EVALUATION OF ANTIFUNGAL ACTIVITY OF SEAWEED EXTRACTS

Avaliação de atividade antifúngica de extratos de macroalgas marinhas

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ABSTRACT

Seaweeds are subject to numerous biological interactions and sometimes to extreme abiotic conditions, so they have developed among other defense mechanisms, the ability to produce biologically active substances. Thus, these organisms produce mainly terpenes and phenols. Among others, the antifungal activity, due to its importance in human and animal health and the production of agricultural products, has been the subject of several studies. In the present work, this activity was investigated in ten seaweeds extracts, by direct bioautography assays, compared to *Colletotrichum lagenarium* and disk diffusion assay, compared to *Aspergillus flavus*. The organisms studied were: *Stypopodium zonale*, *Laurencia dendroidea*, *Ascophyllum nodosum*, *Sargassum muticum*, *Pelvetia canaliculata*, *Fucus spiralis*, *Sargassum filipendula*, *Sargassum stenophyllum*, *Laminaria hyperborea* and *Gracilaria edulis*. *S. zonale*, *L. dendroidea*, *P. canaliculata*, *S. muticum*, *A. nodosum* and *F. spiralis* extracts significantly inhibited the *C. lagenarium* growth, but not inhibited significantly the *A. flavus* growth. The presence of terpenes in all of these extracts was confirmed by thin layer chromatography whereas the presence of phenolic compounds was confirmed only in extracts of *P. canaliculata*, *A. nodosum* and *S. muticum*. In chemical study by column chromatography, followed by gas chromatography/mass spectrometry analysis, the terpenes neophytadiene, cartilagineol, obtusol elatol; and the ester ethyl hexadecanoate were identified in the *L. dendroidea* extract. This is the first report on the activity of seaweed extracts against *C. lagenarium*, a fungus bearing agricultural importance.

Index terms: Etanolic extract, *Colletotrichum lagenarium, Aspergillus flavus.*

RESUMO

As macroalgas marinhas, por estarem sujeitas a numerosas interações biológicas e, por vezes, a condições abióticas extremas, desenvolveram, entre outros mecanismos de defesa, a capacidade de produzir substâncias biologicamente ativas. Assim, esses organismos produzem, principalmente, terpenos e fenóis. Entre outras, a atividade antifúngica, por sua importância na saúde humana e animal e na preservação de produtos agrícolas, tem sido objeto de diversos estudos. No presente trabalho, esta atividade foi investigada em dez extratos de macroalgas marinhas, por ensaios de bioautografia direta, frente ao fungo *Colletotrichum lagenarium* e por ensaio de difusão em disco, frente ao *Aspergillus flavus*. Os organismos estudados foram os seguintes: *Stypopodium zonale*, *Laurencia dendroidea*, *Ascophyllum nodosum*, *Sargassum muticum*, *Pelvetia canaliculata*, *Fucus spiralis*, *Sargassum filipendula*, *Sargassum stenophyllum*, *Laminaria hyperborea* e *Gracilaria edulis*. Os extratos de *S. zonale*, *L. dendroidea*, *P. canaliculata*, *S. muticum*, *A. nodosum* e *F. spiralis* inibiram significativamente o crescimento de *C. lagenarium*, porém, não significativamente, o do fungo *A. flavus*. A presença de terpenos em todos esses extratos foi confirmada por cromatografia em camada delgada e a de compostos fenólicos, apenas nos extratos de *P. canaliculata*, *A. nodosum* e *S. muticum*. No estudo químico por cromatografia em coluna, seguido por análise em cromatógrafo a gás/espectrômetro de massas, foram identificados, em *L. dendroidea*, os terpenos neofitadieno, cartilagineol, elatol e obtusol e o éster hexadecanoato de etila. Esse é o primeiro relato sobre a atividade de extratos de algas frente a *C. lagenarium*, fungo de importância agrícola.

Termos para indexação: Extrato etanólico, *Colletotrichum lagenarium, Aspergillus flavus.*

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INTRODUCTION

Seaweeds are prolific producers of biologically active compounds. Such an ability were developed as defense against numerous organisms which coexist and interact in the same complex environment (BLUNT et al., 2006). According to Smit (2004) the discovery of metabolites with biological activity from algae increased substantially in the last three decades. These substances exhibit an appreciable number of distinct biological activities such as antitumoral, antiviral, antifungal, insecticidal, cytotoxic, phytotoxic, and antiproliferative actions (MACHADO et al., 2010, 2011; KLADI et al 2006; BHAKUNI; RAWAT, 2005). The majority of these compounds are terpenes and polyphenols (BLUNT et al., 2006).

The red macroalgae (Rhodophyta) stands out as the major producer of halogenated compounds above the green and brown macroalgae groups (PEREIRA;

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TEIXEIRA, 1999). Important examples of bioactive halogenated terpenes are (i) laurenditerpenol, isolated from *Laurencia intricata*, which inhibits angiogenesis process (MOHAMMED et al., 2004), and (ii) 7-ethyl desoxiparguerol, isolated from *Jania rubens*, which shows anthelmintic activity and is effective against Ehrlich carcinoma (AWAD, 2004).

Brown algae (Phaeophyta) have shown effectiveness in controlling plant diseases. The laminarin polysaccharide isolated from *Laminaria digitata* is able to elicit host defense responses in plants (KLARZYNSKI et al., 2000). In turn, the extract of the seaweed *Ascophyllum nodosum* stimulates the activity of peroxidases and phytoalexin synthesis in some plants of commercial value, increasing their resistance (LIZZI et al., 1998).

There are few studies about green algae (Chlorophyta). The *Ulva fasciata* extract is able to effectively reduce the number of colonies in powdery mildew, the generic name given to a large number of unicellular bean fungi (STADNIK; MARASCHIN, 2004).

Along with other biological actions, green, brown, and red algae show antifungal activity (BHAKUNI; RAWAT, 2005; BLUNT et al., 2006), which could be a valuable tool in agronomical applications.

As in Brazil, every year, fungi are responsible for worldwide great economic losses in agriculture, since 70% of the plant disorders are provoked by these organisms. Anthracnose, caused by *Colletotrichum* species, is a major post-harvest disease in tropical and subtropical regions of the world (PERES et al., 2002), and is considered one of the most important plant infections caused by fungi. In the case of anthracnose of cucurbits, the causative agent is *C. lagenarium*, firstly named as *C. orbiculare* and *C. gloeosporioides* f. sp. *Cucurbitae* (CORRELL; RHOADS; GEUBER, 1993).

Another cause of economic loss brought about by fungi in agribusiness is the grain contamination by mycotoxins, mainly aflatoxins, produced by *Aspergillus flavus* and *A. parasiticus*. These toxins if ingested may cause toxic hepatitis, hemorrhage, edema, immunosuppression, hepatic carcinoma and death (MARASAS; NELSON, 1987; BALDISSERA et al., 1993). So, the evaluation of algae extracts activity against *C. lagenarium* and *A. flavus*, may present interesting and practical results.

The objective of this work was the search of fungicidal activity of *Stypopodium zonale*, *Laurencia dendroidea*, *Pelvetia canaliculata*, *Sargassum muticum*, *Ascophyllum nodosum* and *Fucus spiralis* extracts against *C. lagenarium* and *A. flavus*, and at establishing a relationship between this activity and the *L. dendroidea* terpenes.

MATERIAL AND METHODS

Algae

Stypopodium zonale and *Laurencia dendroidea* were collected at Buzios, Rio de Janeiro, Brazil by Dr. Wladmir Costa Paradas (Instituto de Pesquisas Jardim Botânico do Rio de Janeiro); *Ascophyllum nodosum*, *Sargassum muticum*, *Pelvetia canaliculata*, *Fucus spiralis* and *Laminaria hyperborea* were collected by Dr. Olga Manuela Matos Freitas from Instituto Superior de Engenharia do Porto, Portugal; *Sargassum filipendula* and *Sargassum stenophyllum* were collected by Dr. Giuliano Buzé Jacobucci from Instituto de Biologia da Universidade Federal de Uberlândia, Brazil and collected at Fortaleza and Pereque Mirim beach, Ubatuba, Brazil; *Gracilaria edulis* were collected by Dr. Anne Q. Hurtado from Integrated Services for the Development of Aquaculture and Fisheries (ISDA) Iloilo City, Filipinas.

Fungi

The strains of *C. lagenarium*, isolated from cucumber, were kindly provided by the Laboratório de Fitopatologia da Escola Superior de Agricultura Luiz de Queiroz (ESALQ) /USP, São Paulo, Brazil. *Aspergillus flavus* strain producer of aflatoxin B1 was isolated from peanut and provided by the Instituto de Biociências /USP, São Paulo, Brazil.

Extraction and isolation

The algae were washed, air-dried, powdered and extracted with ethanol (95%). After removal of the solvent by evaporation under reduced pressure, the crude extracts were submitted to thin layer chromatography (TLC) on AL TLC 20X20 cm, silicagel 60 (Merck), eluted with hexane/AcOEt (7:3) and evaluated by bioautography assay against *C. lagenarium*. To characterize the secondary metabolite classes, the fungitoxic extracts were submitted again to TLC, developed with hexane/AcOEt (7:3) and derivatized with Dragendorff reagent, sulfuric acid (10 %), ferric chroride (10%) and vanillin/H_2SO_4 (MATOS, 1997).

L. dendroidea extract was submitted to liquid chromatography on a silica gel column with a gradient mixture of hexane/CH_2Cl_2/MeOH to give 200 fractions (8 mL each). Fractions were combined according to their TLC pattern to yield 25 sub-fractions. The sub-fractions were submitted to TLC developed with hexane/AcOEt (7:3), and derivatizated with p-hydroxybenzaldeid (MATOS, 1997).

Selected fractions containing terpenoid compounds were submitted to a GC/MS study.

GC/MS analyses were performed in Agilent 6890 series GC system equipped with an OV-5 (30mx0.25mmx0.25 µm, Ohio Valley Specialty Chemical, Inc) capillary column and an Agilent 5573 network selective mass detector.

Antifungal assay

Bioautography assay (against *C. lagenarium*)

This technique was used to determine antifungal activity of the algae extracts against *C. lagenarium*, due to its dark colour. 100 to 0.1 µg, were applied on pre-coated silica gel 60 F254 TLC plates and eluted with hexane/EtOAc (7:3) solution. After evaporation of the solvents, the plate was then sprayed with a spore suspension of *C. lagenarium* and incubated in a moistened chamber, avoiding direct contact of water with the plate, at 25° C for 72 hours. Fungus growth was seen as a grey coloration on the plate, while inhibition zones were white. Nystatin solution (5 µg) was used a reference antifungal.

Disk diffusion assay (against *A. flavus*)

The fungi *A. flavus* were plated onto potato dextrose agar (PDA) and incubated for 10 days at 25° C. The spore suspension used as inoculum was prepared washing cultures with sterile 0.01% Tween 80 solution. Filter paper disk (6.0 mm diameter) containing 10 µL of spores of the *A. flavus* were applied on the PDA in Petri dishes previously inoculated with 10, 25, 50, 150 and 250 mg of the ethanolic algae extract, respectively. The inoculated plates were incubated at 25° C for 5 days. At the end of the period, antifungal activity was evaluated by measuring the zone of inhibition (mm) against the test fungus (MEDEIROS et al., 2011). The commercial fungicide (Benlate 50 WP) was used with positive control. All treatments consisted of three replicates repeated three times and the averages of the experimental results determined.

Statistical analysis

Antifungal experiments were performed in triplicate and data analyzed are mean ± SD subjected to one way ANOVA. Means are separated by Tukey's multiple range tests when ANOVA was significant ($P < 0.05$).

RESULTS AND DISCUSSION

The *P. canaliculata, S. muticum, S. zonale, A. nodosum, F. spiralis* and *L. dendroidea* extracts contained substances that inhibited the growth of *C. lagenarium*, their retention factor values (Rfs) are shown in table 1. There was no *A. flavus* growth using the extracts tested ($p > 0.05$).

The derivatization with Dragendorff by TLC showed absence alkaloids in the six algae extracts with fungitoxic activity. However the possible presence of terpenes and steroids was confirmed by derivatization in sulfuric acid (10%) and vanillin/H2SO4. Phenolic compounds were observed in *P. canaliculata, A. nodosum* and *S. muticum* extracts (Table 1).

Terpenes are produced by representatives from all divisions of seaweeds, being most of them halogenated, as the metabolites isolated from red alga *Laurencia* (Ceramiales, Rhodomelaceae). The genus *Laurencia* is found in tropical and sub-tropical regions around the world and is an extremely rich source of secondary metabolites, mainly sesquiterpenes and C15- acetogenins (BLUNT et al., 2007; SOUTO et al., 2002).

The genus *Stypopodium* is characterized by its ability to produce diterpenes and prenylated hydroquinones (GERWICK; FENICAL ; NORRIS, 1985). According to Van Heemst et al. (1996), aquil phenols have also been found between the components of *S. muticum*, datum in accordance with the positive reaction with ferric chloride observed in this study. Wessels, König and Wright (1999) isolated from *S. zonale* Wessels a diterpene and various sesquiterpenes, which matches with the positive results obtained here for terpenes.

Table 1 – Rfs of the active coumpounds by direct bioautography from algae extracts and Rfs of coumpounds derivatizated with Vanillin/H_2SO_4, H_2SO_4 10% e $FeCl_3$ by TLC.

Extracts	Rf's bioutography assay	$FeCl_3$	H_2SO_4 (10%)	Vanillin/ H_2SO_4 (10%)
P. canaliculata	0.22	-	0.25	-
S. muticum	0.19	-	0.17	-
S. zonale	0.42; 0.32; 0.21; 0.09	0.46	0.35; 0.22; 0.09	0.24; 0.09
A. nodosum	0.54	-	0.50	-
F. spiralis	0.26; 0.19; 0.05	-	0.27; 0.17; 0.03	-
L. dendroidea	0.83; 0.76; 0.55; 0.32	-	0.55; 0.35	0.51; 0.36

L. dendroidea ethanolic extract showed terpenic substances with antifungal activity (Table 1) as seen in its planar chromatographic study. It was subjected to repeated chromatography on silica gel, and GC-MS. In our chromatographic study we could identify the diterpene neophytadiene (2), three sesquiterpenes cartilagineol (1), obtusol (3) and elatol (4) ; and ethyl hexadecanoate (5); 1,1-bis(4-hydroxyphyenyl) ethane (6), isooctyl phythalate (7), phythalic acid (8), and bis (2-etylhexyl) adipate (9) were also identified. The latter are plastic components and currently are found in oceanic waters and accumulated in the algae (Figure 1 and Table 2).

Seaweeds have the ability of accumulating elements from the environment, so they are used as biological indicators in pollution researches (LOBBAN; HARRISON, 1994).

Until now, the only report about cartilagineol antimicrobial activity describes its action against the pathogen *Mycobacterium bovis* (MACHADO et al. 2011). The diterpene neophytadiene is rare in seaweed, having been previously isolated from the ethanolic extract of *Himanthalia elongate* and it showed antimicrobial activity against the fungi *Aspergillus niger*, *Cladosporium cladosporioides* and *C. sphaerospermum* (DE FELICIO et al., 2009).

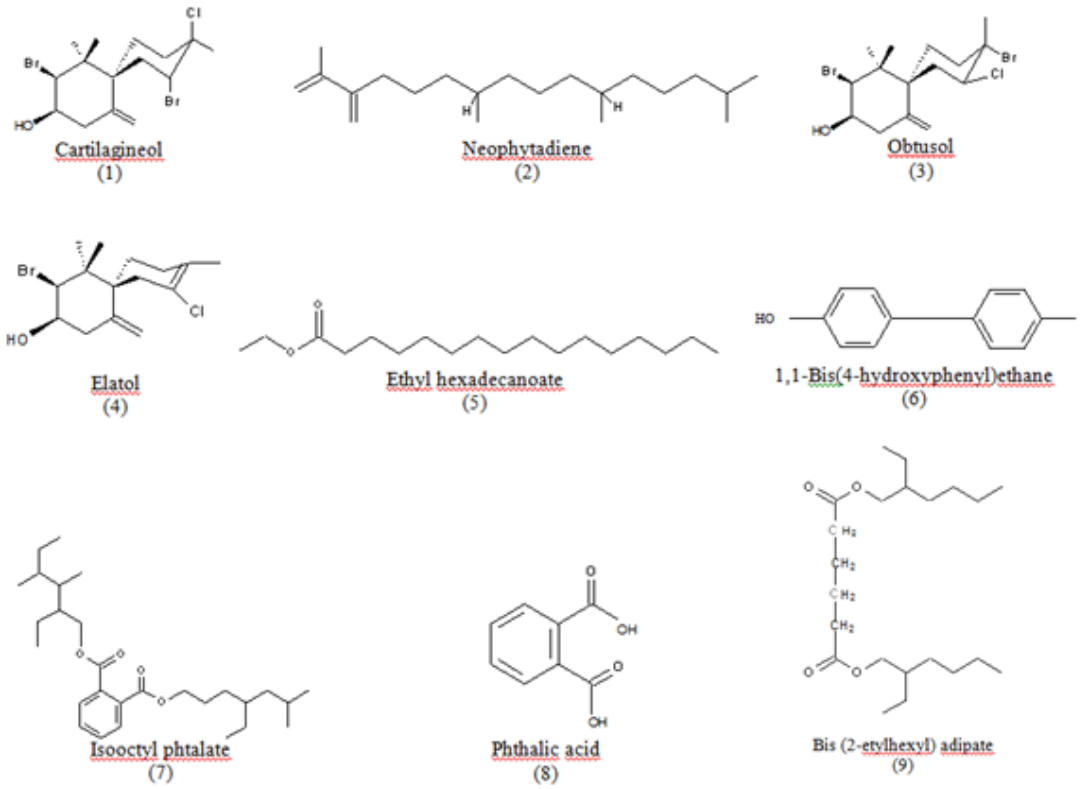

Figure 1 – Chemical structure of compounds identified from *L. dendroidea* extract.

Table 2 – Mass fragments of *L. dendroidea* substances identify by CG/MS.

Time (min.)	Substance	Mass fragments (m/z)
32.795	cartilagineol	334,317, 253, 235, 197, 133, 85
37.015	neophytadiene	278, 137, 123, 95, 82, 68
33.086	obtusol	218, 201, 119, 105, 91, 79, 55
36.764	elatol	319, 299, 253, 235, 153, 85
32.993	ethyl hexadecanoate	284, 255, 239, 199, 185, 101, 88

The obtusol has antimycobacterial and anti-inflammatory activity against *Mycobacterium bovis* (MACHADO et al., 2011), and the elatol inhibited the growth of pathogenic fungi *Mycotypha microspora*, *Eurotium repens*, *Ustilago violacea* and *Fusarium oxysporum* (KONIG; WRIGHT, 1997).

The Rf's of the substances identified as terpenes in the fractions of the *L. dendroidea* extract are similar to those found in the direct bioautography assay against *C. lagenarium* (Table 1), showing correlation of substances belonging to the class of terpenes with substances that showed fungitoxicity, leading to the conclusion that *L. dendroidea* terpenes could be responsible for the algae fungitoxic activity. The thin-layer chromatography was eluted in DCM / MeOH (99:1) and derivatized with p-hydroxy benzaldehyde, confirming the presence of terpenes (Figure 2). The above data showed that terpenes identified in L. dendroidea extract may be responsible for the fungitoxic activity as observed in the direct bioautography tests.

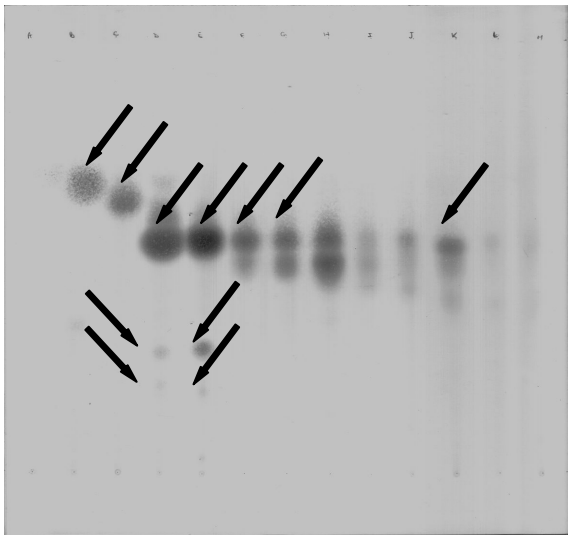

Figure 2 – Terpenes identified by TLC derivatizated with p-hydroxy benzaldehyde of the fractions from *L. dendroidea* extract. The arrows identified the terpenes.

CONCLUSION

The data presented in this work represents the first study on the activity of seaweed extracts against fungi of agricutural importance as *C. lagenarium*, pathogen of importance in the cultivation of cucurbits.

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