Research Article

Extraction of Crude Protein from *Sargassum crassifolium*, Harvested from South Coast of Sri Lanka and Determination of Functional Properties of the Crude Extracts

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Abstract

Sargassum crassifolium is an edible brown-seaweed which contains many bioactive compounds. The study was developed to extract crude proteins using four different methods and determine the functional properties. SDS-PAGE was used to analyze the extracts and best methods were selected. Accordingly, water (1:3), salt (4%), alkaline (1:3) and ethanol (1:4) extractions were selected as the best methods to extract crude protein from S. crassifolium. Among them, salt extract showed the highest protein content ($p<0.05$). Each treatment has shown low intensity band patterns. Ethanol extract showed highest iron chelating and low oxidation levels. Salt extract showed higher DPPH activity while all extracts showed less antibacterial activity ($p<0.05$). Therefore, we can conclude that crude extracts from S. crassifolium showed effective free radical scavenging, metal chelating and low antibacterial activities.

Keywords*: *Antibacterial, Antioxidant, Crude protein, Extraction, Metal chelating

1. Introduction

The world population is continuously growing and it will be reached to a capacity of 9.6×10^9 by 2050 (Gerland *et al.*, 2014). Therefore, the demand for food is increasing. In order to fulfill the growth demand, novel resources and novel methods are required to increase the food production. As a result of increasing the world population, global demand for plant and animal protein are also increasing (Kazir *et al.*, 2019). Marine fauna and flora species have high demand as a food source and health ingredients. Recently, global production of seaweed has become a popular terrestrial agricultural resource due to its high demand as a food source with a different variety of nutrients. It also helps to fulfill the excessive protein demand in the world.

Seaweeds are forms of large, fast-growing, highly productive photosynthetic marine macroalgae that have a simple life cycle and they are highly abundant in shallow

water, near the edge of the world's ocean (Abbott *et al.,* 1992). There are 221 known seaweed species in the world and out of them, 2/3 are reported in food applications (White and Ohno, 1999). The chemical profile of seaweed contains a high nutritional value which is vital for human nutrients, such as proteins with all essential amino acids, minerals and vitamins. In addition to that, they contain bioactive metabolites and numerous different unique compounds (Cardozo *et al.,* 2007; Wong and Cheung, 2000; Cho *et al.,* 2009; Artan *et al.,* 2008; Choi *et al.,* 2009; Zvyagintseva *et al.,* 2005). Calcium and protein content of seaweed varies from species to species, but it contains a low-fat level. Green and red seaweed contain higher protein levels (up to 30%) than brown seaweed (Kolanjinathan *et al.,* 2014). However, the protein content in seaweeds depend on the habitat and factors such as depth. Accordingly, protein content changes among seaweed genera and different species of the same genus (MacArtain *et al.,* 2007).

In Asian countries seaweeds are mainly used to enhance the flavor of food while western countries use it as a food additive or extract (de Carvalho *et al.,* 2009). Seaweeds were widely analyzed by scientists during the past decades and their health promoting benefits have been identified (Scalbert *et al.,* 2005). Literature reviews highlight several disease preventive attributes of sea weeds such as reduction of the risk of inflammation (Ibanez and Cifuentes, 2013), cardiovascular diseases (Kumar *et al.,* 2008; Murray *et al.,* 2018), diabetes (Celikler *et al.,* 2009; Murray *et al.,* 2018; Nwosu *et al.,* 2011), microbial contamination (Lopes, 2014), neurodegenerative diseases (Barbosa *et al.,* 2014). It delivers a multitude of functionalist ranging from a simple nutritional improvement to physiologically complex mechanisms.

Accordingly, seaweed intensively considered as a source to fulfill the growing protein requirement through nutraceuticals and functional food (Wells *et al.,* 2017). Nutraceuticals play a major role to induce favorable physiological functions, to develop the welfare and declines the chances for particular aliment (Mazza, 1998). To promote health conditions of human, prepare the functional food or nutraceuticals that obtain from traditional sources. Since the methods of extraction from seaweed have recently developed and these types of products consumed as a whole food or as a supplement and mostly these are sold as pills or tablets (Liang, 2004).

Seaweed proteins consist of significant amounts of all essential amino acids, which important to the food industry. Seaweed proteins are important for the food industry due to the high levels of all essential amino acids present in them. In the industrial level lecithin and phycobiliprotein are bioactive algal proteins which are used in several industrial applications. Lecithin involved in several biological processes like host-pathogen interaction, cell–cell communication, cancer metastasis and antiviral activities and used to blood grouping and cancer biomarkers (Ziołkowska and

Wlodawer, 2006). Phycobiliprotein used in fluorescent labeling, flow cytometry, fluorescent microscopy and fluorescent immune histochemistry like different activities (Kronick and Grossman, 1983). Algal protein can be used for the production of animal feed which are suitable for farm animals and pets due to this promising prospect for imported commerciality (Becker, 2004). Another application is the production of bioactive peptide which can give physiological health benefits beyond their basic nutritional value (Kawakubo *et al.,* 1997). It contains a multitude of beneficial effects including antihypertensive, anti-oxidative, anti-diabetic etc. (Admassu *et al.*, 2018).

In Sri Lanka, large quantities of marine macroalgae and most frequent seaweeds have been identified. According to recent studies, Sri Lankan water has extremely changing salinity and temperature levels under tropical climatic conditions. It will induce the higher quantity of macroalgae growth around Sri Lanka which is rich with bioactive compounds (Lakmal *et al.*, 2014). *Sargassum crassifolium* is the most economically important species of brown seaweed found in Sri Lanka (Coppejans *et al.*, 2009) which contains essential amino acids and interesting biological properties such as antiviral (Hardouin *et al.*, 2014), antitumor (Nianjun *et al.*, 2004), antioxidant (Zahra *et al.*, 2007; Zaragoza *et al.*, 2008; Khaled *et al.*, 2012), antifouling (Plouguerne *et al.*, 2010), and antifungal (Manivannan, 2011; Khaled *et al.*, 2012). Currently, this seaweed mostly used for the extraction of polysaccharides in the industrial level. At that time algal protein can be considered as a byproduct of the polysaccharide extraction. Because of that, it is in line with 'zero waste vision'. It is important to the environmental and economic levels (Fleurence *et al.*, 1995a). Also, due to their high protein content, seaweed also can act as traditional protein sources such as meat, egg and soybean (Bleakley and Hayes, 2017; Fleurence, 1999b, Bleakly and Hayes, 2017). However, the functional properties of Sri Lankan seaweeds are not much studied. But functional properties of some Sri Lankan seaweed have been studied *in vitro* to identify the potential for pharmacological effects (Lakmal *et al.*, 2014). Therefore, it is important to determine the functional properties of crude proteins of seaweeds in the southern coastal region of Sri Lanka. In recent years, seaweed protein separated with the help of aqueous, acid and alkaline extraction methods as well as enzymatic hydrolysis from dried seaweed powders. At the end of the centrifugation process, the supernatant rich in protein separate and protein recovers by using ultrafiltration, precipitation using ammonium sulfate or chromatographic techniques (Galland *et al.*, 1999).

Enzymes such as protease, cellulose, amylase, endo proteases were used to enzymatic hydrolysis process to degrade the seaweed matrix to release the protein (Kadam *et al.*, 2015). Other than that, chemical hydrolysis or subcritical water hydrolysis have been also explored (Kadam *et al.*, 2015). Eventually, these conventional protein extraction methods are more time consuming and need a large amount of solvents. The purity of the extracted product also limited in these

extraction methods. Isolation and extraction of algal protein from seaweeds are affected by the complex seaweed matrix (Harnedy and FitzGerald, 2013).

The extraction of seaweed compounds can be achieved by using conventional methods or alternative methods (Cikoš *et al.*, 2018). Accordingly, extraction of bioactive compounds from seaweeds has been done by using several types of conventional methods. These conventional methods include, hydro-distillation; soxhlet extraction; maceration; percolation; infusion; decoction; and hot continuous extraction (Simoneau *et al.,* 2000; Vankar, 2004). However, many of the extraction techniques stated above are time-consuming, need comparatively high amount of contaminating solvents which can affect for the sample contamination, losses due to volatilization during concentration procedure, and environmental pollution from solvent waste (Grosso, 2015). Therefore, novel non-conventional extraction technique is currently being studied and developed to expand the extraction yield while minimalizing the time and essential resources. Therefore, the main objective of this study was to develop a protocol to extract crude Algal Protein (AP) from the selected marine macroalgae species, *Sargassum crassifolium*), collected from Southern coastal region and to determine the functional properties of its crude extract.

2. Materials and Methodology

2.1 Methodology

2.1.1 Seaweed Samples Collection, Preparation and Identification

2.1.1.1 Seaweed Samples Collection and Preparation

Seaweed samples (*Sargassum crassifolium*) were collected from Matara at Southern coastal region, Sri Lanka (from August to December months). Seaweed samples, immediately transported to the laboratory of Uva Wellassa University by packing within the sea water filled Low Density Polyethylene (LDPE) bags and Styrofoam boxes. Then, they were washed with running water to remove all unnecessary materials and stored at -21°C in a refrigerator until they were used for the crude protein extraction.

2.2.1.2 Seaweed Sample Identification

Seaweed samples were identified using morphological features according to a published guide (Coppejans *et al.*, 2009).

2.2.2 Proximate Analysis

Samples of *Sargassum* were analyzed separately for the proximate analysis. Crude protein content, moisture content, ash content and crude fat content were determined according to the AOAC official procedures, (2016).

2.2.3 Crude Protein Extraction

Dried and ground seaweed samples were prepared by using following procedure. Raw biomass was oven dried in the hot air oven (Model: SOV140A) and blended in a mixer grinder (Model: HL 1643/04) with a medium power to increase the extraction efficiency. Dried and ground biomass was kept at -20°C until use.

2.2.3.1 Water Extraction Method

The extraction procedure was performed as described by Galland *et al.* (1999), with slight modifications. Briefly, dried and ground macroalgae leaves were suspended in deionized water (1% w/v) and placed under -20°C for overnight. The mixture was then centrifuged (4000 rpm, 20 minutes, 4°C) using a centrifugation machine (Sorvall ST 40R, Thermofisher Scientific, Germany). After centrifugation, the supernatant was separated and deionized water was added to the sediment. The mixture went through another cycle of extraction and supernatants from both cycles were combined and subjected to lyophilization.

2.2.3.2 Salt Extraction Method

The extraction procedure was performed as described by Hatta *et al.* (1988), with slight modifications. Briefly, the sediment of the water extraction was collected and salt solution (10% w/v) added to the sediment and placed under -20°C for overnight. The mixture was then centrifuged (4000 rpm, 20 minutes, 4°C) using a centrifugation machine (sorvall ST 40R, Thermofisher Scientific, Germany). After centrifugation, the supernatant was collected and dialyzed (2 kDa) against deionized water and lyophilized.

2.2.3.3 Alkaline Extraction Method

The extraction procedure was performed as described by Kazir *et al.* (2019), with slight modifications. This protocol is a food-grade process developed herein as follows: dried and ground macroalgae leaves were suspended in NaOH (10% w/v) and placed under -20°C for overnight. The mixture was then centrifuged (4000 rpm, 20 minutes, 4°C) using a centrifugation machine (Sorvall ST 40R, Thermofisher Scientific, Germany). After centrifugation, the supernatant was collected and NaOH added to the sediment. The mixture went through another cycle of extraction and supernatants from both cycles were combined and dialyzed (2 kDa) against deionized water and then lyophilized.

2.2.3.4 Ethanol Extraction Method

The extraction procedure was performed as described by Kandasamy *et al.* (2012), with modifications. Briefly, dried and ground macroalgae leaves were suspended in ethanol solution (50% v/v) and placed under -20°C for overnight. The mixture was then centrifuged (4000 rpm, 20 minutes, 4°C) using a centrifugation machine (sorvall

ST 40R, Thermofisher Scientific, Germany). After centrifugation, the supernatant was separated and an ethanol solution was added to the sediment. The mixture went through another cycle of extraction and supernatants from both cycles were combined and dialyzed (2 kDa) against deionized water. This was followed by a lyophilization.

2.2.4 Yield Analysis

The yield of Algal Protein Concentrate (APC) was analyzed according to the following equation in order to analyze the best ratio of each extraction method.

$$\text{Yield } \% = \frac{\text{Final weight of lyophized freeze dried sample}}{\text{Initial weight of APC}} \times 100 \quad (1)$$

2.2.5 Protein Quantification

Lowry method was applied (Lowry *et al.*, 1951), with slight modifications suggested by Waterborg, (2002), to determine protein content in the purified sample. First, protein standard series was prepared. Then 0.3 ml from the prepared standard series was mixed with 0.3 ml of 2N NaOH and kept in a heating block at 100°C for 10 minutes to hydrolyze the sample. Hydrolysate was cooled and added freshly prepared mixed complex forming reagent [2% w/v of Na_2CO_3, 1% w/v of $CuSO_4 \cdot 5H_2O$ and 2% w/v of sodium potassium tartrate, in the proportion 100:1:1 (by vol.)]. Followed with 0.3 ml of Folin reagent to the mixture and standard at room temperature for 30-60 minutes. Finally, absorbance was taken at 750 nm if the protein concentration was below 500 μg ml^{-1}, or at 550 nm if the protein concentration was between 100 and 2000 μg ml^{-1}. A standard curve of absorbance as a function of initial protein concentration was plotted and it was used to determine the unknown protein concentration.

2.2.6 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS – PAGE)

The extracted crude proteins during extraction process were studied using SDS-PAGE based on the method describe by Sambrook and Russel (2006).

2.2.7 Determination of Functional Properties

2.2.7.1 Antioxidant Properties of Extracts.

2.2.7.1.1 DPPH Radical-Scavenging Activity Assay

The DPPH radical – scavenging activity of crude protein from each method were measured by referring the method described in Jiang *et al.* (2014), with slight modifications. First, 300 μl of DPPH solution (0.1 mm in methanol) and 750 μl of samples were mixed thoroughly. After that it was incubated at room temperature for

30 minutes in a dark environment. At the end of the incubation period absorbance was measured at 518 nm using UV spectrophotometer (SPECTROPHOTOMETER UV-2005). The scavenging effect was expressed using the following formula,

$$\text{Scavenging activity of DPPH}(\%)=\frac{\text{Absorbance of control-Absorbance of test sample}}{\text{Absorbance of control}}\times 100 \quad (2)$$

2.2.7.1.2 Thiobarbituric Acid Reactive Substance Assay (TBARS Assay)

The antioxidant activity of the crude protein was measured by using method (TBARS assay) described by Abeyrathne *et al.* (2014). First, 1 g of corn oil (Purchased from local market) and Tween-20 with 100 ml distilled water were mixed for 2 minutes in an ice bath using homogenizer to prepare an oil- in- water solution. The mixture was incubated under 37°C for 20 minutes. Consequently 1 ml of distilled water,8 ml of oil emulsion and 1 ml of hydrolyzed sample from each enzyme were well mixed in a falcon tube and it was incubated at 37°C for 16 hours in a water bath. Then, 1 ml of incubated sample was transferred to a 15 ml falcon tube consists with 2 ml of 20 mm 15% of TBA/TCA acid solution and 50 µl of 10% butylated hydroxyanisole (SIGMA-ALDRICH, USA) in 90% ethanol. After that the mixture was mixed using a vortex machine and incubated in 90°C in a water bath for 15 minutes to develop a colour. Then it was cooled in an ice bath for 10 minutes and centrifuged at 3000 x g for 15 minutes in 5°C using a centrifuge machine. Finally, absorbance of the supernatant was measured at 532 nm against a blank using a UV spectrophotometer (SPECTROPHOTOMETER UV -2005). Blank was prepared with 1 ml of distilled water and 2 ml of TBA/TCA solution. The amount of TBAR expressed as mg of malondialdehyde per liter of the emulsion.

2.2.7.2 Antimicrobial Properties

2.2.7.2.1 Agar Well Diffusion Method

Antimicrobial property of the agar was determined using the agar well diffusion method described in Abbas *et al.* (2016), with slight modifications. Broth culture of respective bacteria were inoculated on nutrient agar plates. Then sterile cork borer was used to make 4 mm size wells in each plate. Different concentrations of extracts (100 ul) (1250 ppm, 2500 ppm, 5000 ppm, 10,000 ppm and 20,000 ppm) were added into the wells using micropipettes. After that plates were allowed to standby for 30 minutes and plates were incubated at 37°C for 48 hours. Radius (mm) of the bacterial inhibition zones were measured and compared with the positive and negative controls. Augmentin® was used as the positive control and sterilized distilled water was used the negative control.

2.2.7.3 Metal Chelating Activity.

2.2.7.3.1 Fe^{2+} Chelating Activity

Fe^{2+} chelating activity was measured by using the method described in Abeyrathne *et al.* (2014). First, 100 µl of the crude protein, 900 µl of distilled water and 1 ml of 100 ppm $FeSO_4$ were vortex mixed in a 50 ml falcon tube using a vortex machine and then incubated for 15 minutes at room temperature. After that 900µl of 11.3% TCA was added to the mixture and centrifuged at 2500 x g for 10 minutes using a centrifuge machine. Then 1 ml of the supernatant transferred to the culture tube and 1 ml of distilled water, 800 µl of 10% ammonium acetate, 200 µl of ferroin colour indicator (Diluted ferroin colour indicator in ratio of 1:1) were added to it. Then the mixture was vortex mixed using a vortex machine. It was incubated at room temperature for 5 minutes. Absorbance was measured at 562 nm. Fe^{2+} chelating activity was calculated by using following equation,

$$\text{Ferrous chelating activity}(\%)=[1-(\frac{\text{Absorbance of the samples}}{\text{Absorbance of the blank}})]\times 100 \qquad (3)$$

2.2.8 Statistical Analysis

All the data were statistically analyzed by using Minitab 17.0 version statistical software package and Microsoft Office Excels software package and data were expressed as means with standard deviation with minimum three independent measurements. One-way ANOVA and Tukey test were used to analyze the data in functional properties analysis and statistical significance was considered at $p< 0.05$. Three replicates were used for each trial.

3. Results and Discussion

3.1 Proximate Composition of Raw Seaweeds

The chemical composition of seaweed comprises with minerals, vitamins and proteins with all essential amino acids which provides high nutritional value contributing to human nutrients (Cardozo *et al.,* 2007). The nutrient composition of selected seaweed varies with species, geographical location, season and temperature (Fleurence, 1999b). Therefore, this study was done using *S. crassifolium* which was collected from Matara, Southern coast region from August to December months.

Table 1 shows the proximate composition of the sample showed significant differences between moisture, lipid, protein and ash content ($p<0.05$). The proximate analysis shows that crude protein content and moisture content of *S. crassifolium* slightly higher than other compounds. Fat content was relatively low while it contains 3.52%±0.19 of ash. Moisture content is affecting the shelf life and quality of processed seaweed meals as high moisture may hasten the growth of

microorganisms (Ghadikolaei *et al.*, 2012). So, the presence of relatively high moisture content in *S. crassifolium* may decrease the shelf-life of the seaweed. The results of this study agree with the previous studies of Ahmed *et al.* (2012) which describes that macroalgae contain approximately 80-90% of moisture content on a wet weight basis. The presence of a relatively considerable amount of protein is a good source for crude protein extraction which is currently used in food, cosmetic and biomedical industries. High ash content invariably indicates the occurrence of significant amounts of varied mineral components in the seaweed (Matanjun *et al.*, 2008). Low fat and a considerable level of protein contents specify that a proper quantity of raw protein in *S. crassifolium* can be processed into more efficient and nutritional proteins. According to previous studies nutrition composition in seaweeds differs according to influences such as diverse species, environmental conditions, geographic locations, nutrition extraction methods and conditions applied to determine proximate composition (Fleurence, 1999b; Benjama and Masniyom, 2011).

Table 1: Proximate composition of selected species

Species	Proximate Composition (%)			
	Moisture	Crude protein	Crude fat	Ash
S. crassifolium	82.61±0.47%[a]	10.28 ± 0.02 %[b]	2.45±1.01%[c]	3.52±0.19%[c]

a, b,c Means with different superscript letters differ significantly ($p<0.05$)

3.2 Yield Analysis of the Extracts

3.2.1 Yield Analysis of the Extract Using Water, Salt, Alkaline and Ethanol Extraction Methods

The percentage yield of the crude extracts from water, salt, alkaline and ethanol extraction methods of the *S. crassifolium* are shown in Table 2 after freeze drying process According to the table, resulting yields from water extraction method of *S. crassifolium*, were 15.30±1.12%, 37.92±2.47%, 79.27±3.06%, 70.79±3.93% respect to 1:1, 1:2, 1:3, 1:4 treatments ($p<0.05$). According to the statistical analysis, 1:3 ratio treatment was selected as the best food grade treatment extract in terms of simplicity of extraction, non-toxicity, high yield as well as less cost for the extraction process. The results of this study slightly differ with previous findings due to the low purity of extracted crude using different treatments (Kazir *et al.*, 2019). Furthermore, it can be differed with changes of geographical locations and seasonal changes of selected species (Sunarpi *et al.*, 2010).

According to Table 2, salt extract yield percentages were 66.99% ± 5.75 (0%), 65.36% ±3.05 (4%), 67.48% ± 5.71 (6%), 71.63% ± 5.63 (10%) for *S.crassifolium* respectively ($p>0.05$). Among them, 4% treatment was selected as the simple, non-toxic, cheap, and food grade treatment to extract crude from *S. crassifolium*. The salt can pose a problem to the purity of protein. It can be a reason for the changes in the result compared to previous findings. Therefore, further studies are needed to evaluate type of impurities present in the extracted crude protein. Resulting yield from alkaline extracts of *S. crassifolium* were 19.99%±3.00(1:1), 45.14%±2.74(1:2), 68.12%±0.50(1:3), 71.43%±0.52(1:4) respectively ($p<0.05$). Among them 1:3 treatment was selected as the simple and non-toxic treatment. The results of this study did not coincide with the results obtained from the crude protein extraction from seaweed under alkaline treatments (Kazir *et al.*, 2019). This may due to the low purity of extracted crude protein. Furthermore, slight changes can be occurred due to changes of geographical locations and seasonal changes of selected species (Sunarpi *et al.*, 2010). According to Table 2, ethanol extract yield percentages were 13.37%±1.53(1:1), 47.43%±3.21(1:2), 70.64%±4.08(1:3), 84.68%±5.50(1:4) for *S. crassifolium* respectively ($p<0.05$). Among them, 1:4 treatment was selected as simple and non-toxic treatment to extract crude from *S. crassifolium.* Dhanani *et al.* (2017), has mentioned extracted yield percentage depends on the extraction process as well as composition of solvent.

Table 2: Percentage yield of the extracts using water, salt, alkaline and ethanol extraction methods

Extracts	Water				Salts				Alkaline				Ethanol			
Treatments	1:1	1:2	1:3	1:4	0%	4%	6%	10%	1:1	1:2	1:3	1:4	1:1	1:2	1:3	1:4
Yields%	15.30 ± 1.12^{c}	37.92 ± 2.47^{b}	79.27 ± 3.05^{a}	70.79 ± 3.93^{a}	66.99 ± 5.75^{a}	65.36 ± 3.05^{a}	67.47 ± 5.71^{a}	71.63 ± 5.62^{a}	19.99 ± 3.00^{c}	45.14 ± 2.74^{b}	68.12 ± 0.50^{a}	71.43 ± 0.52^{a}	13.37 ± 1.54^{d}	47.43 ± 3.22^{c}	70.64 ± 4.08^{b}	84.68 ± 5.50^{a}

[a, b,c] Means with different superscript letters differ significantly ($p<0.05$)

Yield percentage in seaweeds differs according to the conditions such as diverse species, environmental conditions and it also depends on the applied method of protein determination (Fleurence, 1999b). Meanwhile there are only very limited food grade extraction protocols for macroalgal proteins available so far, and most of them are functioning at laboratory scale. The progress recorded here is very promising, especially in terms of purity and up scalability. Among all the procedures, the highly yielded, cheap, simple and food graded treatments were selected. As a whole, these procedures are up scalable and suitable as a "food-grade" product.

3.3 Protein Quantification and Determination of Crude Proteins from the Extract of *S. crassifolium*

Protein quantification of algae can provide significant information on the chemical structure of algal biomass. The results of this study showed that, there is a significance difference between protein content of different extracts from *S. crassifolium* ($p<0.05$). In *S. crassifolium*, highest protein percent showed by the extract of salt (17.69%±1.39) followed by the water extract (13.34%± 0.97). According to the previous studies, brown seaweed contains small amount of protein percentage varying from 7.8% - 6.9% and *S. vulgare* showed 6.9% protein percentage among other brown seaweeds (Jungbauer and Hahn, 2009). According to the Kazir *et al.* (2019), alkaline extraction method gives higher yielded protein percentage than other extraction method. In this method, major challenge is to separate algal protein due to the presence of high amount of carbohydrate in dry weight of algae (Fleurence *et al.*, 1995).

Another challenge was that algal proteins can be found attached to the cell membrane (Lourenco *et al.*, 2002). Therefore, further studies must be needed to evaluate the types of impurities present in the extracted crude protein. The presence of relatively considerable amount of protein in selected seaweed sample is a good source for crude protein extraction which is currently used in food, cosmetic and biomedical industries.

To characterize the extracted proteins, all extracts were analyzed using 15% SDS-PAGE [Figure 1 and 2]. Majority showed patterns containing approximately 5-8 discrete bands with molecular weight of 50 kDa to10 kDa. Comparable patterns were observed between lanes specially of water, salt and ethanol extract but all bands arrangements were different from others. Meanwhile, 1:3 in salt extract and 1:3,1:4 in ethanol extract showed lower intensity, with low background compared to others.

Table 3: The antioxidant activity of *S. crassifolium* extract from water, salt, ethanol, alkaline extraction methods

Assay method	TBARS Assay / MDA (mg/l)					DPPH radical scavenging activity %				
Extracts	Water	Salt	Alkaline	Ethanol	Control	Water	Salt	Alkaline	Ethanol	Control
Results	$0.34\pm0.02^{a,b}$	$0.35\pm0.05^{a,b}$	0.46 ± 0.06^{a}	0.32 ± 0.06^{b}	0.09 ± 0.01^{c}	30.85 ± 1.87^{d}	58.94 ± 1.52^{b}	33.85 ± 1.64^{d}	51.38 ± 1.35^{c}	79.92 ± 0.70^{a}

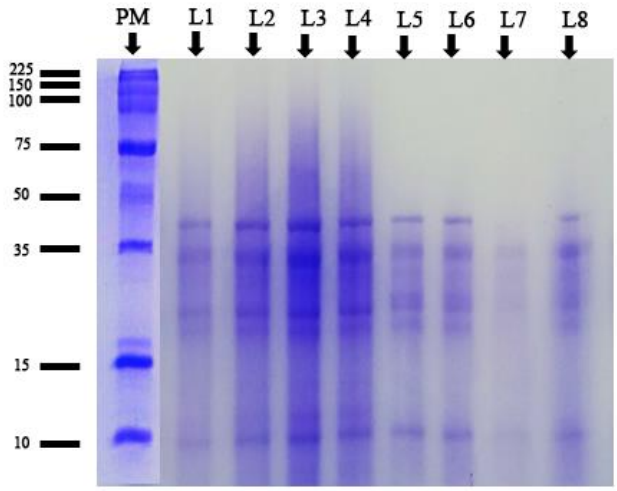

Figure 1: 15% SDS-PAGE gel patterns of Crude protein; Lane 1 = Protein marker; Lane 2, 3, 4, 5 indicates *S.crassifolium* crude protein Extract from water extraction method under different treatments 1:1,1:2,1:3,1:4 respectively. Lane 6, 7, 8, 9 indicates, *S.crassifolium* crude protein Extract from salt extraction method under different treatments 0%, 4%, 6%. 10% respectively.

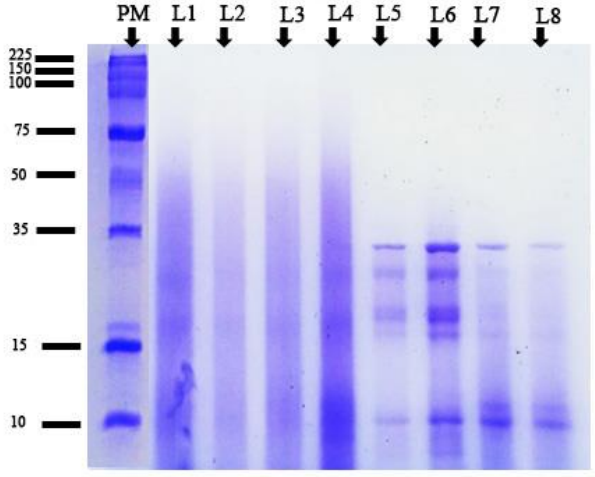

Figure 2:15% SDS-PAGE gel patterns of Crude protein; Lane 1 = Protein marker; Lane 2,3, 4, 5 indicates S.crassifolium crude protein Extract from Alkaline extraction method under different treatments 1:1,1:2,1:3,1:4 and . Lane 6,7, 8, 9 indicates crude protein extracts from ethanol extraction methods under different treatments 1:1,1:2,1:3,1:4 respectively.

Across all lanes, some smearing was observed, possibly due to covalent linking between the proteins and polysaccharides, the latter having much higher molecular

weights than the proteins (Fleurence *et al.*, 1995; Paradossi *et al.*, 1999). In fact, the SDS-PAGE profile of crude protein extracts from different extraction methods for *S. crassifolium* shows that the protein bands were easily resolved with considerable level of smearing and with not too much background, suggesting that crude protein extraction methods might be applicable to a selected seaweed species.

Bands comprising abundant proteins were detected in majority of extracts of different extraction methods in selected seaweed indicating that common proteins are shared among these treatments. Nonetheless, band sizes of low molecular weight (>50 kD) differed, showing variations in protein types across species, indicating that common proteins are shared among these plants. Significant differences in the term of protein bands in electrophoretic patterns were found between different extraction methods (Fleurence *et al.*, 1999a; Rosni *et al.*, 2015).

3.4 Analysis of Functional Properties of Crude Protein

3.4.1 Antioxidant Activity of Crude Protein

Oxidation limits the acceptability of products to customers by creating off-flavour substances with the small molecular weight and by removing essential nutrients and by creating toxic compounds, dimers or lipid and protein polymers (Aruoma, 1998). Saiga *et al.* (2003), revealed that oxidative reactions cause to changes in food products in the rancidity, odour, texture, taste and colour changes parameters and some pathological conditions. In recent investigates, it has been continually asserted that oxidative stress is not limited to free radical induced injury on biomolecules but also involves perturbation of cellular redox status. Oxidative stress-induced pathology contains cancer (Alam *et al.*, 2013: Kinnula and Crapo, 2004), neural disorders (Sas *et al.*, 2008), Alzheimer's disease (Smith *et al.*, 2000), mild cognitive impairment (Guidi *et al.*, 2006), Parkinson's disease (Bolton *et al.*, 2000), alcohol induced liver disease (Arteel, 2003). Therefore, these antioxidants can be considered as important nutraceuticals of many health benefits (Valko *et al.*, 2007).

Antioxidants in food do not necessarily protect biological tissues from free radical oxidative injuries because they have to be transformed into usable tissue forms and interrelate with other substances, in addition to effective variances in concentration. Eventually, they have to have difficulties when absorbing them from the diet (Azzi *et al.*, 2004). The impact of antioxidants on the oxidation of foods are based on their concentration, polarity and the medium (Cuvelier *et al.*, 2000) and also the occurrence of additional antioxidants (Decker, 2002). Proteins and peptides contain different antioxidant properties in amino acids. So, they can inhibit the lipid oxidation by biological designed mechanisms or non-specific mechanisms. Such two forms of antioxidants proteins relate to the endogenous antioxidant ability of foodstuffs (Cuvelier *et al.*, 2000).

Protein also have excellent potential as antioxidant additives in food because they can inhibit lipid oxidation by multiple routes including inactivation of reactive oxygen species, scavenging free radicals, chelation of pro-oxidative transition metals, reduction of hydro peroxides, and alteration of the physical properties of food systems (Elisa, 2008).

Past studies revealed that, synthesis of peptides by hydrolytic reactions appears to be the most successful strategy for creating proteinaceous antioxidants because peptides have significantly higher antioxidant activity than intact proteins (Arteel, 2003). In present study the antioxidant activity of *S.crassifolium* extract from water, salt, ethanol, alkaline were assessed using DPPH free radical scavenging activity assay and Thiobarbituric acid reactive substances (TBARS) assay.

3.4.1.1 TBARS Assay.

The Thiobarbituric Acid Reactive Substances (TBARS) assay is commonly used to measure lipid oxidation and antioxidant activity in food and physiological structures. Thiobarbituric acid reactive substances are formed as a byproduct of lipid peroxidation that can detect by using thiobabutric acid during the TBARS assay (Zeb and Ullah, 2016). In this assay measuring the oxidation by means of measuring malondialdehyde concentration and TBA test response for each extract from different extraction methods were shown in below. The results of the antioxidant effects of each extract showed comparable low concentration of MDA with significance differences ($p<0.05$) [Table 3]. In the present study, control showed 0.096 mg/l ±0.097 malondialdehyde concentration. For *S. crassifolium* with water, salt, alkline and ethanol treatments showed 0.34 mg/l ±0.05, 0.35 mg/l±0.05, 0.46 mg/l±0.06, 0.32 mg/l±0.06 MDA concentration respectively. When compared each extracts of *S. crassifolium*, ethanol extract showed low MDA concentration among other extracts. The results of this study did not coincide with the results obtained from the Chakraborty *et al.* (2013). This may be due to the low purity of extracted and extraction condition applied during the extraction process. Because of the ability in extracted crude protein to scavenge free radicals and inhibiting MDA formation in food it can be used as an effective antioxidant for food industry (Ilknur and Turker, 2018). Although this antioxidant potential as food antioxidants, important to address concerns such as allergenicity and bitter off-flavours, as well as their ability to alter food texture and colour with better quality (Chakraborty *et al.*, 2013). However, this crude protein extract can be used as good preservative to minimize the oxidation in food industry with further studies.

3.4.1.2 DPPH Radical Scavenging Activity

DPPH assay is one of the most popular and frequently employed simple, efficient, relatively inexpensive and quick method among antioxidants assays (Cui *et al.*,

2004). Free radicals are eventually created and experienced exogenously in biological systems, and are known to cause multiple degenerative disorders like mutagenesis, carcinogenesis, cardiovascular disturbances and ageing (Singh and Singh, 2008). The antioxidant compound present in the medium convert $DPPH^{\bullet}$ radical to a more stable $DPPH^{\bullet}$ molecular product by donating an electron or a hydrogen atom. As a result, colour changed from purple to pale yellow of reducing form of $DPPH^{\bullet}$ and this color change is measured by spectrophotometrically in order to quantify the antioxidant activity (Jin *et al.*, 2008). Here, DPPH radical scavenging was showed for the crude protein of *S. crassifolium*, which were produced by water, salt, alkaline, ethanol extraction method.

Average DPPH radical scavenging activity of crude protein were shown in Table 3. The results indicated that all the tested extract of crude protein in this investigation possess radical scavenging properties. According to the above table comparing with the Ascorbic acid (control), it shows significant difference with the extracted samples ($p<0.05$). Considering extracted samples, all extracts were having over 30% scavenging activity. In the present study, control showed 79.92±0.70% DPPH radical scavenging activity. The sequence of antioxidant activity of the *S. crassifolium* extracts was as follows: Salt (58.94%±1.52) > Ethanol (51.38%±1.35) > Alkaline (33.85%±1.64) > Water (30.85%±1.87). When compared each extract of *S. crassifolium*, salt extract demonstrated greater antioxidant potential among other extract. The results of this study coincide with the results obtained from the DPPH radical scavenging activity of the extracts from *S. lomentaria* (Ilknur and Turker, 2018). The results clearly specified that all the tested seaweeds in this investigation possess antioxidant activity. The results suggest that seaweeds possess antioxidant potential which could be effective for future developments in food industry. Although this antioxidant potential as food antioxidants, important to address concerns such as bitter off-flavours and enhance colour texture with better quality (Wilson *et al.*, 2017). However, future researches should be focus used as good preservative to minimize the oxidation in food industry with further studies.

3.4.2 Metal Chelation Activity

Foods generally contain many transition metal forms including iron, copper, and manganese. Such transitional metals aid oxidation processes which trigger unwanted consequences directly or indirectly (Schaich, 1980). Metal chelation behavior is therefore essential in the food industry because metal binding capability of peptides help to prevent oxidation of lipids (Lechevalier *et al.*, 2007).

3.4.2.1 Fe^{2+} Chelation Activity

In the current study, the ferrous chelating activities of extracts from *S. crassifolium* was evaluated. According to the results, *S. crassifolium*, demonstrated lower Ferrous chelation potential among each extracts. Here, Ferrous chelation activity of each

extract showed a significance difference ($p<0.05$). The sequence of Ferrous chelating potential of *S. crassifolium* crude protein extracts were as follows: Ethanol > Water> Salt > Alkaline. For *S. crassifolium* ethanol extract (15.63%± 1.18) showed higher chelation potential than other extracts. Metal chelating capacity was important. Subsequently it reduced the concentration of the catalyzing transition metal in lipid peroxidation (Hsu *et al.*, 2008). Our studies are in accordance with earlier studies which verified that polyphenols derived from seaweeds are potent Fe^{2+} chelators (Senevirathne *et al.*, 2006). Chelation of metal ions can be a mechanism for prevention of free radical reactions (Sugihara *et al.*, 2001). Presence of metal such as ferrous in food increases lipid oxidation (Gutteridge *et al.*, 1982). Hence, in this study crude protein showed considerable level of Ferrous chelating activity, they can be used as an antioxidant for foods with further studies.

3.4.3 Antimicrobial Properties

Microorganisms are naturally presented in the surrounding environment, so they can enter to the food easily during harvesting, slaughtering, processing and packaging (Hatab *et al.*, 2016). Such microorganisms may live under unfavorable food preservation environments such as low temperatures, changed atmospheric packaging, vacuum packaging and resistance to traditional pasteurization (Dimitrijevic *et al.*, 2007**;** Saraiva *et al.*, 2016., Sade *et al.*, 2017). For the inhibition of pathogenic bacteria and extend the shelf-life of food products without the use of chemical preservatives, modern eco-friendly methodologies are thus necessary. Many researchers have recently studied the possible use of certain plant extracts as effective natural preservatives (Fernandez *et al.*, 2005; Suppakul *et al.*, 2016; Clarke *et al.*, 2017)**.** When dietary protein used as preservatives, they are good for the health of customers other than the antimicrobial function (Labadie *et al.*, 2008). It has the benefit of not requiring previously prepared plates, and is often used to assay bacterial contamination of food stuffs (Suppakul *et al.*, 2016). In this assay, different concentration of each crude protein extracts from *S. crassifoliium* used to determine the antimicrobial properties by measuring the inhibition zones after 24 hours.

3.4.3.1 Determination of Antimicrobial Properties of Water, Salt, Alkaline and Ethanol Extracts from *S. crassifolium*

Antimicrobial properties of crude protein derived from water, salt, alkaline and ethanol extractions were evaluated by using the radius (mM) of the zones as shown in Table 4. The results of the antimicrobial effects measurements for each extract showed significance differences ($p<0.05$). All concentrations were showing low potential regarding antimicrobial properties. Crude extracts of seaweed antimicrobial efficacy depend on certain variables such as size, molecular weight charge intensity, sulphate content (in sulphate polysaccharides), and conformation aspects (Val *et al.*, 2001). The diverse potency of these extracts could be due to alterations in their

composition (Etahiri *et al.*, 2003). According to the earlier studies, diverse biomolecules of seaweeds such as protein, fatty acids, polysaccharides, and polyphenols have shown antibacterial activities, which in turn depend on the extraction and sampling methods, as the sampling seasons (Morales, 2008). Salvador *et al.* (2007) studied the antibacterial activity of 82 species of seaweeds in various seasons, and published that the crude extracts of brown and red algae had stronger effects in autumn, while green algae had stronger effects during summer (Lee, 2014). Hence, the results showed potential of using these extracts as antimicrobial agents.

Table 4: Diameter of inhibition zones of the extracted crude proteins.

	Inhibition zone(mm)			
Extracts	Water	Salt	Alkaline	Ethanol
Concentration 20000ppm	6.45 ± 0.11^{b}	$6.26\pm0.23^{c,d}$	$6.15\pm0.13^{b,c}$	6.24 ± 0.02^{b}
10000ppm	6.34 ± 0.11^{b}	6.31 ± 0.02^{b}	6.27 ± 0.04^{b}	$5.85\pm0.13^{b,c}$
5000ppm	5.16 ± 0.04^{c}	$5.17\pm0.02^{c,d,e}$	5.53 ± 0.44^{c}	$5.73\pm0.21^{c,d}$
2500ppm	4.92 ± 0.13^{c}	$5.16\pm0.02^{c,d,e}$	$5.12\pm0.10^{c,d}$	5.13 ± 0.02^{e}
1250ppm	4.50 ± 0.18^{d}	$5.04\pm0.12^{d,e}$	$5.07\pm0.03^{c,d}$	5.11 ± 0.02^{e}
625ppm	4.03 ± 0.03^{e}	5.02 ± 0.01^{e}	5.03 ± 0.01^{d}	5.09 ± 0.01^{e}
Augmentin	8.29 ± 0.23^{a}	8.32 ± 0.07^{a}	8.31 ± 0.02^{a}	8.34 ± 0.12^{a}

4. Conclusion

The water extraction method (1:4 ratio), salt extraction method (10% (v/w)), alkaline extraction method (1:3 ratio) and ethanol extraction method (1:4 ratio) are the best methods to extract crude protein from *S. crassifolium.* Further each crude extract showed effective free radical scavenging, metal chelating and low antibacterial activities.

5. References

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