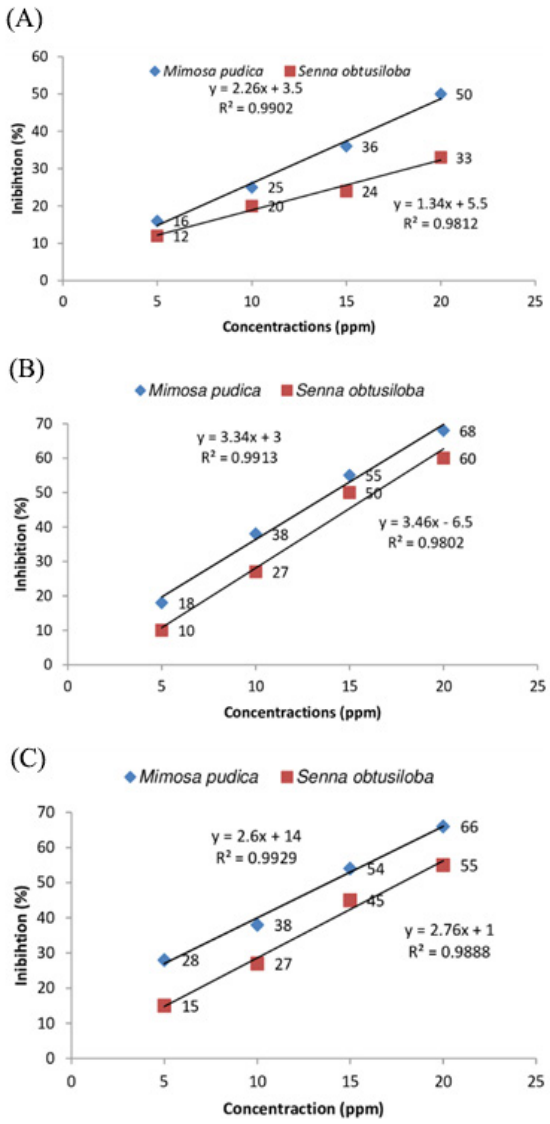

Figure 2 - Potential allelopathic effects of cholesterol (F113) (a) on seed germination, (b) on the development of radicle of seeds, and (c) on the development of hypocotyl of seeds. Data are expressed in percentage of inhibition in relation to the control treatment (distilled water).

inhibition compared to *M. pudica* (malicia) species in the germination, radicle development, and hypocotyl assays: 50%, 68%, and 66%, respectively (at 20 ppm). For *S. obtusiloba* (pasture forest), inhibition occurred on the order of 33%, 60%, and 55% at 20 ppm.

Similar results were obtained by ALVES et al. (2003) when they tested the allelopathic effect of glycosylated steroids; although, the plant species was different. In this case, the experiment occurred on lettuce germination at 0, 100, 200, 400, and 800 mg L^{-1}. The substances did not interfere with lettuce germination regardless of the concentration used. All glycosylated cholesterol concentrations inhibited both radicle and hypocotyl development as a function of dose. This confirms the lower sensitivity in seed germination of invasive species.

Studies of allelopathic steroid activities are rare in the literature. Recent studies have shown that natural substances present in rice (*Oryza sativa*) have herbicide activities including free and glycosylated steroids (e.g., CHUNG & AHMAD, 2010).

The effect of cholesterol (F113) was much higher for germination of invasive species as seen in radicle and hypocotyl development. The most significant inhibition effects obtained by the cholesterol substance (F113) was for the development of the radicle with 55% and 68% for *M. pudica* at concentrations of 15 and 20 ppm, respectively. For *S. obtusiloba*, the percentage of inhibition was 50% and 60% for 15 and 20 ppm, respectively.

We cannot affirm the possible mechanism of allelopathic action of cholesterol, but once in the soil, cholesterol or its derivatives formed by the action of biotic and abiotic factors can alter the local microbiota, favoring groups that may impair weed growth.

New research is needed to study the actions of others metabolites present in *P. brasiliense*. This use of extracts and metabolites isolated from marine macroalgae can transform marine macroalgae into a tool for weed control.

CONCLUSION

Cholesterol with abundant sterols were obtained from the marine macroalgae *Plocamium brasiliense* and showed allelopathic effect on the germination of *M. pudica* and *S. obtusiloba*. The inhibition effects were obvious on the development of radicle and hypocotyl. This article is the first report of a cholesterol with allelopathic activities.

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DECLARATION OF CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

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