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EVALUATION OF ANTI-INFLAMMATORY, ANTIDIABETIC, CYTOTOXIC ACTIVITY OF KAPPAPHYCUS ALVAREZII

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ABSTRACT

In recent years, a significant number of novel metabolites with potent pharmacological properties (antioxidant, antitumor, anti-HIV etc.) have been discovered from the marine organisms. *Kappaphycus alvarezii* (Doty). Being an invasive in the Gulf of Mannar after 2000 AD, has high content of steroid, phenol etc., this work was the first report of its medical application. The present study investigated the medicinal valve of red alga *Kappaphycus alvarezii* using *in vitro* studies. The algal methanol extract was screened for its antidiabetic evaluated by α- amylase; anti-inflammatory activity using hyaluronidase inhibition and cytotoxicity against the stannous chloride in *E. coli* AB 1157. Chemical structures of the isolated compound from the thalli of *K. alvarezii* were established by spectral techniques (UV, 125 H NMR). The methanol extract *of K. alvareziis* showed potent α-amylase inhibition activity than anti-inflammatory activity. The screening results suggested that the algal can be processed further analysis using animal models.

KEYWORDS: **antidiabetic; anti-inflammatory; *Kappaphycus*; phytochemicals, proton NMR**

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INTRODUCTION

Kappaphycus alvarezii (Doty [1]) (Rhodophyta, Gigartinales) synonymously known as *K. cottonii* is a red alga. This alga is an introduced species and a noxious aquatic weed in Hawaii. However, recent molecular studies have revealed the most abundant species in Hawaii reported to cause an invasion on the coral reefs is actually *Eucheuma denticulatum* and not *K. alvarezii* [2]. It is one of the most important sources of carrageenan used in a variety of commercial applications as gelling, thickening, and stabilizing agents, especially in food products such as frozen desserts, chocolate milk, instant products, yogurt, jellies and in sauce preparation. Aside from these functions, it is used in pharmaceutical formulations, cosmetics, and industrial applications such as mining [3]. *K. alvarezii* has been introduced into many different tropical places, since it is the main source of carrageen in the world. Several thousand coastal people can be benefited from its cultivation. Most notably, it is increasingly being cultivated on the Southeast coasts of India. *Kapppahycus spp. h*ave been reported to display antitumor activity [4], heavy metal chelation [5, 6], nitrate reductase activity [7], *in vivo* antioxidant activities [8], *in vitro* antioxidant activities [9, 10], antigenotoxicity against mercury induced DNA damage in fish [11]. Phytochemical studies on species demonstrated the presence of high protein, steroids, carotenoids, vitamins and minerals etc.,[8, 12]. Literature is scanty regarding the phytochemical and other application such as anti-inflammatory, antidiabetic activities studies. The present study was taken up to gather further knowledge regarding the effects of *K. alvarezii* extract on above mentioned activities, in order to establish pharmacological possibilities for its application. Furthermore, the present study evaluated the mutagenic effects of alga using *E. coli AB 1157*. Thus, the aim of the present study was to elucidate the potential beneficial role of marine algae as a food using *in vitro* test.

MATERIALS AND METHODS

Sample preparation

The marine alga *Kappaphycus alvarezii* was collected during August 2010, from the Mandapam coast (latitude 9˚ 17' N, longitude 79˚ 22' E), Gulf of Mannar. The sample was identified by Scientist in charge, at the Centre for Marine and Fisheries Research Institution (CMFRI), Mandapam and voucher specimen (DMCSKA01) was maintained in the Department museum. Adult male albino rats of Wister strain (240 ± 20 g) were obtained from the Animal Behavior Department, School of Biological Sciences, Madurai Kamaraj University, Madurai, India.

Algal extract preparation

The thalli of *K. alvarezii* were cut into pieces, sun dried and powdered in a grinder to 40-mesh size powder. The algae was extracted methanol water, vacuum dried and stored at -20^{0}C until use. The extract was filtered through Whatman No. 4 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 dried under vacuum and the dried extract was used for exploring its potential activity.

Phytochemical screening

Phytochemical screening of various extracts was carried out according to the standard methods as described by Trease and Evans [13] and Harbour [14] for alkaloids, tannins, flavonoids, steroids, saponins and cardiac glycoside. The residue was purified in silica column with mobile phase, Ethyl acetate: chloroform: acetone 3:4:3. The purified residues were characterized in proton NMR.

Anti-inflammatory activity by hyaluronidase inhibition assay

The assay was performed according to Ling *et al.,* [15] and Sigma Protocol. The assay medium consisting of 3 - 5U hyaluronidase (from Sigma –Aldrich, Bangalore) in 100 µl 20 mM sodium phosphate buffer pH 7.0 with 77 mM

sodium chloride, 0.01% BSA was preincubated with different concentrations of the test compound (in Dimethyl sulfoxide; DMSO) for 15 min at 37^{0}C. The assay was commenced by adding 100µl hyaluronic acid (from Sigma - Aldrich, Bangalore; 0.03% in 300 mM sodium phosphate, pH 5.35) to the incubation mixture and incubated for a further 45 min at 37^{0}C. The undigested hyaluronic acid was precipitated with a 1ml acid albumin solution made up of 0.1% bovine serum albumin in 24 mM sodium acetate and 79 mM acetic acid, (pH 3.75). After standing at room temperature for 10 min, the absorbance of the reaction mixture was measured at 600 nm. The absorbance in the absence of enzyme was used as the reference value for maximum inhibition. The inhibitory activity of test compound was calculated as the percentage ratio of the absorbance in the presence of test compound *vs.* absorbance in the absence of enzymes. The enzyme activity was checked by control experiment run simultaneously, in which the enzyme was pre-incubated with 5 µl DMSO instead, and followed by the assay procedures described above. Compounds were tested in a range of 10 -100 µg in the reaction mixture. Indomethacin was used as reference standard.

Anti-diabetic activity using amylase inhibition assay

The inhibition assay was performed using the chromogenic DNSA method [16]. The total assay mixture composed of 1400 µl of 0.05 M sodium phosphate buffer (pH 6.9), 50 µl of amylase (Diastase procured from HiMedia, Mumbai, Cat No. RM 638) and extracts at concentration 100, 250 and 1000 µg were incubated at 37°C for 10 min. After pre-incubation, 500 µl of 1% (w/v) starch solution in the above buffer was added to each tube and incubated at 37°C for 15 min. The reaction was terminated with 1.0 ml DNS reagent, placed in boiling water bath for 5 min, cooled to room temperature and the absorbance measured at 540 nm. The control amylase represented 100% enzyme activity and did not contain any sample of the analysis. To eliminate the absorbance produced by the sample, appropriate extract controls with the extract in the reaction mixture in which the enzyme was added after adding DNS. One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of maltose from starch per min under the assay conditions. The IC_{50} value was defined as the concentration of α-amylase inhibitor to inhibit 50% of its activity under the assay conditions. The inhibitory/induction property shown by the sample was compared with that of control and expressed as percent induction/inhibition. This was calculated according to the following formula.

$$\% \text{ inhibition/induction} = \frac{\text{Activity in presence of compound}}{\text{Control Activity}} \times 100$$

Cytotoxicity test

The *E.coli* AB 1157, a wild-type strain, proficient to repair damage in the DNA is considered for this study. Initially, the stock culture of bacteria was revived by inoculating in broth medium and grown at 37ºC for 18 h. The LB Agar plates (Tryptone-10 g, NaCl-10 g and Yeast extract 5 g, Agar 20 g in 1000 ml of distilled water) were prepared and wells were made in the solidified LB agar plate. Each plate was inoculated with 18 h old cultures (100 µl, 10^{-4} cfu) and spread evenly on the plate. After 1 day of incubation, the colonies of viable cells were counted and the percent of the survivors were calculated. The sensitivity of bacteria to Stannous chloride $SnCl_2$ (mutagen) was also checked in the plate test. The survival fraction of the bacterial was calculated by dividing N/N0, where N is the number of viable bacterial cells after

experimental time, N0 is the number if viable cells at the initial time.

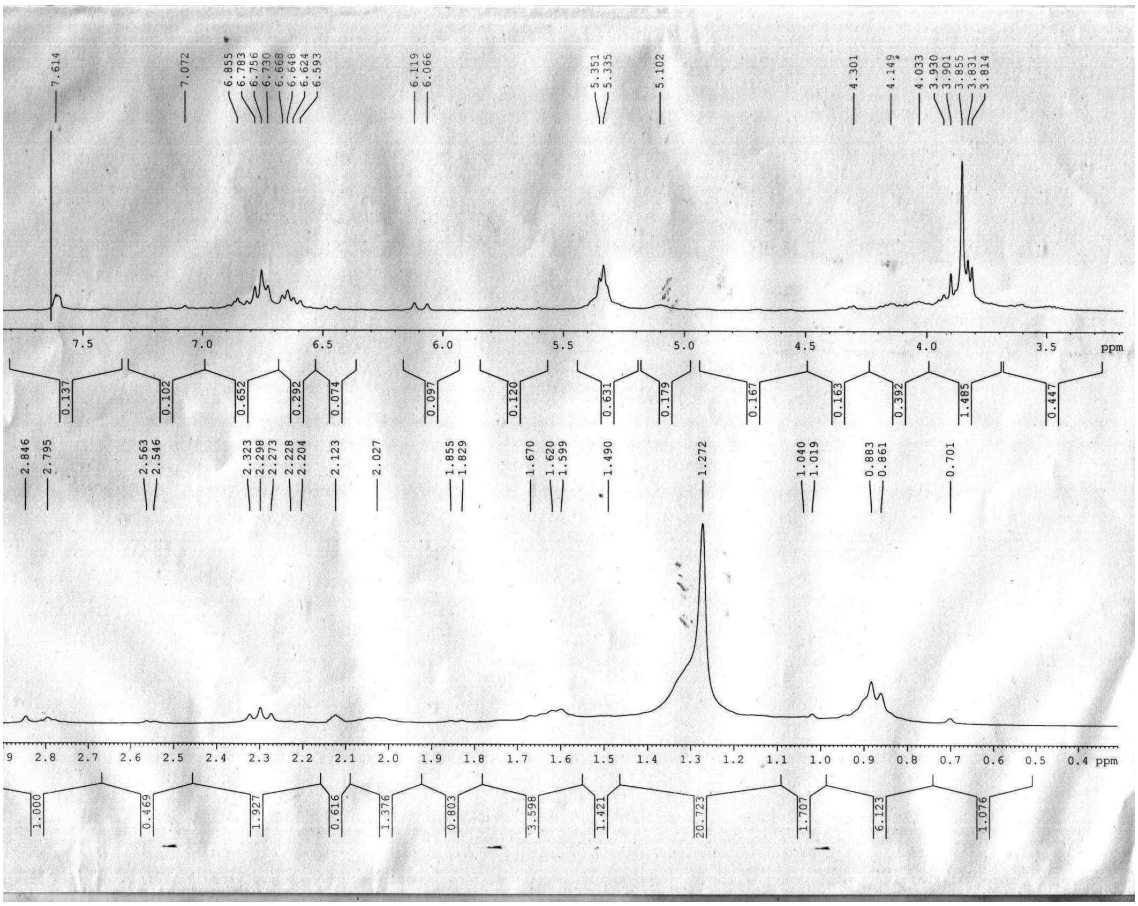
STATISTICAL ANALYSIS

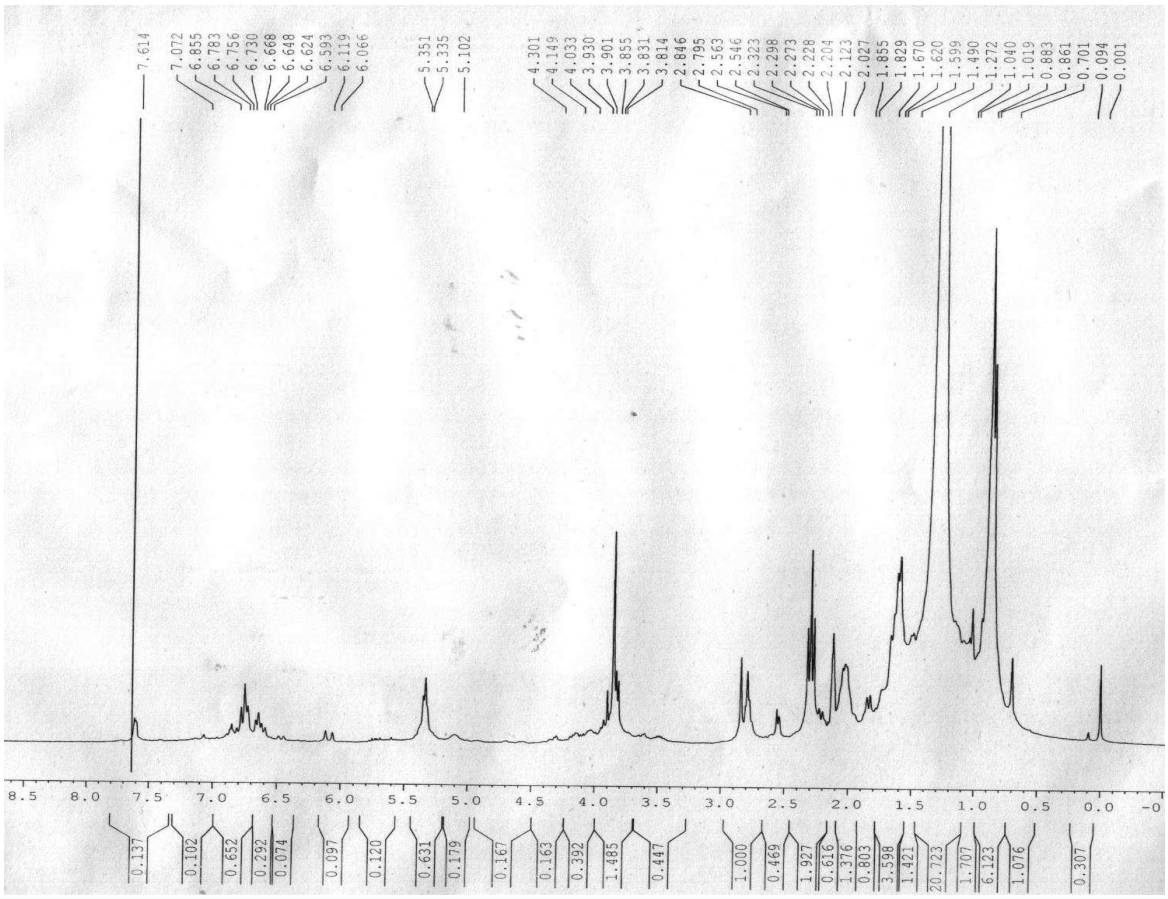
The results were expressed as mean ± SEM of three independent experiments. The one-way ANOVA test was used to analyze the result and $P<0.05$ was considered significant.

RESULT

Phytochemical screening

Phytochemical screening study of *K. alvarezii* extract revealed that the extract had significant quantity Alkaloids, Flavonoids, Steroids, Tannins and absence of Terpenoids. Total phenolic content was 28.47 mg GAE (standard, Gallic acid equivalent)/100 g dw; Protein was 1.623 ± 0.04 mg/g fresh weight; Carotene was 6.24 mg/100 gm. The extract was characterized by thin layer chromatography using Silica gel coated TLC plates, (Merck) with mobile phase, Ethyl acetate: chloroform: acetone 3:4:3. Chromatograms were evaluated under UV light at 600 to 660nm to detect the presence of steroids. The presence of steroid if any was further confirmed by spraying the plates with hot vanillin in sulfuric acid.





Figures 1a and 1b
Proton NMR of K. alvarezii extract which exhibited anti-inflammatory and anti-diabetic activity

The bioactive fractions against DPPH free radicals were dried. Residues of 30mg were dissolved in acetone solvent for proton NMR studies. The residue of *K. alvarezii* has a bunch of signals in the aliphatic region of the ^{1}H NMR spectrum (Figure 1a and 1b). As there were no responsible signals in the aromatic or olefinic region, the presence of flavonaoids in the extract was safely ruled out. Absence of signal in the regions between 4 to 6 indicated the absence of glycosides or unsaturated terpenoids. Obviously the signals in the region 0.5 to 2.0 ppm suggest the presence of steroidal identity in the extracts. The signal around 3.8 ppm could be due to the hydrogen geminal to oxygen probably hydroxyl group present in the steroidal nucleus. From the spectral data it was concluded that the compounds exhibiting activity are probably oxygenated steroids.

Anti-diabetic and anti-inflammatory activity

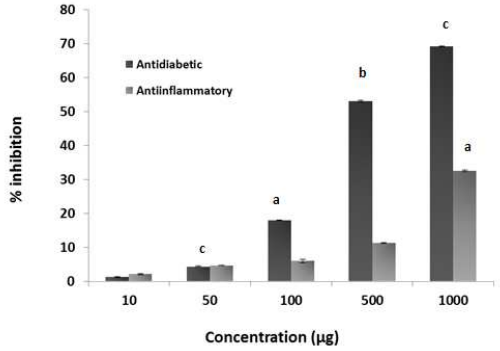


Figure 2

Anti-diabetic and anti-inflammatory activity of red alga K. alvarezii. Data was given in mean ± SD; n=5. Where letter denotes a (p >0.05); b (P > 0.01); c (P > 0.001) compared to control.

The anti-diabetic and anti-inflammatory activities of *K. alvarezii* were depicted in Figure 2. The test concentrations (10, 50, 100, 500, 1000 µg/ml) showed a concentration-dependent reduction in α amylase and hyaluronidase enzymes during our *in-vitro* studies. The maximum inhibition was observed for 1000 µg/ml concentration for both assays. The algal anti diabetic activity varied from 1.28 to 69.3% (with IC_{50} 481.2 ± 0.2 µg; R^2=0.9228) and its potential i luction of hyaluronidase enzymes ranged between 2.13 to 32.56 % (with IC_{50} 1.23 mg; R^2=0.749).

Cytotoxicity using E.coli AB 1157

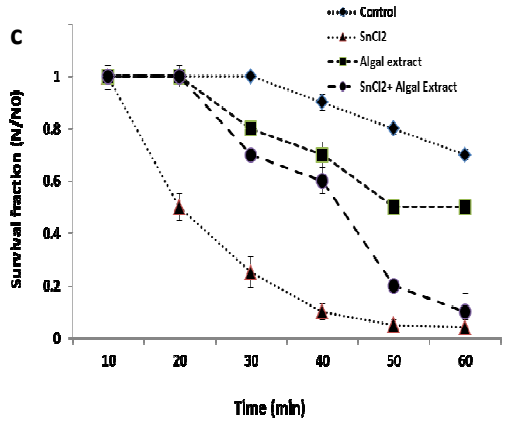


Figure 3

Effect of the *K. alvarezii* extract on the inactivation induced by stannous chloride on *E. coli* AB1157. Exponential growth of *E.coli* suspended in saline and treated with stannous chloride for different incubation times (min) related with the presence or absence of the extract and with the extract alone. Data was given in mean ± SD; n=5.

The survival level of *E.coli* AB 1157 was shown in Figure 3 shows the obscure action of $SnCl_2$. Moreover, it is verified that the presence of the algal extract was not able to protect the cultures against the cytotoxic effects of the reducing agent studied. The survival of the *E. coli* AB1157 culture was not influenced due to the treatment with the algal extract, when compared to the control. Minimum inhibitory concentration noted for *K. alvarezii* and $SnCl_2$ was 350 and 62.5 µg respectively.

DISCUSSION

The NMR data of *K. alvarezii* showed the presence of steroids group, unlike that reported by Vallinayagam *et al.,* [17] on proton-NMR signals corresponding to Poly unsaturated esters (7.263 ppm, 5.371 ppm and 1.254 ppm) in *Sargassum wightii*, (7.239 ppm, 5.322 ppm and 1.227 ppm) in *Gracilaria edulis*, and Poly saturated alcohol (7.23 ppm 1.256 and 1.226 ppm, 0.827ppm) in *Padina gymnospora.* These signals were absent in the *K. alvarezii* NMR data confirming the presence of oxygenated steroidal identity and absence of all other groups as reported by Vallinayagam *et al.,* [17]. 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 and oxyphytosterols, as present in the human body, may be derived from absorption of oxidized sterols present in the food, as well as from endogenous origin [18]. Oxysterols have been ascribed a number of important roles in connection with cholesterol turnover, atherosclerosis, apoptosis, necrosis, carcinogenisis, inflammation, immunosuppression and development of gallstones [19]. 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. 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. Blackberry fruits [20] and Green tea polyphenols [21] were reported to have anti-inflammatory activity. Our report coincides with the ayurveda formulation *Triphala guggulu* [22] and betal leaf [23]; but less than reported in *Ulva lactuca* and *Sargassum crassifolia* [24].

The plant-based α-amylase inhibitor offers a prospective therapeutic approach for the management of diabetes [25]. The α - amylase constitutes a family of endoamylases that catalyse the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glycosidic bonds [26]. Therefore, the antidiabetic effect of alga might attribute to its inhibitory effect against a- amylase that retarding the digestion of carbohydrate to delay the postprandial rise in blood glucose. Our *in vitro* studies demonstrated an appreciable α-amylase inhibitory activity, where further experiments can be performed in animal models to confirm the hypoglycemic activity and anti-inflammatory activity. It is a safe and effective intervention for diabetes. In recent years there has been greater interest in investigating compounds originating from plants and their effects on DNA. Stannous chloride ($SnCl_2$) is used in nuclear medicine as a reducing agent to obtain technetium - 99 m radiopharmaceuticals. It has been reported that natural products might reduce the genotoxic and cytotoxic effects related to $SnCl_2$.It is suggested that, possibly, this extract could prevent: (i) the direct action of the stannous ions on the cultures, and/or (ii) the indirect action of $SnCl_2$ through the generation of free radicals. These results are in accordance with what has been previously reported in a similar study using rutin [27]. Other authors have reported that some vegetal extracts protect the *E. coli* strain AB1157 against the damage caused by stannous ions [28 - 30].

CONCLUSION

In summary, inhibition of hyaluronidase and α amylase activity by the algal extract may be an important mechanism underlying its chondroprotective effects. Notably, it, is a weak inhibitor of both hyaluronidase. Future studies will address the active molecules of alga which cause hyaluronidase.

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