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Article

Inhibitory Effects of the Lipophilic Extracts and An Isolated Meroditerpene of Brown Alga in Pasture Weeds in the Eastern Amazon Region

Efeitos Inibidores dos Extratos Lipofílicos e de um Meroditerpeno Isolado de Alga Marrom em Plantas Daninhas de Pastagem na Região da Amazônia Oriental

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ABSTRACT - Two lipophilic extracts and atomaric acid (**1**), an isolated natural product, were obtained from the marine brown alga *Stypopodium zonale* (Dictyotaceae) to identify and characterize their potential inhibitory effects on the seed germination, radicle elongation, and hypocotyl development of the weeds *Mimosa pudica* and *Senna obtusifolia*. The extracts were prepared with hexane and dichloromethane, and atomaric acid (**1**) was isolated from hexane extract by way of conventional chromatographic methods. During a 15 days period, germination bioassays were performed at 25 °C with a 12 h photoperiod, whereas radicle elongation and hypocotyl development were assayed at 25 °C with a 24 h photoperiod. After, Petri dishes 9.0 cm in diameter were coated with qualitative filter paper, 25 seeds were placed in a germination chamber, while six pregerminated seeds were placed in the Petri dish for 2-3 days. After 10 days, radicle and hypocotyl extension were measured; and the inhibitory potential of the extracts was assessed at 10 ppm and that of the atomaric acid at 5, 10, 15, and 20 ppm. In both *M. pudica* and *S. obtusifolia*, dichloromethane extract achieved the greatest rates of inhibition during seed germination (34% and 22%, respectively), radical germination (38% and 30%, respectively), and hypocotyl development (29% and 22%, respectively). At a concentration of 20 ppm, atomaric acid (**1**) also demonstrated reduced inhibitory potential, with mean values of 58.67% for *M. pudica* and 48.67% for *S. obtusifolia*.

Keywords: phytotoxins, *Stypopodium zonale, Mimosa pudica, Senna obtusifolia.*

*RESUMO - Dois extratos lipofílicos e o ácido atomárico (**1**), um produto natural isolado, foram obtidos da alga parda marinha **Stypopodium zonale** (Dictyotaceae) para identificar e caracterizar seus potenciais efeitos inibitórios na germinação de sementes e no alongamento de radícula e do hipocótilo das plantas daninhas **Mimosa pudica** e **Senna obtusifolia**. Os extratos foram preparados com hexano e diclorometano, e o ácido atomárico (**1**) foi isolado do extrato em hexano por métodos convencionais em cromatografia. Durante 15 dias, os bioensaios de germinação foram realizados a 25 °C e fotoperíodo de 12 horas, enquanto os bioensaios de alongamento da radícula e do hipocótilo foram realizados a 25 °C e fotoperíodo de 24 horas. Posteriormente, placas de Petri de 9,0 cm de diâmetro foram revestidas de papel-filtro, 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 dos extratos foi avaliado a 10 ppm, e o do ácido atomárico, a 5, 10, 15 e 20 ppm. Em ambos, **M. pudica** e **S. obtusifolia**, o extrato em*

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*diclorometano alcançou maiores percentuais de inibição que o extrato em hexano durante a germinação das sementes (34% e 22%, respectivamente), alongamento da radícula (38% e 30%, respectivamente) e desenvolvimento do hipocótilo (29% e 22%, respectivamente). Na concentração de 20 ppm, o ácido atomárico (**1**) também demonstrou potencial inibidor, com valores médios de 59% para **M. pudica** e 49% para **S. obtusifolia**.*

Palavras-chave: fitotoxinas, *Stypopodium zonale*, *Mimosa pudica*, *Senna obtusifolia*.

INTRODUCTION

For farmers wide, weeds present a costly problem, one which requires cost-effective strategies for reducing pasture infestation in order to ensure more productive agriculture and longer-living crops. At the same times, such strategies need to reduce not only the negative environmental impacts of aggressive pesticides but also social dissatisfaction with the use of pesticides incited both nationally and internationally (Lobo et al., 2008; Souza Filho et al., 2009a, b). Using natural products as weed inhibitors could be one such strategy to control the cost of combating weeds that infest crops and to reduce discontent with the use of chemicals that contaminate natural resources, threaten wildlife, and people's homes and risk the quality of the agricultural products.

Since 2010, the marine natural product of Brazilian seaweeds have been studied for their effects on the seed germination, radicle elongation, and hypocotyl development of the weeds *Mimosa pudica* L., and *Senna obtusifolia* (L.) Irwin & Barneby, locally called "malícia" and "mata-pasto", respectively. In previous studies, researchers have demonstrated the inhibitory effects of the crude extract and different fractions of monoterpenes produced by the marine red alga *Plocamium brasiliense* (Greville) Howe & Taylor (Fonseca et al., 2012), as well as of a mixture of the diterpenes pachydictyol A and isopachydictyol A isolated from the acetone extract of the brown alga *Dictyota menstrualis* (Hoyt) Schnetter, Hörning, & Weber-Peukert (Fonseca et al., 2013). Their promising results have encouraged us to continue research with other extracts and natural products produced by marine algae.

The brown marine alga *Stypopodium zonale* (Lamouroux) Papenfuss is known to produce several bioactive meroditerpenoids (Penicooke et al., 2013; Soares et al., 2015, 2016). In our study, we used hexane and dichloromethane extracts from *S. zonale*, as well as the major metabolite, to create lipophilic extracts and a meroditerpene, atomaric acid (**1**), for use in bioassays focused on countering the seed germination, radicle elongation, and hypocotyl development of weeds *M. pudica* and *S. obtusifolia* in the eastern Amazon region.

MATERIAL AND METHODS

Our project received a permit for research with scientific purposes (no. 3534), in January 2012, from at SISBIO/ICMBIO - (Authorization System and Information on Biodiversity/Chico Mendes Institute, of the Ministry of the Environment, Brazil)

First, we obtained the algae *S. zonale* by scuba diving to a depth of 0.5-3.5 m at Enseada do Forno, Búzios, in Rio de Janeiro, Brazil, during September 2010. After we cleaned intact samples of alga from epiphytic organisms and washed them with seawater, one of us (VLT) identified the samples as *S. zonale*. Thereafter, we deposited the exsiccate in the herbarium of Rio de Janeiro State University (HRJ 8643).

We successively extracted the air-dried material alga (158 g dry weight) with 100% *n*-hexane and 100% dichloromethane at room temperature in the shade at a rate of 4 × 1.5 L for 7 days in each solvent. After combining the extracts, we let the solvents evaporate under reduced pressure, which yielded two brownish residues (3.5 g in *n* hexane and 7.0 g in dichloromethane).

We subjected the partial hexane crude extract (2.5 g) to silica gel column chromatography eluted with pure *n*-hexane (500 mL), *n*-hexane/EtOAc (7:3, 700 mL), and pure MeOH (200 mL)

and monitored fractionation by using thin layer chromatography (TLC, silica gel, *n*-hexane/EtOac, 7:3). We recorded ^{1}H-(300 MHz) and ^{13}C-NMR (75.5 MHz) spectra on a Varian Unity Plus 300 spectrometer using TMS (Tetramethyl silane) as internal standard. For column chromatography, we used silica gel 60 (Merck, 70-230 and 230-400 mesh) and dextran Sephadex LH-20 gel (Sigma–Aldrich), whereas for TLC (Thin Layer Chromatography), we used silica gel 60 GF_{254} aluminum support plates (Merck). TLC-plates showed spots of aromatic substances in ultraviolet light, and after being salted with 2% sulfate solution in sulfuric acid followed by heating at 100 °C for 3 min, they revealed showed yellowish brown spots. Fractions eluted with *n*-hexane/EtOAc (7:3) containing impure substance **1** were combined nd purified by Sephadex LH-20 gel chromatography of dextran eluted with 100% MeOH to yield pure substance **1** (113 mg).

Next, we evaluated the effects of the potentially phytotoxic extracts and atomaric acid (**1**) on the seed germination and growth of the radicle and hypocotyl of the weeds *M. pudica* and *S. obtusifolia*. We collected seeds of the weeds in cultivated pastures in the municipality of Castanhal, Pará (07°20'53" S, 50°23'45" W) 68 km from the state capital of Belem, in Brazil's eastern Amazon region. To break seed dormancy, we cleaned and treated the seeds with concentrated sulfuric acid (Souza Filho, 1998).

For 15 days, we monitored the seed germination bioassays for the activity of hexane and dichloromethane extracts, which involved the daily counting and elimination of germinated seeds. Following the method of Duran et al. (1985), we considered seeds to have germinated if they presented root extensions of less than 2.0 mm. We developed the bioassays under controlled conditions with a constant temperature (25 °C) and a photoperiod of 12 (i.e., for seed germination) or 24 h (i.e., for radicle elongation and hypocotyl development) in a germination chamber with cool white fluorescent lamps and a luminous flux of 10 μmol m^{-2} s^{-1}. Each Petri dish, 9.0 cm in diameter and lined with filter-quality paper, received 3.0 mL of the solution prepared with extracts. We prepared each solution with the same solvent (i.e., hexane or dichloromethane) in which we obtained the extracts. After the solvent evaporated, each plate received 36 seeds and distilled water in the same amount of solution in order to maintain the original concentration (Souza Filho et al., 2009b). To evaluate the extracts, we used a concentration of 10 ppm of each extract and replaced the water in the volume of 3.0 mL. We performed the seed germination bioassays in triplicate and the control treatment consisted only of distilled water.

Likewise, we performed the seed germination bioassays with atomaric acid (**1**) under the same conditions used in the bioassays for seed germination with hexane and dichloromethane extracts. Once placed for approximately 2-3 days in a Petri dish, six pregerminated seeds grew for 10 days, after which we measured the radicles and hypocotyls. Unlike in the bioassays for seed germination with hexane and dichloromethane extracts, we tested the atomaric acid (**1**) in concentrations of 5, 10, 15, and 20 ppm.

We fully randomized the experimental design for all bioassays in a hierarchical model with three replications. Data analysis comprised variance analysis (i.e., *F* testing) and comparing means with the aid of Tukey's test ($p < 5\%$), all in the statistical program SAS (SAS, 1989).

RESULTS AND DISCUSSION

We evaluated the inhibition effects of two lipophilic extracts and the atomaric acid of *S. zonale* on the development of two weeds from the Amazon region. We identified the atomaric acid by comparing data from Hydrogen and Carbon Nuclear magnetic resonance spectroscopy data (^{1}H NMR and ^{13}C NMR) reported in previous studies (Wessels et al., 1999; Soares et al., 2007). Table 1 presents our results of dates^{1}H- and ^{13}C NMR (APT experiment) with atomaric acid (**1**).

Analyses with TLC and NMR, as well as of comparisons with previously analyzed substances and extracts (Dorta et al., 2002, 2003; Soares et al., 2007, 2015, 2016; Wessels et al., 1999), revealed that the hexane extract contained meroditerpenes primarily, followed by fatty acids and steroids, whereas the dichloromethane extract exhibited larger proportions of oxidized meroditerpenes, steroids, and pigments. Although we detected meroditerpenes 1–5 in the hexane and dichloromethane extracts, because compound 1 was the most abundant, we isolated it only (Figure 1).

Table 1 - ^{1}H- and (300 MHz; in $CDCl_3$) and ^{13}C NMR (75 MHz; in $CDCl_3$) of atomaric acid (1)

C/H	δ_C[(1)]	δ_H (m; J *Hz*)[(2)]
1	35.34	2.84 (1H; d; 14.2); 2.26 (1H; d; 14.2)
2	40.62	
3	35.44	1.72 (1H; m)
4	25.35	1.25 (1H; s); 1.89 (1H; m)
5	36.57	1.49 (2H; d; 1.8)
6	38.98	
7	41.93	1.39 (1H; dd; 6.0; 12.0)
8	22.47	1.74 (1H; m); 1.53 (1H; m)
9	23.54	2.40 (1H; dd; 8.9; 13.0); 1.97 (1H; d; 12.6)
10	123.47	
11	52.92	2.32 (1H; m)
12	24.95	1.81 (1H; m); 1.58 (1H; m)
13	33.38	2.26 (2H; m)
14	176.02	
15	132.92	
16	20.43	1.68 (3H; s)
17	20.80	1.67 (3H; s)
18	17.89	1.03 (3H; s)
19	20.39	0.94 (3H; s)
20	15.80	1.16 (3H; d; 7.0)
1’	126.76	
2’	114.50	6.69 (1H; d; 3.0)
3’	152.58	
4’	113.25	6.54 (1H; d; 2.9)
5’	125.65	
6’	146.77	
7’	16.75	2.22 (3H; s)
8’	55.56	3.73 (3H; s)

[(1)] The values are ppm downfield from TMS, and assignments were made by ATP experiment. [(2)] The values are ppm downfield from TMS, *J* values (in Hz) in parentheses.

We analyzed an aliquot of each extract at a concentration of 1%, the results of which appear in Table 2. The dichloromethane extract had a moderate inhibitory effect on the germination of seeds, ranging from 34% to 22% inhibition for *M. pudica* and *S. obtusifolia*, respectively. By contrast, the more apolar extract presented rates of inhibition ranging from 14% to 1.8% for *M. pudica* and *S. obtusifolia*, also respectively. Such results indicate that the dichloromethane extract was more active than the hexane extract against the germination of seeds of *M. pudica* and *S. obtusifolia* and contains chemical components capable of inhibiting the germination of those.

At the same time, the effect of each extract on radicle elongation (Table 2) suggests that the dichloromethane extract contains active components that inhibited radicle elongation by 38% and 30% in *M. pudica* and *S. obtusifolia,* respectively. The apolar extract achieved rates of inhibition ranging from 18% to 14% for *M. pudica* and *S. obtusifolia* inhibition, also respectively. Similar to the results regarding seed germination, the hexane extract achieved rates ranging from 16% to 14% for the respective inhibition of radicle elongation in *M. pudica* and *S. obtusifolia.*

Last, the effect of the extracts on the hypocotyl development of the weeds exhibited the same pattern observed in our earlier assays. Again, the dichloromethane extract was the most effective (29% for *M. pudica* and 22% for *S. obtusifolia*). Ultimately, the dichloromethane extract of *S. zonale* tested was more efficient against *M. pudica* in all experiments (Table 2). In addition, the low water solubility of both extracts could explain the weak results obtained for rates of inhibition.

Table 3 depicts results regarding the inhibitory activity of atomaric acid (**1**) at concentrations of 5, 10, 15, and 20 ppm on the seed germination of *M. pudica* and *S. obtusifolia.* At 20 ppm,

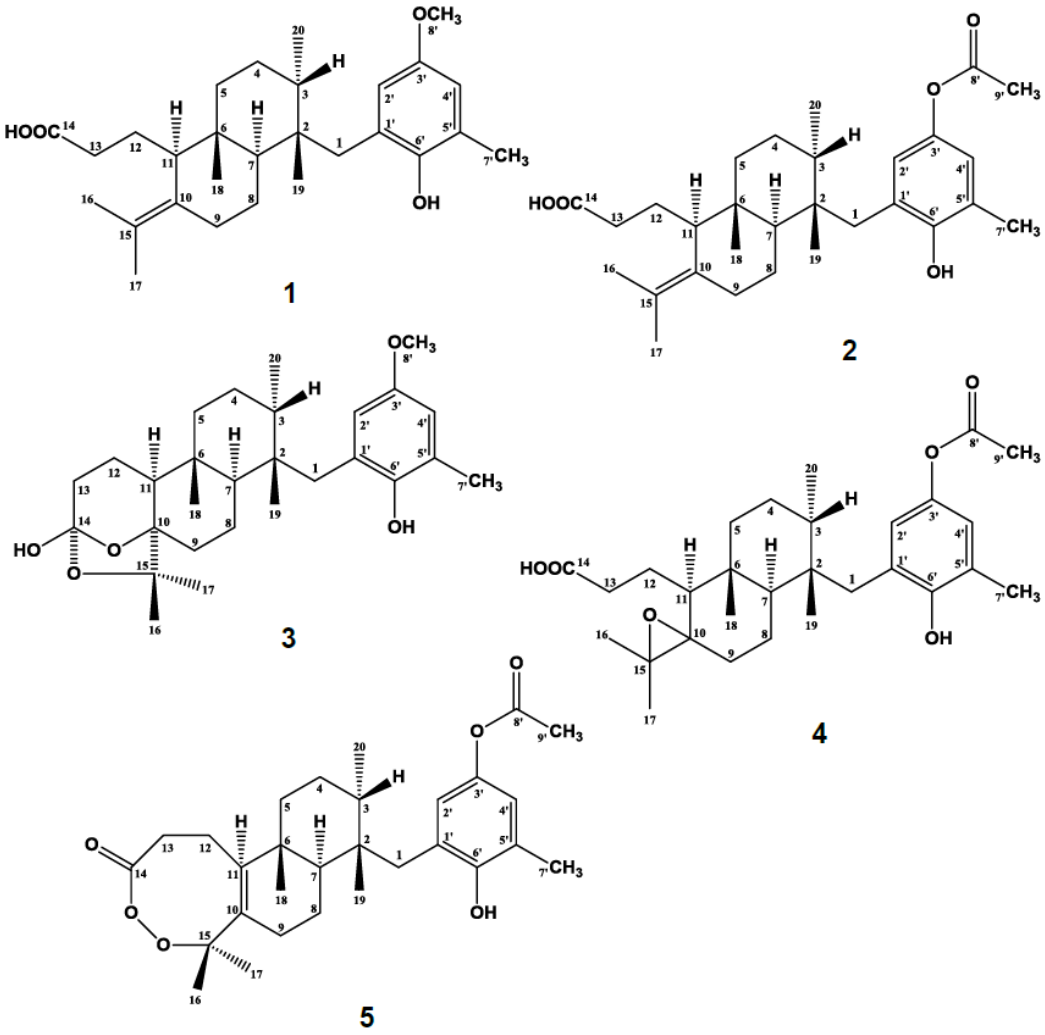

Figure 1 - Meroditerpenes from the brown seaweed *Stypopodium zonale*.

Table 2 - Inhibitory effects of the extracts (Concentration of 1%) of the seaweed *S. zonale* on seed germination; radicle elongation and hypocotyl development of two species of weeds *M. pudica* and *S. obtusifolia*

Algal Eextract	Seed germination	Radicle elongation	Hypocotyl development
Mimosa pudica			
n-Hexane	18	16	22
Dichloromethane	34	38	29
Senna obtusiloba			
n-Hexane	14	14	20
Dichloromethane	22	30	22

The data are expressed as percentage of inhibition compared to the control (%).

atomaric acid (**1**) achieved the greatest inhibition of the germination of *M. pudica* and *S. obtusifolia* seeds at rates of 36 and 27%, respectively. The inhibitory effects on the germination of those seeds, however, depended on the concentration of atomaric acid (**1**), since the phytotoxic effects increased significantly as the concentration increased from 5 to 20 ppm.

The effect of each concentration (i.e., 5, 10, 15 and 20 ppm) of atomaric acid (**1**) on radicle elongation appears in Table 4. The greatest inhibition against *M. pudica* and *S. obtusifolia* at respective rates of 69% and 37%, occurred with a concentration of 20 ppm. Again, however, the inhibitory effects on both weeds depended upon the concentration of atomaric acid (**1**).

Table 5 presents the results of atomaric acid's (**1**) inhibitory effects on the hypocotyl development of the seeds. As in the previous experiments, the inhibitory effect increased along with the concentration of atomaric acid (**1**). In a concentration of 20 ppm, the atomaric acid (**1**) showed the greatest inhibitory effect against hypocotyl development in *M. pudica* (59%) and *S. obtusifolia* (49%).

Table 3 - Inhibitory effects of atomaric acid (**1**) on the seed germination of sensitive plant (*M. pudica*) and (b) mata-pasto (*S. obstusifolia*) (Data expressed as percentage inhibition compared to control treatment – distilled water)

Atomaric acid concentration (ppm)	Inhibitory effects (%) ± standard error	
	Musa pudica	*Senna obtusifolia*
5	8.33 (±1.53)	5.33 (± 0.58)
10	13.67 (±1.53)	7.33 (± 0.58)
15	19.67 (±3.06)	11.33 (±1.53)
20	35.67 (± 1.15)	27.00 (±2.00)

Altogether, our results reveal that hexane and dichloromethane extracts from *S. zonale* have potent phytotoxic activity against *M. pudica* and *S. obtusifolia.* Comparative analysis of the phytotoxic bioassays on seed germination, radicle elongation, and hypocotyl development showed that the extract in dichloromethane was more active than the one in hexane. At the same time, the atomaric acid demonstrated satisfactory inhibition in a concentration of 20 ppm against *M. pudica* and *S. obtusifolia.* The results of the three experiments indicate, however, that the inhibitory effects depended upon the concentration of atomaric acid (**1**) in the two weeds. Such findings suggest the effective inhibitory potential of both the dichloromethane extract and the atomaric acid (**1**).

Table 4 - Inhibitory effects of the atomaric acid (**1**) on elongation radicle (a) sensitive plant (*M. pudica*) and (b) mata-pasto (*S. obtusifolia*) (Data in percentage of inhibition (%) in relation to the control treatment distilled water)

Atomaric acid concentration (ppm)	Inhibitory effects (%) ± standard error	
	Musa pudica	*Senna obtusifolia*
5	10.67 (±1.53)	6.00 (±1.00)
10	27.00 (±2.00)	11.67 (±1.53)
15	39.33 (±1.53)	29.67 (±3.06)
20	69.00 (±3.00)	37.00 (±1.00)

Table 5 - Inhibitory effects of the atomaric acid (**1**) on development hypocotyl (a) sensitive plant (*M. pudica*) and (b) mata-pasto (*S. obtusifolia*) (Data in % inhibition in relation to the control treatment distilled water)

Atomaric acid concentration (ppm)	Inhibitory effects (%) ± standard error	
	Musa pudica	*Senna obtusifolia*
5	3.33 (±1.53)	3.67 (±0.58)
10	10.7 (±2.08)	9.67 (±1.53)
15	39.67 (±2.52)	25.67 (±1.53)
20	58.67 (±3.06)	48.67 (±1.53)

Other benthic seaweed extracts, including s hexane, dichloromethane, ethyl acetate, and ethanol-water extracts from the red seaweed *Plocamium brasiliense* (Fonseca et al. 2012), as well as fractions and diterpenes isolated from the brown alga *Dictyota menstrualis* (Fonseca et al., 2013), have also achieved promising results against *M. pudica* and *S. obtusiloba.* Results obtained with the extracts, fractions, and natural products of benthic seaweeds underscore the importance of research to identify alternative weed inhibitor such as herbicides, as well as highlight seaweeds as a promising alternative for the management of weeds in pasture.

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