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Study on metabolic compounds of *Kappaphycus alvarezii* and its *in-vitro* analysis of anti-inflammatory activity

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Amino acids, Alkaloids, Lipids, Triterpanoids, Hyaluronidase, *Kappaphycus alvarezii*

A B S T R A C T

The aqueous extract of *Kappaphycus alvarezii* was evaluated for antimicrobial activity and Minimum inhibitory concentration against human pathogens. The phytochemical analysis showed the presence of alkaloids, saponin, phenols, steriods, protein, phytosterols, aminoacids, sugars, reducing sugars, flavonoids, tannins and absence of terpenoids and anthraquinone. *Kappaphycus alvarezii* possesses significant anti-inflammatory activity probably due to inhibition of hyaluronidase enzyme. Therefore, it was worthwhile to review its anti inflammatory property to give an overview of its status to scientist both modern and ancient.

Introduction

Among marine organisms, seaweed is a promising candidate for drug production because it is relatively easy to obtain adequate, reliable, and most importantly, renewable supplies by aquaculture. They are rich source of structurally diverse bioactive compounds with valuable pharmaceutical potential (Ravikumar 2002, Sureshkumar 2002). This study deals with algae from sea host. Marine algae, the *Kappaphycus alvarezii* synonymously known as red algae is one of the most important sources, used in a variety of commercial applications as gelling, thickening, and stabilizing agents, especially food products such as frozen desserts, chocolate milk, instant products, yogurt, jellies, and in sauce preparation. *Kappaphycus alvarezii* is a tough, fleshy, firm marine algae ("seaweed") up to 6 feet in length.

Kappaphycus has been used in herbal medicine for its anti-inflammatory, diuretic, choleretic and hemostatic properties and has been approved for food use in South eastern Asia in Japan, Korea, and Taiwan. At present, there is a trend to use functional foods that provide health benefits by reducing the risk of chronic diseases, enhancing the ability to manage them and promoting better performance, consequently improving the quality of life. The richness of

the algae in minerals, vitamins, bioactive substances, proteins, lipids and polyphenols with antibacterial, antiviral and antifungal properties points to the potential as a supplement in functional foods or as a raw material for the extraction of bioactive compounds.

In India, where the use of spice is common, *K. alvarezii* powder has been indicated as an ingredient for the preparation of spice to enhance the nutritional quality because of the ash, the protein and crude fiber content, the high amount of Vitamin E and the small amounts of niacin and Vitamin B2. Kappa carrageenan derivatives from *Kappaphycus striatum*, especially the sulfated derivative at a high dose, exhibited enhanced antitumor activity compared to carrageenan oligosaccharides and promoted immunostimulatory activity, including on macrophage phagocytosis, and cellular immunity, especially on spleen lymphocyte proliferation in mices. Natural sulphated polysaccharides are capable of affecting the early stages of carcinogenesis and neoplastic progression in the period prior to the appearance of an invasive malignant tumor. This substance can be used as a drug carrier for therapy of malignant tumors and as a prophylactic with low toxicity, decreasing the side effects of cytostatic drugs.

The present study deals with the metabolic compound analysis of *Kappaphycus alvarezii*, antimicrobial activity, Mininimal Inhibitory Concentration against human pathogens and its *in-vitro* anti inflammatory activity analysis by Hyalurnidase inhibition assay.

Materials and Methods

The marine algae *Kappaphycus alvarezii* was collected from Pudhukottai District. The sample was identified by Scientist in charge, at the Centre for Marine and Fisheries Research Institution (CMFRI), Mandapam. The collected algae were stored in shade provision in the laboratory for further analysis.

Extraction of algal sample

The thalli of *K. alvarezii* were cut into pieces, shade dried and powdered in a grinder to 40- mesh size powder. The extraction was obtained by double distilled water. The extraction was filtered though Whatman No. 1 filter paper to obtain a particle free extract. The residue was re-extracted twice and filtered (to elute/collect the excess suspended molecules). The extracts were pooled, concentrated and freeze dried at -20^0C and the dried extract was used for exploring its further potential activity.

Phytochemical screening

Phytochemical screening of aqueous extract was carried out according to the standard methods as described by Trease and Evans (1989).

Test for alkaloids

0.5gm of lyophilized algal extract (sample) was stirred with 5ml of 1% aqueous hydrochloric acid on steam bath and filtered; 1ml of each of the filtrate was treated with a few drops of Mayer's reagent, Dragendorf's reagent and picric acid solution. Precipitation with any of the reagents was taken as preliminary evidence for the presence of alkaloid in the extract.

Test for saponins

About 0.5gm of sample was shaken with water in a test tube. Frothing, which persist on warming was taken as preliminary evidence for the presence of saponins.

Test for tannins

About 0.5gm of sample was stirred with 1ml of distilled water, filtered and a few drops of 1% ferric chloride was added to the filtrate. A blue-black, green or blue-green precipitate was taken as the evidence for the presence of tannins.

Test for anthraquinone

About 0.5gm of sample was taken and 5ml of chloroform was added and shaken for 5min. The extract was filtered and filtrate was shaken with equal volume of 10% ammonia solution. A pink violet or red colour in ammonical layer indicates the presence of anthraquinone.

Test for flavanoids

About 0.5gm of sample was treated with 2ml of 2% sodium hydroxide solution. An intense yellow colour turned to colourless on the drop wise addition of dilute acid. This indicates the presence of flavanoids.

Test for phenol

Equal amount of ferric chloride was added to the sample. Deep bluish green colour indicates the presence of phenol.

Test for steroids and terpenoids

About 200mg of sample was boiled in 10ml of chloroform and mixture was filtered. And 2ml of filtrate was added to 2ml of acetic anhydride and 2ml concentrated sulphuric acid. Blue green ring indicates the presence of steroids and red colour indicates the presence of terpenoids.

Salkowsky test

0.5gm of sample was dissolved in 2ml of chloroform. 2ml of concentrated sulphuric acid was carefully added to form a lower layer (chloroform layer). A reddish-brown colour at the interface indicates the presence of a steroidal ring.

Test for phytosterols

About 5mg of sample was filtered and add a few drops of acetic anhydride to the filtrate. Then add concentrated sulphuric acid through the walls of the test tube. The formation of brown coloured ring shows the presence of phytosterols in it.

Test for proteins

To 2mg of sample, 2ml of Millon's reagent was added and observed for two minutes for the formation of white precipitate. On gentle heating which may turned to red indicates the presence of proteins in it.

Test for amino acids

To 2mg of sample, 2ml of ninhydrin reagent was added. Violet colour indicates the presence of amino acids/ proteins in it.

Test for sugars

About 0.5 mg of the sample dissolved in water was taken. The volume was made up to 1 ml with distilled water. 4 ml of the anthrone reagent was added. It was heated for 10 minutes in boiling water bath with lids closed. The tube was cooled rapidly. Blue black colour indicates the presence of sugars.

Test for reducing sugars

To the 5ml of Benedict's reagent, 2mg of sample was added and boiled for 5min in boiling water bath. Red precipitate indicates the presence of reducing sugar.

Anti-microbial activity

Anti-microbial activity was determined by a Muller-Hinton agar method. The target bacterial strains used for screening antibacterial activity are *Staphylococcus aureus, Escherichia coli, Enterococci sp, Streptococcus pyogenes, Proteus, Pseudomonas, Actinomycetes, Candida albicans*. The bacterial strains were inoculated on nutrient broth and incubated for 24 h at 30±1°C. The sensitivity test of aqueous extract was determined using agar disc diffusion method (Bauer *et al.,* 1966). Wells are made in Muller Hinton agar plate using cork borer (5 mm diameter) and inoculums containing bacteria were spread on the solid plates with a sterile swab moistened with the bacterial suspension. Twenty and forty micro-liters of the working suspension/solution of extract and same volume of water for control were filled in the wells with the help of micropipette. Plates were kept for some time till the extract diffuse in the medium and incubated at 37°C for 24 h. After incubation, the plates were observed for the zone of inhibition (ZI), the diameter of the inhibition zone was measured and recorded.

Minimum Inhibitory Concentration

To determine Minimum Inhibitory Concentration (MIC) 1ml of nutrient broth was taken in 5 test tubes. Different sets were prepared for each bacterium. Different concentrations of algal extract ranging from 1mg to 5mg/ml were added into test tubes. To this 50µl of an overnight broth culture of each bacterium were inoculated in the respective tubes and the tubes were incubated for 24 hours at 37°C. One tube was inoculated with 1ml sterile nutrient broth with the 50µl of an overnight broth culture of each bacterium and placed at +4°C in a refrigerator overnight to be used as standard for the determination of complete inhibition. MIC is expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube. Because very faint turbidity may be given by the inoculum itself, the inoculated tube kept in the refrigerator overnight may be used as the standard for the determination of complete inhibition.

Thin layer chromatography

Preparation of chromo plate

The glass slides were cleaned and dried in hot air oven. Slurry was prepared by mixing silica gel with double the volume of distilled water in a clean beaker. One drop of slurry was placed on the slide by using another slide edge, the drop of slurry was scattered all over to make thin film. The slides were kept as such for few minutes. Then the chromo plates were activated by heating in hot air oven at 120°C for 30 min.

Loading of sample

The slides were allowed to cool at room temperature and marked about 2 cm from the bottom as the origin. The working suspensions were loaded at the center of the each slide above from the edge.

Development of chromatogram

The development tank was saturated with suitable solvent systems according to Eskil Hultin (1966).

Alkaloids : Benzene/ Methanol-80:20
Flavonoids : Chloroform/Methanol-70:30
Lipid : Chloroform/Methanol/water-10:10:3
Triterpeniods : Acetic acid/water-1:3

Aminoacids : Butanol/Acetic acid/Water- 4:1:1

The slides were kept in the tank without touching baseline by solvent. The final solvent front was marked and the slides were dried.

Spot visualization

For visualization of flavonoids 1% ethanolic solution of aluminium chloride was used and viewed under 560nm UV light. Alkaloids and triterpenoids were visualized under UV light and they were visible as yellow and orange fluorescent spots. Few pieces of iodine crystals were kept in the tank and covered with glass plate to saturate the tank with iodine vapor for detecting lipids. The plate was then kept in iodine vapor saturated tank and left for few hours and brown colored spots were visualized. For amino acids ninhydrin was sprayed on the plate and observed for purple color spots.

Retrieval of the active compound

Spots on the preparative silica gel slides were scratched with the help of clean and dry spatula and collected in beaker containing appropriate solvents (Joshi, 2011) and left overnight. The content in the beaker was stirred and filtrated through Whattman no. 1 filter paper. The filtrate was collected in clean and dry beaker. The filtrate containing active compound was used for the determination of antimicrobial effect against *in vitro* analysis of anti inflammatory activity.

Anti-inflammatory activity by hyaluronidase inhibition assay

3–5 U hyaluronidase in 100µl in 20mM sodium phosphate buffer (pH7) with 77mM $NaCl_2$, 0.1% BSA was pre-incubated with different concentration of the active compound and aqueous extract of *K. alvarezii* for 15mins at 37^{o}C. Assay was commenced by adding 100µl hyaluronic acid (0.03% in 300mM Sodium Phosphate (pH 5.35), to the incubation mixture and incubated for further 45mins at 37^{o}C. The undigested hyaluronic acid was precipitated with a 1ml of acid albumin solution made up of 0.1% BSA in 24mM sodium acetate and 79mM acetic acid (pH3.75). The mixture was kept at room temperature for 10 mins and the absorbance of the reaction mixture was measured at 600nm in colorimeter. Absorbance in the absence of enzyme was used as reference value of maximum of inhibition. Inhibitory activity of test compound versus absorbance in the absence of the enzyme was used for calculation. Indomethocine was used as reference standard.

Results and Discussion

Phytochemical screening

Phytochemical screening study of *K. alvarezii* extract revealed that the extract had significant quantity alkaloids, saponin, phenols, Steriods, protein, phytosterols, aminoacids, sugars, reducing sugars, flavonoids, steroids, tannins and absence of terpenoids, anthraquinone (Table 1).

Antimicrobial activity

Preliminary Anti-bacterial activity screening was reported to the algal sample. 20 and 40 µl of the working suspension/solution of algal extract and same volume of distilled water for control were filled in the wells with the help of micropipette. After incubation, the plates were observed for the zone of inhibition [ZI], the diameter of the inhibition zone was measured and recorded (Table 2 and Figure 1).

Minimum Inhibitory Concentration

Secondary screening of *K. alvarezii* against pathogens revealed the minimum inhibitory concentration of algal extract. The result was tabulated (Table 3).

Separation of active compounds

The chromatographic observation revealed the presence of amino acids, alkaloid, flavonoids, lipids and triterpenoids. Fig.2 showed the result for the thin layer chromatography. Table 4 represented the aqueous extract containing amino acids, alkaloids, flavonoids, lipids and triterpenoids with the Rf value of 0.09, 0.19, 0.65, 0.69, 0.87.

Hyaluronidase inhibition assay-*In vitro* anti inflammatory activity

Standard graph was plotted for indomethiocine using colorimetric reading at 600 nm. The reduction levels of Hyaluronidase enzyme in *in-vitro* studies were observed. The maximum inhibition was found in the crude aqueous extract (1.26 µg/ml) followed by amino acids 1.01 µg/ml, triterpenoids 0.86 µg/ml, alkaloids 0.69 µg/ml and flavonoids 0.54 µg/ml were observed and tabulated in Table 5.

Algae are very popular sea vegetables, and many people consume this vegetable as a health food in China, Japan, and South Korea. Recent studies have examined the biological and pharmacological activities of marine algae and it was shown to be a potentially prolific source of highly bioactive secondary metabolites and could be used for the identification of novel pharmaceutical agents (Ali, 2010).

Several bioactive compounds such as phlorotannins, diterpenes, polysaccharides, phytosterols, and phytopigments have been isolated from algae and many of these compounds have been demonstrated to possess numerous biological activities, including antioxidant, cytotoxic (Guardia *et al.,* 1999), hepatoprotective (Kim *et al.,* 2005), antiviral (Barbosa *et al.,* 2004), antifungal (Perry *et al.,* 1991) and antidiabetic properties (Lee *et al.,* 2004).

The data of *K. alvarezii* showed the presence of steroids group, unlike that reported by Vallinayagam*et al.,* 2009. These signals absent in the *K. alvarezii* confirming the presence of oxygenated steroidal identity and absence of all other groups as reported by Vallinayagam *et al.,* 2009. Sterols are important structural components of cell and organelle membranes of higher organisms. They regulate membrane fluidity and permeability as well as membrane-associated metabolic processes. Oxysterols have been ascribed a number of important roles in connection with inflammation, immunosuppression and development of gallstones (Panda 2009).

Hyaluronidase is an enzyme that degrades hyaluronic acid and chondroitin sulfate which are components of the extracellular matrix of connective tissue. By degrading the components of connective tissue, hyaluronidase promotes the spread of inflammatory mediators throughout these tissues, thereby contributing to the pathogenesis of inflammatory diseases such as allergic effects, migration of cancer cells, inflammation and the increase in permeability of the vascular system.

Although the oceans are a rich source of bioactive compounds, with hundreds of patents describing new bioactive marine natural products have been filed. Several marine natural products are currently in pre-clinical and clinical evaluation. Now-a-days plant based drugs for inhibiting cancer,

inflammation and glucose were emerging from different natural sources. Inflammation is a common phenomenon and it is a reaction of living tissues towards injury. Our report coincides with the ayurveda formulation. Our *in-vitro* studies demonstrated an appreciable anti-inflammatory activity, where further experiments can be performed in animal models to confirm the anti-inflammatory activity.

Table.1 Phytochemical analysis

Phytochemical constituents	**Observation**
Alkaloids	+
Saponin	+
Tannins	+
Anthraquinone	-
Flavanoids	+
Phenol	+
Steroids	+
Terpenoids	-
Phytosterols	+
Proteins	+
Amino acids	+
Sugars	-
Reducing Sugars	-

'+'= presence, '-'= absence of inhibition

Table.2 Antimicrobial activity of *K. alvarezii*

Test organisms	**Distilled water Zone of inhibition(in diameter)**	
	20µl	**40µl**
Salmonella typhi	0.6	1.3
Bacillus sp.	0.2	0.5
Escherichia coli	0.6	1.3
Enterococci sp.	0.5	1.2
Staphylococcus aureus	1.0	1.6
Proteus	0.7	1.8
Pseudomonas	0.9	2.1
Actinomycetes	0.4	1.2
Streptococcus pyogenes	0.6	1.6
Candida albicans	0.5	1.5

Table.3 Minimum inhibitory concentration of *K. alvarezii*

Test organisms	Distilled water Zone of inhibition(in diameter)				
	1mg	**2mg**	**3mg**	**4 mg**	**5mg**
Salmonella typhi	0.40	0.46	0.53	0.67	0.79
Bacillus sp.	0.39	0.45	0.56	0.68	0.82
Escherichia coli	0.41	0.57	0.63	0.73	0.79
Enterococci sp.	0.36	0.47	0.58	0.69	0.81
Staphylococcus aureus	0.40	0.43	0.54	0.59	0.72
Proteus	0.37	0.57	0.72	0.86	0.95
Pseudomonas	0.34	0.47	0.64	0.81	0.97
Actinomycetes	0.49	0.54	0.68	0.70	0.87
Streptococcus pyogenes	0.24	0.39	0.47	0.53	0.68
Candida albicans	0.45	0.51	0.68	0.72	0.84

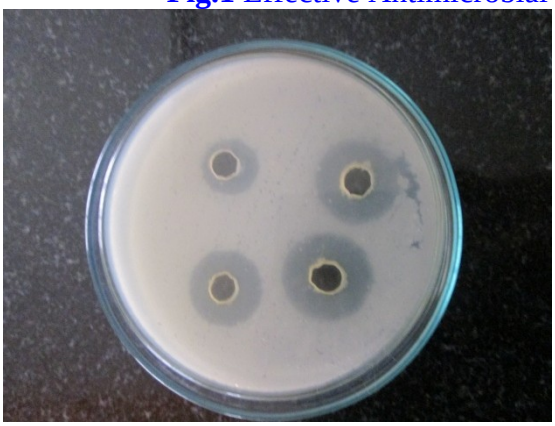
Table.4 Rf values of Phytochemicals in TLC

Phytochemical Components	**Rf values**
Flavonoids	0.65
Alkaloids	0.19
Lipids	0.69
Terpenoids	0.87
Aminoacids	0.09

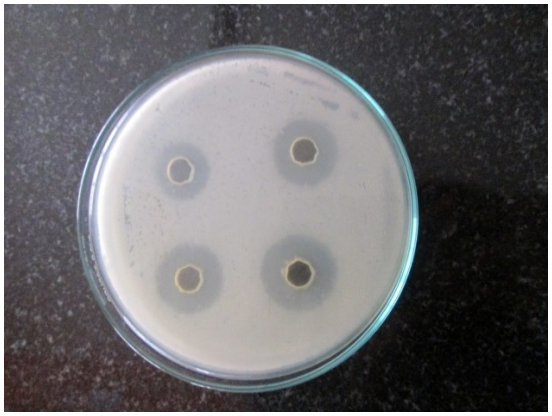
Table.5 Hyaluronidase inhibition assay

Samples	**Hyaluronidase Inhibition µg/ml**
Aqueous extract	1.26
Alakloids	0.69
Flavonoids	0.54
Triterpenoids	0.86
Amino acids	1.01

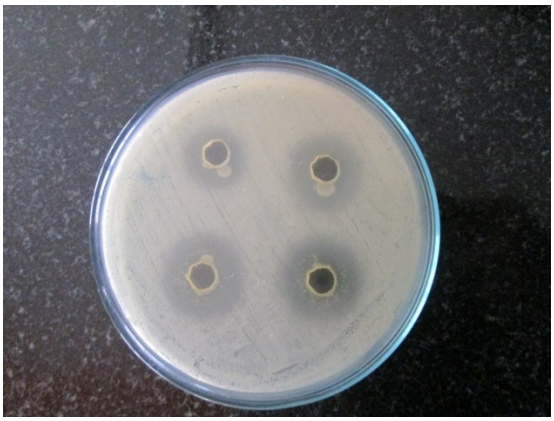
Fig.1 Effective Antimicrobial activity of *Kappaphycus alvarezii*



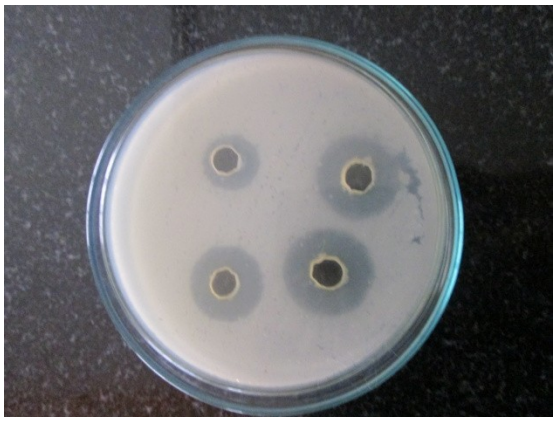
Salmonella typhi



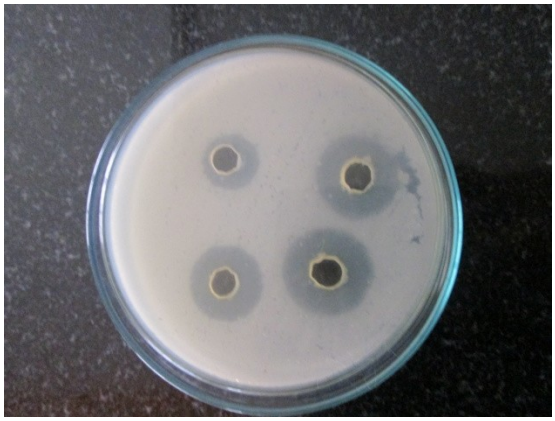
Candida albicans



Streptococcus pyogenes

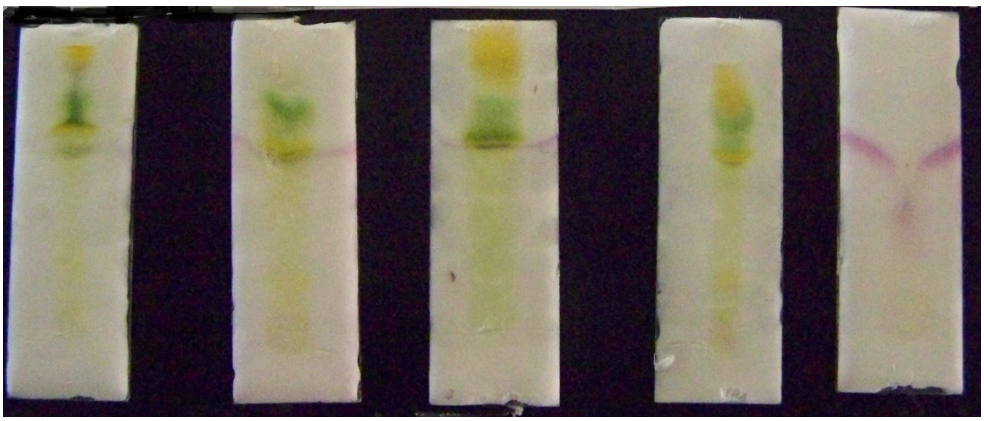


Proteus sp.,



Staphylococcus aureus

Fig.2 Separation of active compounds by thin layer chromatography



1 2 3 4 5

1- alkaloids, 2-flavonoids, 3- lipids, 4- triterpenoids, 5-aminoacids

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