Phytochemical analysis and antiphytopathogenic activity of *penicillus capitatus* lam. (ulvophyceae; udoteaceae) extracts against plant pathogenic microorganisms

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The chemical constituents of *Penicillus capiatatus*, was successively extracted with petroleum ether, diclorometane, acetone and methanol and cyclohexane, chloroform, ethyl acetate and methanol. Broth microdilution method was used to study samples antimicrobial activities against six bacterial strains and six fungi. The phytochemical analysis revealed the presence of flavonoids, triterpenoids and phenolic compounds in all extracts. Volatile oils were present only in cyclohexane extract and other metabolites such as alkaloids, steroids, tannin, coumarins and saponins were absent. To phytopathogenic bacteria best results were shown by the cyclohexane extract against *Xanthomonas campestris* pv *campestris*, *X. campestris* pv *malvacearum* and *X. campestris* pv *viticola*, whereas *Pectobacterium caravotorum* subsp. *caravotorum* showed more resistance to organic extracts of *P. capitatus.* For antimicrobial activity against phytopathogenic fungi best results were shown with hexane extract against *Fusarium solani*, whereas *Aspergilus flavus* showed more resistance to organic extracts of *P. capitatus*. This is first report of the effect of organic extracts of green alga *P. capitatus* against bacteria and fungi phytopathogens. Our investigations are an example of the potential for obtaining new sources of antimicrobial agents.

Keywords: Antimicrobial activity; algal extract; seaweeds; bioactive compounds; plant pathogens.

1. Introduction

Decrease in agricultural productivity can be attributed to a variety of reasons, however, pathogen infections plays a significant role in crop losses. As agricultural production has intensified over the past few decades, increased the use of agrochemicals, however, conventional chemical pesticides negatively adversely affect the environment and nontarget organisms. Bioactive natural compounds has represented great potential as alternative method of plant pathogen control [1].

The marine environment representing approximately half of the global biodiversity, consists of a rich diversity of chemical classes. Marine algae are known for their potential as a source of bioactive compounds. These chemical compounds have been reported as promising bioactivities, including the antimicrobial activity [2, 3]. Seaweeds are considered as diverse source of secondary metabolites characterized by a broad spectrum of biological activities. Some compounds showed cytostatic, antiviral, antihelminthic, antifungal and antibacterial activities from macroalgae [4]. Many biologically active important compounds such as alginate, carrageenans and agar as phycocolloids are isolated from seaweeds and used for medicinal purpose and some of them are under investigation for development of new drugs [5].

Harder [6] was the first to observe antimicrobial substance in seaweeds. Then until 1970s, no large scale screening of antimicrobial activity was carried out. The seaweeds are bestowed with varied source of bioactive natural products that exhibits antimicrobial properties against plant pathogens [7, 8 ,9]. Although thousands of bioactive compounds have been discovered, the need for novel therapeutic compounds is still urgent in view of the emergence of a number of new diseases and the resistant strain of microorganisms. In agriculture, intensive application of synthetic pesticides caused damage to the ecological state of the agricultural system. Pesticides of biological origin are generally less toxic, affect only the target pest and closely related organisms and are effective in very small quantities which decompose quickly. Published literature reports on the diverse bioactivities of seaweeds, but the antibacterial efficacy of seaweeds against plant pathogens are comparatively a new concept and a few attempts have been made in this regard [7, 10]. The genus *Penicillus* Lam., one of the marine macroalgal genera belonging to the class Ulvophyceae, Udoteaceae Family, at present consists of 14 taxa at specific and infraspecific level. They are all distributed in tropical and subtropical regions and two species

are reported to Brazilian coast, *P. capitatus* Lam. and *P. pyriformis* A. Gepp ex E. Gepp. Only *P. capitatus* is widely distributed throughout the above tropical Atlantic area [11]. *P. capitatus* shows an antimicrobial activity against *Bacillus subtilis*, in a study realized in Yucatan, Mexico [12] while Soares *et al*. [13] found triacylglycerols and fatty acids as the major componentes in dichlorometane extract. *P. capitatus* showed high activity against HSV-1 virus. This genus had high concentrations of polysaccharides and fatty acids. These compounds may be responsible for the observed activity and indicate that this genus is an ideal target for investigating presence of biomolecules for various medical and industrial applications.

It is very urgent to identify alternatives to chemical pesticides for plant protection without sacrificing the productivity and profitability of agriculture. Due to the side effects of chemical pesticides, sustainable crop production through eco-friendly management is essentially required in the present scenario. Thus, the present study was aimed to explore the phytochemical constituents of different extracts of *P. capitatus* and yours antimicrobial activity against phytopathogenic and clinical microorganisms.

2. Material and Methods

2.1 Collection of algal material

Penicillus capitatus was collected from Praia do Paiva, Cabo de Santo Agostinho, Pernambuco state, Brazil (08º 15'10.50" S e 34º 56'51.80" W) in March 2016. The algae were cleaned using brush for the removal of the epiphytes with distilled water. *P. capitatus* was air dried for two weeks at room temperature. The dried material was coarsely powdered and stored in polyethylene bag until it was used for screening. The seaweed was taxonomically identified at the Dárdano de Andrade-Lima Herbarium (IPA), from Instituto Agronômico de Pernambuco (IPA) and voucher specimens were deposited (IPA 91015).

2.2 Extracts Preparation

The powdered material (100 g) was successively extracted with different solvents with increasing polarity [cyclohexane, chloroform, ethyl acetate and methanol (Extraction 1) and petroleum ether, dichlorometane, acetone and methanol (Extraction 2)] and placed into the Soxhlet apparatus. The material was refluxed for about 36 to 48 hours until saturation and the resulting extracts were evaporated in a rotary flash evaporator. The obtained extracts were collected in a clean Petri dish and weighed.

2.3 Phytochemical profiling

The phytochemical screening of the extract was performed by thin-layer chromatography (TLC) on silica plates (60F254, aluminum backed, 200 µm layer thickness, 8.0 x 5.0 cm, Merck, Darmstadt, Germany). The presence of alkaloids, triterpenes, steroids, cinnamic acid derivatives, aglycone and flavonoid heterosids, hydrolysable tannins, and proanthocyanidins were investigated using the adequate development systems and revealers listed in Table 1(Harborne, 1998). After development, the plates were air dried and sprayed with the revealers in a fume hood.

2.4 Microorganisms cultures

Plant pathogenic bacteria such as Acidovorax citrulli, Pectobacterium carotovorum subsp. carotovorum, Ralstonia solanacearum and Xanthomonas campestris pv. campestris, X. campestris pv malvacearum and X. campestris pv viticola, were sampled from the Culture Collection of the Phytobacteriology Laboratory of the Agronomic Department of Universidade Federal Rural de Pernambuco, Brazil. All the tested bacterial species were maintained on nutrient yeast dextrose agar (NYDA). The six phytopathogenic fungi used during the growth experiments were as follows: Aspergillus flavus, Fusarium moniliforme, Fusarium oxysporum, Fusarium solani, Rhizopus sotolonifer and Verticillium lecanii. The samples of mycelium necessary for the in vitro experiments were taken from cultures grown in slants and kept at 26^0C for 72 h on Potato Dextrose Agar (PDA, Difco).

2.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC)

The minimal inhibitory concentration (MIC) was determined by a microdilution broth susceptibility assay. Bacteria were cultured overnight at 30ºC. The test samples of the extracts were dissolved in 10% DMSO. Dilutions were prepared in a 96-well microtiter plates to get final concentrations ranging from 0 to 50mg/ml. Finally, 20 µl of inoculum (10^6CFU/ml) was inoculated onto the microplates and the tests were performed in a volume of 200 µl. Plates were incubated at 30º C for 24 h. The standard reference drug, chloraphenicol, was used as a positive control for the tested plant pathogenic bacteria. The lowest concentrations of tested samples, which did not show any visual growth after macroscopic evaluation, were determined as MICs, which were expressed in mg/mL. Using the results of the MIC assay, the concentrations showing complete absence of visual growth of bacteria were identified and 50 µl of each culture broth was transferred

onto the agar plates and incubated for the specified time and temperature as mentioned above. The complete absence of growth on the agar surface in the lowest concentration of sample was defined as the MBC. Each assay in this experiment was replicated three times.

2.6 Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

A microplate method [14] was used with slight modifications to determine minimal inhibitory concentration (MIC) values of plant extracts. Plant extracts were serially diluted, ranging from 1/2 up to a 1/100 dilution from the crude extract. In each well, 100 µL of each extract dilution was mixed with 100µL of the fungal spore suspension (2×10^6 spores mL^{-1} in fresh PDB). The microplates were incubated for 2-3 days at 27 °C with daily monitoring. All experiments were done in triplicate. The MIC readings were performed spectrophotometrically with a microplate reader at 595 nm. MICs values were calculated by comparing growth in control wells and the extract blank, which consisted of uninoculated plates. The MIC of the extracts was defined as the lowest concentration of plant extract that caused growth inhibition of more than 90% at 48 h, as compared to the control.

The in vitro fungicidal activity (MFC) was determined as described by [15]. After 72 h of incubation, 20 µL was subcultured from each well that showed no visible growth (growth inhibition of over 98%), from the last positive well (growth similar to that for the growth control well), and from the growth control (extract-free medium) onto PDA plates. The plates were incubated at 27 °C until growth was seen in the growth control subculture. The minimum fungicidal concentration was regarded as the lowest extract concentration that did not yield any fungal growth on the solid medium used.

2.7 Classification of antimicrobial activity

In this work, The MIC and MBC or MFC of the extracts were classified according to follows criteria: The activity was classified as high (≤ 12.5mg/mL), moderate (12.5 to 25 mg/mL), low (50 to 100 mg/mL) and very low (above 100mg/mL). The activity was classified as high (≤ 12.5mg/mL), moderate (12.5 to 25 mg/mL), low (50 to 100 mg/mL) and very low (above 100mg/mL).

2.8 Evaluation of bactericidal/fungicidal and bacteriostatic/fungistatic capacity

The action of an antibacterial on the bacterial strains can be characterized at two parameters as Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) or Minimum Fungicidal Concentrations (MFC). According to the ratio MBC or MFC/MIC, we can apperceive antibacterial or antifungal activity. If the ratio MBC/MIC=1 or 2, effect is bactericidal but if the ratio MBC/MIC=4 or 16, effect is bacteriostatic.

3. Results and Discussion

In this report, we investigated the chemical composition of crude organic extracts of *P. capitatus*. The phytochemical analysis performed on eight extracts of *P. capitatus* revealed the presence of several secondary metabolites (Table 2). Flavonoids, triterpenoids and phenolic compounds were present in all extracts. Volatile oils were present only in hexane extract and others metabolites such as alkaloids, steroids, tannin, coumarins and saponins were absent in all extracts.

The antibacterial activity of *P. capitatus* extracts were examined against six bacterial phytopathogenic and six fungi phytopathogenic causing damage in major crops. The MIC tests of *P. capitatus* organic solvent extracts against 12 phytopathogenic microorgaisms were carried out using the microdilution technique. The MIC values of eight extracts ranged from 1.562 to 50 mg/mL (Table 3 to Table 6). To phytopathogenic bacteria the lowest MIC was for 1.562 mg/mL *cyclo*hexane extracts against *Xanthomonas campestris* pv *campestris*, *X. campestris* pv *malvacearum* and *X. campestris* pv *viticola*, whereas *Pectobacterium caravotorum* subsp. *caravotorum* showed more resistance to organic extracts of *P. capitatus* with a MIC of 25 mg/mL (Table 3). The generally low activity of the extracts against the gram negative organisms may be due to the fact that gram negative bacteria possess an outer membrane and a periplasmic space, both of which are absent in gram positive bacteria. The outer membrane of gram negative bacteria is known to present a barrier to the penetration of numerous antibiotic molecules. In addition, the periplasmic space contains enzymes which are capable of breaking down foreign molecules introduced from outside [16]. When we looked for antimicrobial activity against phytopathogenic fungi the lowest MIC was for 6.25 mg/mL *cyclo*-hexane extract against *Fusarium solani*, whereas *Aspergilus flavus* showed more resistance to organic extracts of *P. capitatus* with a MIC of 50 mg/mL (Table 4). Padmakumar and Ayyakkannu [17] screened antifungal activities of 80 marine algal species and did not find a single algal extract was active against *Aspergillus flavus*. Similarly, Lavanya and Veerappan [18] reported that dichloromethane and ethanol extracts of *Sargassum dentifolium*, *Laurencia papillosa* and *Janio corniculata* had no activity against *Aspergillus flavus*.

The defined stringent end point criteria for “activity”, suggest that extracts should be considered efficacious if they exhibit MIC values ≤ 12.5mg/mL. In this context, all extracts from Extraction 2 showed high activity for phytopathogenic bacteria (Table 5 and 6). The *n*-hexane and methanol extracts, for *Pectobacterium carotovorum* subsp. *carotovorum*,

showed moderate activity (Table 3). To the antifungal activity, chloroform, ethyl acetate and methanol extracts, from first extraction, to *Aspergillus flavus* showed low activity, and methanol extract (Extraction 1) to *Fisarium moniliforme, Rhizopus sotolonifer* and *Verticillium lecanii* showed moderate activity (Table 4). Other extracts showed high activity. To extracts from Extraction 2, approximately 40% showed moderate activity (Table 6).

It is difficult to correlate the antimicrobial activity of an extract to a compound class due to their complexity and variability. Nevertheless, researchers have reported on the relationship between the chemical composition of the extracts and their antimicrobial activity. These results indicate that bioactive molecules involved in the antimicrobial activity have different characteristics. The extracts obtained with different organic solvents contain compounds distributed according to their polarity. In line with our results, several works also reported antimicrobial activity in the different organic solvents [19, 20, 21, 22, 23, 24] which indicates the vast array of metabolites that can be involved in the antimicrobial activity.

Genovese *et al.* [25] reported that the marine biodiversity and associated chemical diversity constitute an unlimited reserve of bioactive substances in the field of bioactive products. Seaweeds provide a rich source of structurally diverse secondary metabolite. Several studies have demonstrated that seaweeds are an excellent source of components such as polysaccharides, tannins, flavonoids, phenolic acids, bromophenols, and carotenoids that exhibits different biological activities [5].

Investigations of antimicrobial chemical defenses in green algae of the genus *Penicillus* indicate that these abundant macroalgae also harbor potent defenses against fungal pathogens. From *P. capitatus*, Puglisi *et al*. [26] isolated two novel triterpene sulfate esters, capisterones A and B, with antifungal activity against *Lindra thalassiae* at natural whole-tissue concentrations. Engel *et al*. [27] screened seaweeds from Bahamas against marine pathogens and saprophytes. Among seaweeds screened, *P. capitatus* and *Halimeda copiosa* were the only to inhibit the growth of all assay microorganisms. Of the many active extracts identified in that survey, *P. capitatus* have thus far been chosen for further study. Different results were found by [12]. The authors conducted a screening against *Bacillus subtilis*, *Streptococcus faecalis* and *Micrococcus luteus*. The organic extracts from *P. capitatus* were only active against *B. subtilis*.

Variation in antimicrobial activity of seaweed may be due to the method of extraction, solvent used in extraction and season at which samples were collected. Several diferent organic solvents have been used to screen algae for antimicrobial activity. Different solvents have been reported to have the capacity to extract different phytoconstituents depending on their solubility or polarity in the solvent [28].

The extracts used in this study, had stronger antibacterial than antifungal activities. The probable reason is the difference in the composition and permeability of their cell walls. The cell walls of gram-positive bacteria are made of peptidoglycans and teichoic acids, while the cell walls of gram-negative bacteria are made of peptidoglycans, lipopolysaccharides, and lipoproteins [29]. The lipid portion of the outer membrane of gram-negative bacteria is poorly permeable to antimicrobials, hence the reason for their greater resistance. The cell wall of fungi consists of polysaccharides such as glucan and hitchin and it is poorly permeable. This observation is in accordance with many other studies, focused on antimicrobial activity which has demonstrated that structure and permeability of the cell wall are reasons for the different sensitivities in gram-positive bacteria, gram-negative bacteria and fungi.

In conclusion, this study indicates that *Penicillus capitatus* produces a high variability of compounds, some them with high antimicrobial activity wich makes them interesting for programs screening natural products. We found bioactivity in all extracts of *P. capitatus* against all microorgnisms tested but *n*-hexane extracts was more active (lowest MIC). These results indicate the potential for obtaining new sources of antimicrobial agents. Further works will emphasize the isolation and characterization of active principles responsible for antimicrobial activity and action mechanisms of these green algae.

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Conflicts of Interest The authors declare have not any conflicts of interest.

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Table 1 Development systems and revealers used for analysis by thin-layer chromatography of secondary metabolites in *P. capitatus* extracts.

Secondary metabolites	Development system	Revealer
Alkaloids	EtOAc/HCOOH/AcOH/H2O (100:11:11:26 v/v)	Dragendoff's reagent
Triterpenes and steroids	EtOAc/HCOOH/AcOH/H2O (100:0.5:0.5:0.5 v/v)	Lieberman-Burchard's reagent
Aglycone and flavonoid heterosids	EtOAc/HCOOH/AcOH/H2O (100:11:11:27 v/v)	Neu's reagent
Proanthocyanidins	EtOAc/HCOOH/AcOH/H2O (100:11:11:26 v/v)	Vanilin-chloridric acid
Cinnamic acid derivatives	EtOAc/HCOOH/AcOH/H2O (100:11:11:27 v/v)	Neu's reagent

Table 2 Classes of secondary metabolites present in the organic extracts of *Penicillus capitatus*.

Chemical compounds	Extraction 1				Extraction 2			
	Cychlohexane	Chloroform	Ethyl Acetate	Methanol	Petroleum Ether	Dichloro Metane	Acetone	Methanol
Flavonoid	+	+	+	+	+	+	+	+
Triterpenoid	+	+	+	+	+	+	+	+
Alkaloid	–	–	–	–	–	–	–	–
Coumarin	–	–	–	–	–	–	–	–
Tanin	–	–	–	–	–	–	–	–
Phenolic compounds	+	+	+	+	+	+	+	+
Volatile oils	+	–	–	–	–	–	–	–
Saponin	–	–	–	–	–	–	–	–

+Presence; - Absence