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Primary Phytochemical Analysis Of *Kappaphycus Sp.*

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ABSTRACT: *Kappaphycus sp*., an edible seaweed from the sea coast of Rameswaram, India was analysed for its primary phytochemical analysis. Chemical composition was carried out for estimation of proteins, fatty acids, β-carotene and sterol compounds. β-carotene was estimated through high performance liquid chromatography where as fatty acids and sterol compounds were determined using gas chromatography technique. From the standard graph, the protein was estimated as 0.169gm/ml indicating that the protein content is quite high in red algae. Sterols were estimated on the basis of chromatographic peak areas for each with respect to total sterol peak area. The predominant sterol identified is cholesterol. From the qualitative analysis of β-Carotene, it was observed that one compound is present in large besides other impurities. Results of this study suggest the utility of *Kappaphycus sp.* for various nutritional products for use as health food or nutraceutical supplement.[Journal of American Science 2009:5(2) 91-96] (ISSN: 1545-1003)

Key word: Kappaphycus sp, Antibacterial, Protein, Fatty acid, Sterol, β-carotene

1. Introduction

Marine organisms are a rich source of structurally novel and biologically active metabolites. Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry. Seaweeds have some of the valuable medicinal value components such as antibiotics, laxatives, anticoagulants, anti-ulcer products and suspending agents in radiological preparations. Fresh and dry seaweeds are extensively consumed by people especially living in the coastal areas. From the literature, it is observed that the edible seaweeds contain a significant amount of the protein, vitamins and minerals essential for the human nutrition [Fayaz et al., 2005]. Marine sources are receiving much attention mainly because of the contents of functional ingredients such as polyunsaturated acids, β-Carotene and their pigments Carotenoids, sulphated polysaccharide (antiviral), and sterols (antimicrobials). Among the different compounds with functional properties, antioxidants are the most widely studied. Sterols are an important family of lipids, present in the majority of the cells. Because of different routes of synthesis, sterols from plants, fungi and animals show marked differences. Brazilian red algae have been found to have phenolic substances. Oxidative stress is an important factor in the genesis of pathology, from cancer to cardiovascular and degenerative disease. Marine algae are the excellent source of bioactive compounds such as carotenoids, dietary fibre, protein, essential fatty acids, vitamins and minerals [Viron et al., 2000, Sanchez-Machado et al., 2002, Fayaz et al., 2005]. Fayaz et al. (2005) suggested the utility of *Kappaphycus alvarezzi* for various nutritional products including antioxidant for use as health food or nutraceutical supplement. Sanchez-Machado et al. (2004) found that the predominant sterol was desmosterol in red seaweeds (87-93% of total sterol content). Tasende (2000) confirmed that fatty acids and sterol of algal class, families and sometimes even species are characteristics to those particular taxa and could be useful as chemotaxonomic. Cholesterol generally plays a structural role in the cell membrane [Nabil and Cosson, 1996]. Clinical studies have demonstrated that dietary intake of plant sterols (as part of the normal diet, or as a supplement) may help reduce blood cholesterol levels [Dunford and King, 2000]. Sterols are thus among the nutritionally important lipids that need to be routinely determined in foods. Plant sterols have been quantified by gas chromatography [Govindan and Hodge, 1993, Jeong and Lachance, 2001] or by HPLC with UV detection [Indyk, 1990] or evaporative light scattering detection. However, few studies have presented techniques for parallel determination of different sterols. Further, it is observed that gas chromatography/mass spectrometry techniques are widely employed for identification of sterols. Microalgal metabolites have attracted attention for two main reasons, first, because they are the source of toxins in harmful algal blooms and secondly because they are a potentially rich source of new drug candidates. Many of the microalgal metabolites have chemical structure and possess interesting biological activity. Present investigation aims at estimation of proteins, β-carotene, fatty acids and sterol compounds from *Kappaphycus sp*.

2. Materials and methods

Sample was collected from the sea coast of Rameshwaram, Tamil Nadu, India in the form of dry and living sample. Algae samples were cleaned at epiphytes and necrotic parts were removed. Samples were rinsed with sterile water to remove any associated debris. Sample was kept under sunshade for 7 days. After drying the sample, it was ground thoroughly to powder form. The powder was then used for the primary estimation of proteins, fatty acid, β-carotene, sterol compounds and antimicrobial test. This powder was stored in cold conditions in an airtight container and analysis was carried out within three months of processing. Total protein content, fatty acids, sterols and beta carotene analysis in *Kappaphycus sp*. were determined by the respective methods.

2.1 Quantitative estimation of proteins

One gram of sample was taken and homogenized in a prechilled mortar and pestle with ice cold Tris buffer (0.1M pH-7). The extract was centrifused at 8000 rpm for 10 minutes and the supernatant was collected. It was added with 10% TCA (twice the volume) and kept overnight at 40°C. The sample was centrifuged at 8000 rpm for 10 minutes and the pallet was collected, dissolved in Tris buffer and was used for the estimation of protein. The final protein extract was used to measure the absorbance at 595nm.The absorbance was recorded 3 times by taking 1ml each at 595 nm. It is found out to be 0.316, 0.312 and 0.320. The average was taken as 0.316.

2.2 Analysis of fatty acid composition

Dried samples (2.50 g) were extracted with trichloromethane-methanol (2.1v/v) in a soxhelt apparatus for 48 h. The extract was evaporated under vacuum. Ethanol with 6% KOH was added to the residue and the reaction mixture was saponified by refluxing at 100^0C for 1 h with pyrogallol. The mixture was cooled, concentrated under reduced pressure and thereafter H_2O and ethanol were added and extraction with ether was repeated three times. The aqueous alkaline fraction was acidified with 6N HCl to pH 1 and then extracted several times with hexane. The organic fraction was dried over anhydrous Na_2 So_4 and evaporated under reduce pressure.

The fatty acid fraction was dissolved in methanol-H_2 So_4 (97.5:2.5 v/v) with pyrogallol methylated. The fatty acid methyl esters were extracted with hexane:ether (2:1 v/v). The organic fraction was dried over anhydrous Na_2 So_4 and concentrated under vacuum. The fatty acid methyl esters were purified by silica thin layer chromatography (TLC) and analyzed on a Varian 3400 CX gas chromatograph equiped with a flame ionization detector and a OV-1 Interchin column (30 m X 0.32mm). The column temperature was programmed from 180 to 200^0 C at a rate of 1 ^{0}C min^{-1} and from 200to 240^0 C at a rate of 2^0C min^{-1}. The injection temperature was 270 ^{0}C and the detector temperature was 300^0C. The gas carrier was helium at a pressure of 1 bar. Methyl esters were identified by comparison of the retention times with those of heptadecanoic acid and by comparison with authentic standards.

2.3 Analysis of sterol composition

Dried samples (2.50g) were extracted in 2:1 (v/v) dichloromethane-methanol using a Soxhlet apparatus for 24 h. The extract was concentrated under reduced pressure and fractionated, in order to obtain total sterols, by preparative TLC on Silica gel plates (0.25 mm)developed in the first dimension in 92:8 (v/v) hexane-ethyl acetate (18 cm length of run) to separate steryl esters (SE), and, in a second dimension in 90:10:0.5 (v/v) dichloromethane-methanol-water(12 cm length of run) to separated free sterols (FS), acylated steryl glycosides (AGS) and steryl glycosides (GS) (*27*). The SE, FS, AGS and GS bands were located according to Rf values of standards simultaneously chromatographed: cholesterol palmitate, cholesterol (Sigma), Cholesterol-3-0-6-palmitol, palmitoyl-glucopyranoside and cholesterol-3-0-glucopyranoside (Matreya, Pleasant Gap, USA). Spots of standars were visualized with Liebermann-Burchard reagent (Rf=0.95, 0.07, 0.05 and 0.35 respectively). The different bands were scrapped off and eluted with dichloromethane SE and FS with 2:1 (v/v) dichloromethane-methanol for AGS and GS. The SE were saponified by 1 h reflux with 6% (w/v) methanolic KOH with 0.5% (w/v) pyrogllol. The AGS and GS were separately hydrolyzed in a 1% (v/v) ethanolic $H_2SO_4$by refluxing for 4 h. Sterols were dried in a stream of nitrogen gas and purified by silica gel TLC plates developed in dichloromethane with colesteryl acetate as the standard. Identification was determined as mentioned above. Resulting total steryl acetates were analyzed on a Carlo Erba 2900 gas chomatograph coupled with a Jeol JMS-D300 mass spectrometer. Gas chromatography was carried on 30 m x 0.32 mm fused silica capillary column with a 0.1 mm film of DB5. The column was operated at a pressure of 0.5 bars of helium. Temperature was increased from 260 to 278^0 C at a rate of 2^0 C min^{-1} then to 300^0 C at a rate of 5^0C min and held at 300^0 C for 10 min. The injector temperature was held at 275^0 C and the detector temperature 300^0 C. Electronic impact mass spectra was measured at 70 eV and an ionization temperature of 150^0C. Identifications were based on the retention times of the steryl acetates relative to cholesterol acetate and their mass spectra. For quantification, 5 a cholestane was added to each

sample as internal standard. Relative abundances of sterols were calculated by measurements of spectra which were proportional to content.

2.4 Extraction of β-carotene

β-carotene was extracted following the method described by Hart and Scott (1995) with some modifications. In this method triplicate samples of approximately 5 g were taken for – β-carotene extraction. The samples were extracted with tetrahydrofuran/methanol (1:1, THF:MeOH) followed with petroleum ether (40±60◦C) and antioxidant, 0.1% butylated hydroxytoluene. (BHT). During the extraction, 10% NaCl was added to give a better separation between the organic and aqueous phase. The aqueous phase was extracted twice with peteroleum ether and the washings were added together. Saponification was performed with addition of 40% KOH/MeOH to.
tetrahydrofuran/methanol (1:1, THF:MeOH) followed with petroleum ether (40±60◦C) and antioxidant, 0.1% butylated hydroxytoluene. (BHT). During the extraction, 10% NaCl was added to give a better separation between the organic and aqueous phase. The aqueous phase was extracted twice with peteroleum ether and the washings were added together. Saponification was performed with addition of 40% KOH/MeOH to the extract, with a flow of nitrogen gas and was kept in the dark at room temperature for an hour. Saponification eliminated chlorophyll pigments and hydrolyzed carotenoids esters which would interfere in the HPLC chromatographic process. The carotenoid was extracted from the KOH/MeOH phase with petroleum ether and washed with distilled water until pH was neutral. The extract was dried by rotatory vacuum evaporation and was diluted again with petroleum ether and dichloromethane to a volume of 5 ml. **β**-carotene content in the sample extract was determined by reverse phase high performance liquid chromatography (HPLC) method. Recovery experiments were also performed in which standard solution of beta-carotene was added to the tested sample.

2.5 High performance liquid chromatography Separation of β-carotene

Quantitative analysis on the amount of β -carotene present was performed using HPLC μa reverse phase column, Waters m-Bondapak C18 column (30cmX3.9 mm i.d.) operated at 30^{o}C. The column was preceded by a Waters Guard-Pak pre-column module housing a disposable Guard-Pak pre-column insert packed with the same material as that in the analytical column. A Waters 510 pump was used to deliver the mobile phase which was a ternary mixture of acetonitrile, methanol, dichloromethane MeCN:MeOH:DCM) 75:20:5 v/v/v, containing 0.1% BHT and 0.05% trie-thylamine (TEA), a solvent modifier and prepared fresh daily. The flow rate was 1.0 ml/min. Solvents for liquid chromatography were of HPLC grade. All solvents for use as the mobile phase in HPLC were filtered through a 0.45 mm cellulose membrane filter and degassed using an ultrasonic bath. β -carotene standard was purchased from Sigma Chemical Company and a concentration 0.2 mg/ml was prepared diluted in the mobile phase and 20 μl injected into HPLC. Peak responses were determined at 450 nm with a variable wavelength programmable photodiode array UV detector (Waters 994) and Waters 520 printer plotter. β-carotene peak was identified by its retention time and compared with that of pure β-carotene standard. Twelve sample extracts were analyzed. Thin layer chromatography (TLC) and UV± vis absorption spectrophotometry were also used to aid in the identification of β -carotene.

Statistical Analysis

For all analysis (fatty acid, sterols), the mean and standard deviation of replicate trials were performed and values were reported as ±SEM.

3. RESULTS AND DISCUSSION

3.1 Quantitative analysis of Protein

Quantitative analysis of protein was carried out and the values of optical density corresponding to standard concentration are provided in Table 1. From Table 1, it can be observed that the protein content is quite high in red algae. It can serve as functional food with vital nutritional and biological values.

Table 1 Standard Concentration versus Optical Density table for Protein

OD value for the protein at 595 nm	
Concentration of protein(μg)	**Optical Density(OD)**
10	0.101
20	0.111
30	0.122
40	0.132
50	0.143
60	0.149
70	0.157
80	0.164
90	0.169
100	0.175

From the standard graph the protein was estimated as 0.169gm/ml (Table 1). Fayaz et al. (2005) mentioned that the protein content is 16.24±0.04 in *Kappaphycus alvarezzi,* were determined by standard AOAC methods.

The difference could be due to geographical and seasonal variations. Strong variations of protein content were observed according to the season in the following species; palmariapalmata, 22.0%±1.3, porphyra, 25.8%±1.5, chondruscrispus, 25.5%±1.1, gracilaria, 22.5%±1.1.

3.2 Analysis of Fatty acid composition

Fatty acid analysis shows that the compounds present in *Kappaphycus sp*. are, Caproic acid, Caprylic acid, Methyl heptanonaite, Butanoic acid, Ethanoic acid, Octadecic acid, Octadecatrienoic acid, 4-Methylo atanonaite (Fig. 1). The relative percentages of identified fatty acids are presented in Table 2. From the identified eight fatty acids, Caprylic acid contributes highest composition. The relative quantity of Octadecatrienoic acid is in lesser quantity among all identified fatty acids. The composition of fatty acid may be useful for taxonomic purposes.

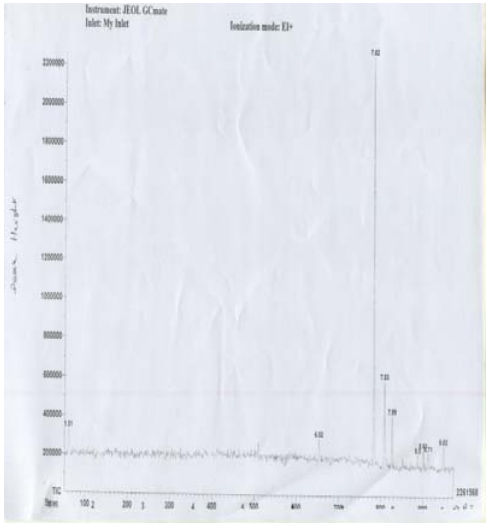

Fig. 1 Fatty Acid Estimation (Gas Chromatography)

Table 2 Relative levels of fatty acid in *Kappaphycus sp*.

Fatty acid	Mean±S.E.
Caproic acid	32±1.15
Caprylic acid	82±1.64
Methyl heptanonaite	26±0.123
Butanoic acid	15±1.123
Ethanoic acid	11±0.28
Octadecic acid	11.1±0.12
Octadecatrienoic acid	6.1±0.058
4-Methylo atanonaite	9±0.18

Fayaz et al. (2005) conducted experiments on red algae and found that gas chromatography analysis of sample extract showed the presence of fatty acid mainly containing heptadecanoic acid is 34.24%. This difference may be due to environmental effect. The quantitative estimation of the fatty acid content of c.crispus revealed that gametophytes had larger amounts of fatty acid (0.71±0.01) than sporophytes (0.622±0.004) (*11*). Many micro algae have been shown to be a good source of LCPUFA (*32*). An investigation on the fatty acid content of brown algae from Indian coast has shown the presence of only nine fatty acids, palmitic acid as the major constituent and the absence of lauric acid in the alga is notable and distinct [Dhamotharan, 2002]. Jaysankar and Kulandaidelu (1999) have shown variations in the levels of fatty acid in different variations and species of gracilaria. Dhamotharan (2002) have shown quantitative variations in the fatty acid content of sargassum collected during winter and monsoon months.

3.3 Estimation of Sterols content

Table 3 shows the percentage sterol composition of the seaweeds analysed, estimated on the basis of chromatographic peak areas for each with respect to total sterol peak area. Sterols in the non-saponifiable fractions of the seaweed samples were identified by comparison of retention times and UV absorption spectra with those obtained for corresponding standards (cholesterol, Methylenecholesterol, stigmasterol, camposterol, and β-sistosterol). For determination of retention times, the reference standards were injected both individually and as a mixture (Fig. 2). From Table 3, it can be observed that the predominant sterol is cholesterol. Cholesterol has previously been found to be present at high concentrations in Caribbean red seaweeds (Govindan and Hodge, 1993) and similar observations were reported by Beastall et al., (1971). The content of stigmasterol is less among all the sterols identified in *kappaphycus sp*. Rao et al. (1991) have shown similar variations in the sterol content of the algae. Goad and Goodvin (1972) observed that the proportion of cholesterol and desmosterol can vary sample to sample in a given species with some samples containing only cholesterol or both c27 sterols. Sanchez-Machado (2004) determined in canned or dried brown seaweed and red seaweeds. The predominant sterol was fucosterol in brown seaweeds (83 to 97% of total sterol content) and desmosterol in red seaweeds (87 to 93% of total sterol content) [Dhamotharan, 2002].

Rival variation in sterol composition among different seaweeds possibly reflecting physiological differences under or environmental influences. Marine algae have shown to be good source of unsaponofiable, non toxic sterols that have medicinal value [Orcut, 1970]. The studies of Combaut et al., (1985) have shown cholesterol to be the predominant sterol of red algae and fucosterol in brown algae. Rao et al., (1991) have shown higher levels of sterols in dried samples of three species of sargassum as compared to fresh and frozen samples. In the present study, according the relative distribution

of sterols cholesterol content is high. Stigmasterol is less.

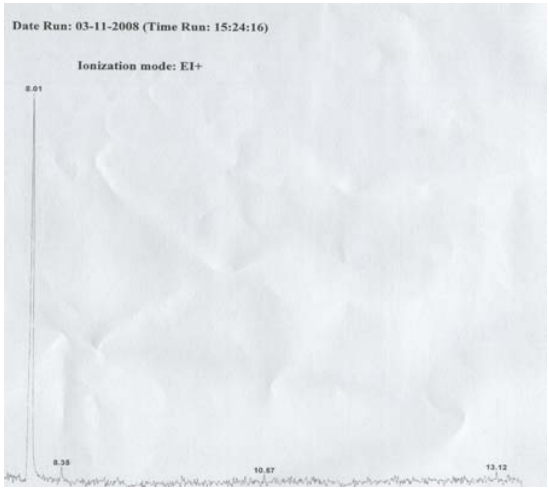

Fig. 2 Sterol Estimation (Gas Chromatography)

3.4 Analysis of β-Carotene

From HPLC result of β-Carotene shows that one compound is present in large besides other impurities (Fig. 3). The red colour of these algae results from the pigments phycoerythrin and phycocyanin; this masks the other pigments, Chlorophyll *a* (no Chlorophyll b), β-carotene and a number of unique xanthophylls. The main reserves are typically floridean starch, and floridoside; true starch like that of higher plants and green algae is absent. The walls are made of cellulose and agars and carrageenans, both long-chained polysaccharide in widespread commercial use. There are some unicellular representatives of diverse origin; more complex thalli are built up of filaments. Fayaz et al., (*1*) determined HPLC chromatogram of β-Carotene in extracted sample of *Kappaphycus alvalerez*. It showed the β-Carotene peak (RT=9.11/minute). The concentration of β-Carotene was 5.26 mg/100 gm sample. In the present study, the β-Carotene peak is observed to be 12.94 /minute. The concentration of β-Carotene is 2.5 mg/50 gram sample. Difference between the RT values of the above study and in the present study may be due to the influence of environmental conditions of the habitat over the physiology and biochemistry of the algae in the marine eco system, which indicates by the seasonal and geographical variations observed in the proximate composition of the algae. Dave et al. (1987) and Dhamotharan (2002) have studied 29 genera of red algae from Gujarat coast of India and showed monthly variations in their crude protein levels. Variations may be due to seasonal and biochemical composition of the algae.

Table 3. Relative distribution of Sterols

Sl. No.	Sterol	Mean± S.E.
1	Camposterol	1.03±0.11
2	Cholesterol	83.44±0.33
3	Stigmasterol	0.44±0.02
4	β-Sistosterol	0.75±0.03
5	Methylenechole sterol	0.65±0.01

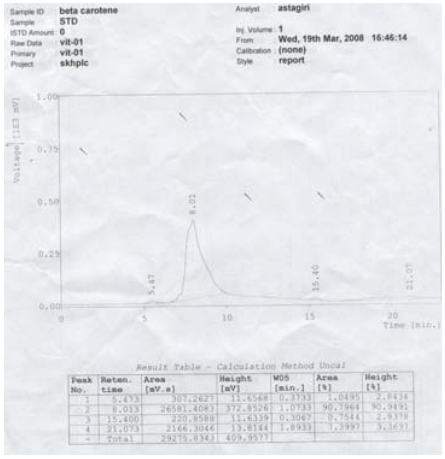

Fig. 3 β-Carotene Estimation (HPLC)

4. Conclusion

Investigation was carried out on primary metabolites of *Kappaphycus sp*. The metabolites include proteins, fatty acids, sterols and β-Carotene. From the studies, it is concluded that the *Kappaphycus sp*. located in the sea coast of Rameswaram, India, can be used as antibacterial product and serve as a food product, since it contains rich proteins, exhibits high cholesterol content, presence of one large compound of β-Carotene and variety of fatty acids. From the findings, it is also observed that the primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry. Results of this study suggest study that the utility of *Kappaphycus sp.* for various nutritional product for use as health food or nutritional supplement. The remarkable differences between our results and the results obtained in the previous studies may be due to several factors. Because of the intraspecific variability, occasionally related to seasonal variations as observed in the literature and at the same time test materials have trace impurities. Finally, we conclude that macro algae from the coast of Tamilnadu are potential source of bioactive compounds and should be investigated for antibiotics. However, further work is required to identify the bioactive compounds which will show antioxidant activity against fungi and viral related pathogens..

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