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International Journal of Current Research in Life Sciences
Vol. 07, No. 04, pp.1420-1425, April, 2018

ISSN: 2319-9490

RESEARCH ARTICLE

ANTIMICROBIAL POTENTIAL OF *KAPPAPHYCUS ALVAREZII* AGAINST PLANT PATHOGENS

***Deepa, V. H., Anushree Acharya, Pinky Gupta, Rakesh Kumar and Payel Sarkar**

Department of Microbiology, Ramaiah College of Arts, Science and Commerce, Bangalore, Karnataka, India

Received 10th February, 2018; Accepted 18th March, 2018; Published Online 06th April, 2018

ABSTRACT

Seaweeds are one of the important living resources of the marine ecosystem. They are the macroscopic algae that are considered as renewable sources of various bioactive compounds and produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities. Fresh and dry seaweeds are extensively consumed by the people living in coastal areas as it contains carotenoids, dietary fibers, proteins, essential fatty acids, vitamins and minerals which are highly essential for human nutrition. Seaweeds have wide applications in pharmacological researches because of their antimicrobial and anti-oxidative properties. *Kappaphycus alvarezii*, a red alga (Division Rhodophyta), having various nutritional products including carrageenan and antioxidants, is used as food or neutraceutical supplement. They have showed effective antimicrobial activity against various human pathogens but not much is known about their antimicrobial potential for use against plant pathogens. In this study, we focused on the antimicrobial activity of *Kappaphycus alvaezii* against six plant pathogenic fungi (*Fusarium oxysporum, Aspergillus oryzae, Penicillium chrysogenum, Phytophthoracapsici, Colletotrichumgloeosporoides, Geotrichumcandidum*) and two plant pathogenic bacteria (*Xanthomonas punicae and Ralstonia solanacearum*). *Kappaphycus alvarezii* was extracted by four different organic solvents such as methanol, ethanol, acetone and chloroform. The antimicrobial activity was done by well diffusion method. In this study, we have shown that *K. alvarezii* hasantimicrobial potential against the tested plant pathogens. Ethanol was found to be the best solvent for extraction and retention of the antimicrobial activity of this red alga.

Key words: Kappaphycus alvarezii, Red algae, Plant pathogens, Antimicrobial activity.

Citation: Deepa, V. H., Anushree Acharya, Pinky Gupta, Rakesh Kumar and Payel Sarkar, 2018. "Antimicrobial potential of *kappaphycus alvarezii* against plant pathogens" *International Journal of Current Research in Life Sciences*, 7, (04), 1420-1425.

INTRODUCTION

Seaweeds are important sources of various bioactive compounds (Bouhlal et al 2010; Kayalvizhi et al 2012). Fresh and dry seaweeds are extensively consumed by the people living in coastal area as it contains carotenoids, dietary fibers, proteins, essential fatty acids, vitamins and minerals which are highly essential for human nutrition. (Fayaz et al 2005) Seaweeds have been screened extensively to isolate lifesaving drugs or biologically active substances all over the worlds. *Kappaphycus alvarezii* (belongs to Rhodophyta) is the elk horn sea moss, a species of red algae. This alga grows to two meters long and is green or yellow in color. *Kappaphycus alvarezii* is a tough, fleshy, firm marine alga which can grow up to 6 feet in length. Its coarse thalli measures about approximately ½ inch in diameter. The thalli are heavy with straight major axis that lacks secondary branches near the tips. It is frequently and irregularly branched. *Kappaphycus* species are among the largest tropical red algae with a high growth rate (can double in biomass in 15 -30 days).

Corresponding author: **Deepa, V. H.,**
Department of Microbiology, Ramaiah College of Arts, Science and Commerce, Bangalore, Karnataka, India.

Kappaphycus alvarezii, is used as food or neutraceutical supplement because of its various nutritional products including Carrageenan and antioxidant. It is one of the most important commercial sources of carrageenans, a family of gel-forming, viscosifying polysaccharides. Currently, most carrageenan is commercially extracted from K*appaphycus alvarezii*, which contains mainly kappa carrageenan with low levels of iota carrageenan. Earlier studies have reported that seaweeds can be used for development of new drugs against cancer, microbial infection and inflammation. Seaweeds are considered as diverse source of Secondary metabolites characterized by a broad spectrum of biological activities. Many biologically active compounds such as Alginate, carrageenans and agar as phycocolliods are isolated from seaweeds and used for medicinal purpose and development of new drugs. Gram-positive organisms are more susceptible to the seaweed extracts and this susceptibility of Gram positive bacteria to algal extracts is due to the difference in their cell wall structure and their composition. *K. alvarezii* showed effective antimicrobial activity against various human pathogens such as *Bacillus subtilis Staphylococcus aureus, Lactobacillus acidophilus, Escherichia coli, Pseudomonas aeruginosa*, *Proteus mirabilis and Vibriocholarae* in earlier

studies (Pushparaj *et al.,* 2014, Madhavaranialwarsamy, 2014). Different parts of the thalli differ in their antimicrobial potential (Fayaz *et al.,* 2005). It also showed effective inhibition against Mycobacterium tuberculosis (Mayakrishnan *et al.,* 2015). It also showed antifungal activity against the human pathogenic fungi such as *Aspergillusfumigatus, Candida albicans, Epidermophyton sp., Microsporumcanis*and *Trichophytonverrucosum* (Rajasulochana *et al.,* 2013).

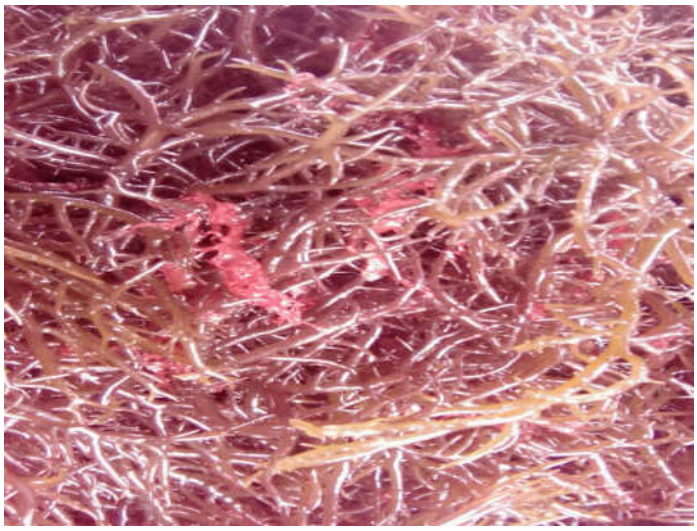


Figure 1. ***Kappaphycus alvarezii***

Although some work has been done on the effectiveness of *K. alvarezii* against human pathogens, not much is known about their potential in controlling plant pathogens. Plant pathogens cause plant diseases that reduce a grower's ability to produce crops and can infect almost all types of plants. *Aspergillus, Penicillium, Phytophthora, Colletotrichum, Geotrichum, Fusarium* are the most common phytopathogenic Fungi. Most plant pathogenic bacteria are rod-shaped (bacilli). In order to be able to colonize the plant they have specific pathogenicity factors. Five main types of bacterial pathogenicity factors are known: uses of cell wall–degrading enzymes, toxins, effector proteins, phyto hormones and exo polysaccharides. *Erwinia, Xanthomonas, Ralstonia, Agrobacterium* and *Pseudomonas* species are the most common Plant pathogenic bacteria. One study has shown that *K. alverazii* effectively inhibits the growth of *Xanthomonasoryzae* (Venkatesh *et al.,* 2011). The chemical pesticides used against plant pathogens causes severe hazardous health problems to the consumers. It is time to find out an effective alternative for the chemical pesticides and fertilizers. In this present study, we have demonstrated the antimicrobial activity of *K. alvarezii* against six plant pathogenic fungi and two bacteria. In future, our workwill pave a way to an alternative method to control plant pathogens in agriculture without any health issues to the consumer as *K. alvarezii* is already being used in the food industry.

MATERIALS AND METHODS

Sample Collection

The sea weed *Kappaphycus alverazii* was collected from the Kottaipattinam seashore, Pudhukottai district, Tamilnadu, India. The collected seaweed was washed with seawater and then with fresh water. The extraneous matters were removed and shade dried and cut into small pieces and powdered in a mixer. The powdered sample was stored in freezer for further study.

Extract Preparation from the Seaweed Powder

5 g of the powdered seaweed were extracted in 200 ml of different solvents, such as Methanol, Ethanol, Chloroform and Acetone, by soaking overnight at room temperature. After incubation, the extract was filtered by using Whatmann no 1 filter paper and dried completely by solvent evaporation method. The dried extracts were dissolved in 1 ml of same solvent and tested for the antimicrobial activity against the plant pathogens.

Collection of Plant Pathogens

The plant pathogenic Fungi and Bacteria were collected from Gandhi Krishi Vignana Kendra, Bangalore, and Karnataka. The Fungal samples(*Fusarium oxysporum, Aspergillus oryzae, Penicillium chrysogenum, Phytophthoracapsici, Colletotrichumgloeosporoides, Geotrichumcandidum*) were sub cultured on Potato Dextrose Agar medium (Hi Media), *Xanthomonas punicae* on Nutrient agar media (Hi Media M001) and the *Ralstonia solanacearum* on TTC media (Nutrient Agar media enriched with TTC solution). The organisms were confirmed by the morphological, microscopic and biochemical tests.

Determination of Antimicrobial Activity

The antimicrobial activity was carried out by using agar well diffusion method. The solvents like Methanol, Ethanol, Chloroform and Acetone were used to re dissolve the seaweed extract and were tested against the plant pathogens in three different concentrations. Overnight grown bacterial broth cultures (0. 1 ml) were transferred to sterile petriplates with Nutrient agar media (*Xanthomonas punicae*) (Hi Media laboratories M001), Triphenyl Tetrazolium Chloride media (*Ralstonia solanacearum*)(Nutrient agar medium added with TTC solution) and was spread with a sterile spreader to create a lawn. Well sporulated fungal cultures are swabbed over the solidified PDA media with the help of sterile swab and kept undisturbed for 15 minutes to adsorb to the media About 5 mm diameter well was made in each plate with the help of a cork borer. Different concentrations of algal extract were prepared (50 µg/ml, 100 µg/ml, 200 µg/ml) and 100 µl of each concentration was added to the well by using sterile pipettes and the well added with the solvent alone act as control. These plates were incubated at 37^0C for 24 hours *(Ralstonia solanacearum*), 28^0C for 48 hours (*A. oryzae,F. oxysporum,G. candidum,C. gloeosporoides, P. chrysogenum and Phytophthora capsici*) and 28^0C for 24 hours (*Xanthomonas punicae*)and the zone of incubation around the well was measured (including the well) in nearest millimeter.

Determination of Minimum Inhibitory Concentration (MIC) of Algal Extracts

The overnight cultures were prepared by inoculating the plant pathogens in respective broth like Potato Dextrose broth (fungi), Nutrient broth (*Xanthomonas*)and Triphenyl Tetrazolium Chloride broth (*Ralstonia*)and incubated at 28 ^{0}C (fungi and *Xanthomonas*) and 37 ^{0}C (*Ralstonia*) for 24 hours. The overnight grown cultures (10 µl) are added to the respective broth (890 µl) and redissolved algal extract (100

µg/ml) was added to this mixture. This set up was incubated at 28 ^{0}C (fungi and *Xanthomonas*) and 37 ^{0}C (*Ralstonia*) for 24 hours and the OD values are measured at 640 nm in colorimeter. The minimum concentration at which there was a drop in OD value was considered as the MIC.

RESULTS AND DISCUSSION

Plant pathogens were characterized by morphological, microscopic and biochemical tests

The plant pathogenic fungi were collected from Gandhi Krishi Vignana Kendra University, Bangalore and confirmed by colony morphology and staining. The bacterial cultures were confirmed by gram staining and biochemical tests such as indole test, starch hydrolysis, methylred, Voges Proskauer, gelatin liquefaction (*Xanthomonas punicae*) and sugar fermentation test (*Ralstonia solanacearum*). The results are tabulated (Table 1).

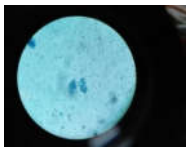
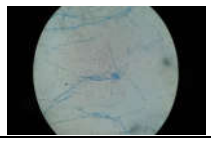
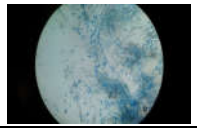
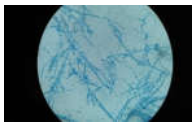
***Kappaphycus alvarezii*was extracted by using different solvents**

The alga was collected from Kottaipattinam seashore, dried and powdered and extracted by using methanol, ethanol, acetone and chloroform after overnight incubation. The fresh algal extract did not show effective antimicrobial activity.

***Kappaphycus alvarezii* showed antimicrobial activity against the tested plant pathogens**

Antimicrobial activity was analyzed using the well-diffusion method (Figure 2). The extracted alga was dissolved in the respective solvent (50µg/ml, 100µg/ml, 200µg/ml,) and 100µl was added to the well. The control wells were added with respective solvent. The algal extract showed effective antagonistic activity by producing clear zone of inhibition compared to that of the solvent. The antimicrobial activity of the selected seaweed *Kappaphycus alvarezii* (extracted with four different solvents like methanol, ethanol, acetone and

Table 1. Characterization of fungal and bacterial plant pathogens

Sl No.	Organism	Colony features	Microscopic fields of view
1	*Aspergillus oryzae*	Initially Black colonies with white periphery. later turns to yellowish colonies	
2	*Penicillium chrysogenum*	The colonies are cotton-like in texture. The colonies usually begin as a whitish color and, over time, produce green, bluish green coloured conidia	
3	*Phytophthora capsici*	Colonies are cottony with slightly petaloid pattern	
4	*Colletotrichumgloeosporoides*	Grey,cottony mass .Reverse dark greyish colour.	
5	*Geotrichum candidum*	Creamy white colonies on PDA media with a butyrous texture with a velvety, suede-like or ground glass/matt appearance.	
6	*Fusarium oxysporum*	On Potato Dextrose Agar media, *F.oxysporum* grew rapidly to produce of white-floccose(cottony) colonies with the aerial mycelia becoming tinged in purple. The reverse was a rather non-descript pale to yellow	

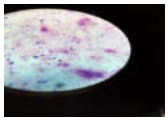
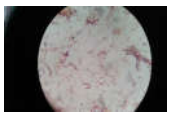
Sl.No	Organism	Confirmatory tests	Results	Gram staining
7	*Xanthomonas punicae*	1.Indole test 2.Methyl red test 3.Voges proskauer 4.Gelatin liquefaction 5.Starch hydrolysis	Negative Negative Negative Positive Positive	
8	*Ralstonia solanacearum*	1.KOH test 2.Sugar Fermentation test a. Glucose b.Dextrose c.Sucrose d.Mannitol	Positive Positive Positive Positive Positive	

Table 2: Antimicrobial activity as determined by the diameter of zone of inhibition for different solvent extracts of *Kappaphycus .alvarezii* against the respective bacterial pathogens. The values represent the difference in the diameters of test (solvent extract of the alga) and solvent only control wells. *The values that are bolded indicate the maximum inhibition. CI: Complete inhibition in both control and test

Name of the Bacterial Pathogen	Concentration µg/ml	Diameter zone of inhibition in (mm)			
		Methanol Extract	Ethanol extract	Acetone extract	Chloroform extract
Xanthomonas punicae	50	3.3	4.0	1.4	10.4
	100	7.0	0.3	2.6	9.3
	200	12	27	9.6	21.6
Ralstonia solanacearum	50	7.3	6.3	2.3	C I
	100	6.0	8.3	4.3	C I
	200	3.3	3.7	4.0	C I

Table 3. Antimicrobial activity as determined by the diameter of zone of inhibition for different solvent extracts of *Kappaphycus alvarezii* against the respective fungal pathogens. The values represent the difference in the diameters of test (solvent extract of the alga) and solvent only control wells. *The values that are bolded indicate the maximum inhibition. CI: Complete inhibition in both control and test

Name of the Fungal Pathogen	Concentration µg/ml	Diameter zone of inhibition in (mm)			
		Methanol extract	Ethanol extract	Acetone extract	Chloroform extract
Aspergillus oryzae	50	1.7	6.3	3.3	-7.3
	100	1.6	3.0	3.6	6.0
	200	3.4	2.7	7.4	-0.3
Colletotrichum gleosporoides	50	2.3	0.3	2.3	9.3
	100	2.4	6.0	2.7	2.4
	200	4.3	4.0	6.0	C I
Fusarium oxysporum	50	-3.7	2.7	7.0	C I
	100	1.4	7.3	2.0	C I
	200	8.7	3.0	2.7	C I
Geotrichum candidum	50	3.7	1.7	3.3	C I
	100	7.3	0.7	5.3	C I
	200	9.7	5.7	10.7	C I
Phytopthora capsici	50	6.7	0.0	-2.0	C I
	100	0.7	13.7	3.4	C I
	200	0.0	19.3	3.3	C I
Penicillium crysogenum	50	2.3	0.7	0.4	0.3
	100	4.3	8.0	4.7	-1.7
	200	3.3	10.6	4.2	0.1

Figure 2. Representative plate showing the well-diffusion method used. Here, *X.punicae* is showing zone of inhibition in ethanol extract of *Kappaphycus alvarezii.* The difference between test (algal powder dissolved in solvent) and control (only solvent) wells have been evaluated

chloroform) against six plant pathogenic fungi and two bacteria is tabulated in Tables 2 and 3. The highest activity of 27 mm based on highest concentration (200 µg/ml) was recorded *in K. alvarezii* extracted by ethanol against *Xanthomonas punicae* and the lowest activity of 2. 7 mm against *Aspergillus oryzae* by ethanol extract and *Fusarium oxysporum* by Acetone extract.

Chloroform extract of *K. alvarezii* exhibited effective antibacterial activity against *Xanthomonas punicae* in all three tested concentrations (50 µg/ml 100 µg/ml 200 µg/ml) as 10. 4mm, 9. 3mm and 21. 6 mm respectively. R. *solanacearum, G. candidum, F. oxysporum and Phytophthora capsici* were completely inhibited in all the three concentrations by both solvent chloroform and algal extract, this indicates that

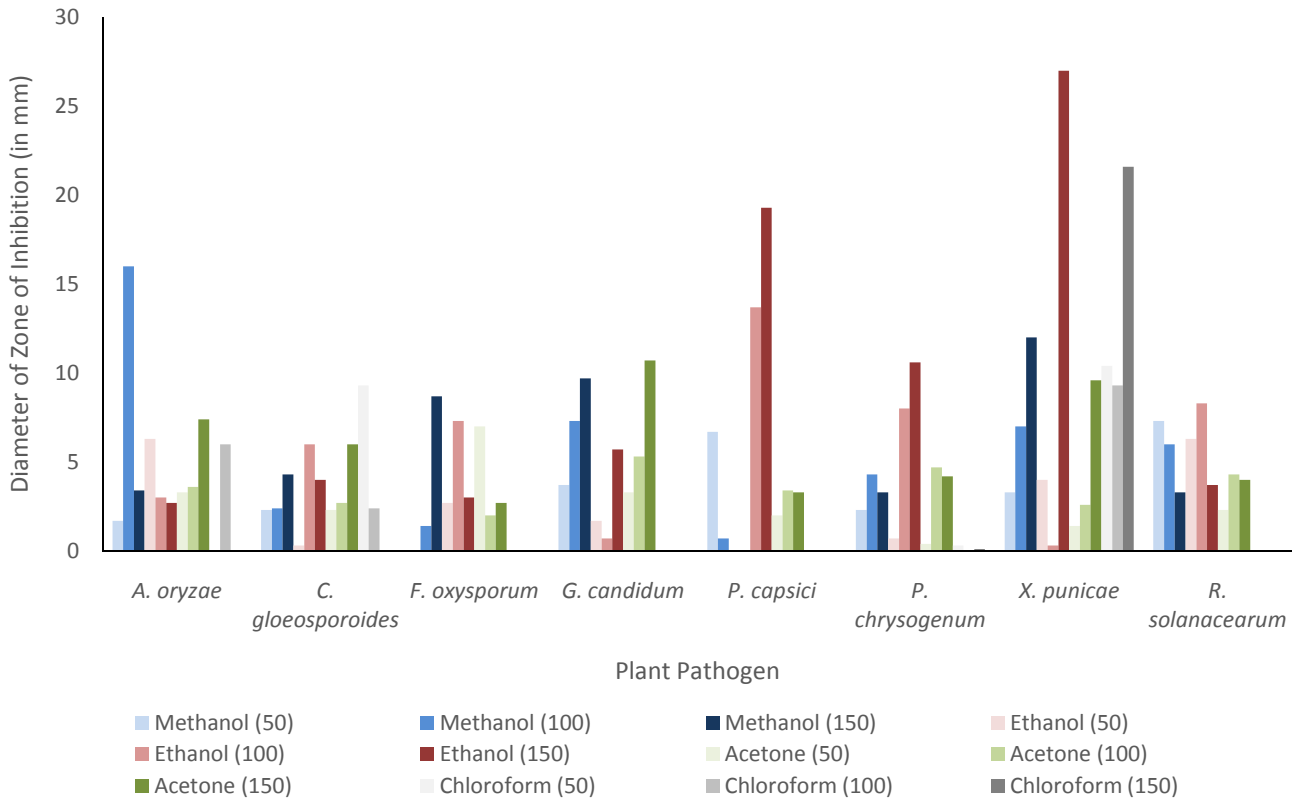


Figure 3. Graph showing antimicrobial activity, expressed as zone of inhibition, of the indicated solvent extracts of *K.alvarezii* against the indicated plant pathogens. Numbers in parenthesis indicate the algal concentration of the solvents

chloroform itself have effective inhibition against these organisms. The highest activity (10. 4mm) in lowest concentration (50 µg/ml) was seen against *Xanthomonas punicae* by the chloroform extract. These results clearly show that *K. alvarezii* exhibits antimicrobial activity against the tested plant pathogens in one or more extract solvents (Figure 3). Chloroform extracts results could not be analyzed effectively in this study because there was inhibition of growth in both test and control wells for most of the tested organisms. This could be due to the inherent antimicrobial activity of chloroform (Martos *et al.,* 2013).

The ethanol extracts of*Kappaphycus alvarezii*was found to be the most effective of the tested solvents

In our study, it was observed that *K. alvarezii* extracted by ethanol was found to be significantly effective against all organisms. This shows the efficiency of ethanol to extract the antimicrobial substance from the algae. The difference between the inhibition zone of control and test shows that the antimicrobial activity of this alga is more than the solvent. Acetone and methanol also exhibited inhibition against all the organisms but comparatively lesser. The MIC ofthe ethanol extract of *K. alvarezii*against *Fusarium oxysporum, Aspergillus oryzae, Penicillium chrysogenum, Phytophthoracapsici, Colletotrichumgloeosporoides, Geotrichumcandidum*, *Xanthomonas punicae and Ralstonia solanacearum* was determined to be 30 µg/ml, 50 µg/ml, 30 µg/ml, 30 µg/ml, 30 µg/ml, 30 µg/ml, 20 µg/ml and 20 µg/ml, respectively. Thus, if one solvent has to be chosen for universal application against common plant pathogens, from our studies we can determine this to be ethanol. Ethanol, also being safe to be consumed or be present in agricultural or food products in trace amounts, can be safely used for plant pathogen treatment in agriculture.

Conclusion

We started this study with the expectation of determining whether the antimicrobial activity of the seaweed *K. alvarezii* could be effectively used against common plant pathogens, which could then serve as an alternative for the chemical treatments in agriculture in future. In this present study, the results confirmed that the seaweed *K. alvarezii* have a significant potential to counter the growth of plant pathogens under tested laboratory conditions. So, further research on identification of the particular antimicrobial substance and its purification is needed. Also, the dosage and methods of application need to be evaluated in future studies. Thus, this study paves way to further improvements in chemical-free agricultural practices.

Acknowledgement

This work was supported by the Gokula Education Foundation and Department of Microbiology, Ramaiah College of Arts, Science and Commerce. We thank the department and the college for their support. We are highly grateful to the fishermen in Kottaipattinam coastal area, Pudhukottai (Dist.), Tamil Nadu, for providing the algal samples. We are also thankful to the Dean and Head of the department of GKVK University, Bangalore, Karnataka who provided us with the plant pathogens.

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