doi.org/10.36721/PJPS.2022.35.2.REG.561-570.1

Elemental analysis of carrageenans isolated from *Hypnea musciformis* red algae of Karachi coast using SEM-EDX

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Abstract: The carrageenans with high molecular weight, crude and dialysed polysaccharide fractions obtained from *Hypnea musciformis* red algae of Karachi coast in Pakistan. The elemental composition was determined by using SEM-EDX technique which is one of the modern, reliable and accurate techniques. After analyzing multiple mineral elements were detected in different quantities. The numbers of elements found in crude extracts were greater than that in the dialysed extracts. All extracts contained the higher concentrations of C followed by O (except dialysed acidic extract). Among other elements, Cl and K were present in the highest amounts (>25.0%) in dialysed acidic extracts. However, Al was detected in low concentrations in only crude aqueous and acidic extracts. The mineral concentrations ranges were 30.34-52.46%, 17.00-43.46%, 0.63-4.05%, 0.49-3.35%, 0-0.33%, 0.74-17.92%, 0.59-25.31%, 0.58-25.46%, 1.10-6.72%, 0-2.60% for C, O, Na, Mg, Al, S, Cl, K, Ca and Zn in these crude and dialyzed extracts respectively. The study confirmed the presence of major elements such that Na, Mg, Ca, K and Zn in high quantities. However, there was no toxic element identified like Cd, Hg and Pb which show that these carrageenans are safer to utilize in food and pharmaceutical industries.

Keywords: Carrageenans, polysaccharides, mineral elements, SEM-EDX, *Hypnea musciformis*, seaweeds.

INTRODUCTION

The consumption of seaweed and seaweed related products have gained worldwide high reputation importance over the last few years. Marine algae have been used as supplements for both human and animal as meals. The potential health benefits of seaweed and seaweed extracts have been well documented (Brownlee *et al*., 2012). Marine macro algae is one of the important sources of medical and nutrition materials in the world (Mohammadi & Asbchin 2011). Seaweed polysaccharides are being used in the food industry and having particular technological importance due to their Broad Spectrum of functionality with particular emphasis on gelation and viscosity enhancement (Alba and Kontogiorgos, 2019). Seaweeds possess a low lipid content and enrich in polyunsaturated fatty acids. This characteristic enables more attractive, as they are a healthy, nutritive and low-caloric food (Pereira, 2018).

Karachi has a large coastal area of Arabian Sea and produces a hugeamount of marine algae of numerous varieties including red, brown and green algae. *Hypnea musciformis* of class red algae (rhodophyceae) is widely distributed at Karachi coast and it is a rich source of industrially viable phycocolloids (polysaccharides). Carrageenans are the sulphated polysaccharides extracted from *H. musciformis* and widely used as functional ingredients in the food and dairy products as stabilizing, thickening and gelling agents (Murano *et al*., 1997; Thomas 1997., Puvanenthiran *et al*., 2003). They have strong electrolyte character due to their sulphate groups.

Beside texture property, the protein reactivity of these phycocolloids have led to their wide applications in other industries such as pharmaceutical, cosmetics, beverages and agriculture sectors. These polysaccharides are found as active elicitor of plant defense mechanism and also used as biostimulant of plant growth and development (Bi *et al*., 2010; Khan *et al*., 2009). Our research group is the first to test carrageenans as an elicitor against various crop plants (Bi & Iqbal 1998; Bi *et al*., 2008). Seaweeds are considered a nutrient-rich food as they are a good source of minerals, vitamins (A, B1, B2, B9, B12, C, D, E and K), essential minerals (calcium, iron, iodine, magnesium, phosphorus, potassium, zinc, copper, manganese, selenium and fluoride), dietary fibers (Pereira L. 2018; Shannon *et al* 2019) and also contain protein, essential amino acids and polyphenols, which exhibit antioxidant and anti-inflammatory properties (Panzella, Napolitano 2017).

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Ample data is available on physicochemical and viscoelastic properties of milk based gel and carrageen an mixtures, we also reported physicochemical characterization, structural analysis, gel permeation and anion exchange chromatography and thermodynamic studies of carrageen an from *H. musciformis* of Karachi coast (Bi *et al*., 2006 & 2007; Arman & Qader 2012). An O-methylene phosphonic κ-carrageenan-based polymer electrolyte, O-methylene phosphonic κ-carrageenan (OMPC), was successfully synthesized using formaldehyde and phosphorous acid. The structure of OMPC was confirmed via FTIR, ^{1}HNMR, ^{31}PNMR and SEM-EDX analyses. The OMPC shows potential as a polymer electrolyte membrane due to its considerably high ionic conductivity when hydrated (Joy Wei Yi Liew, 2017).

Further research should focus on improving the segmental motion of polymer chains in the membrane and strengthening the hydrated membrane. However, there was no work reported on the ultra structural features and elemental composition of these carrageen an polysaccharides. The present study describes the surface chemistry and elemental analysis of these polysaccharides by using SEM-EDX technique. This research study probably the first approach for the elemental analysis of carrageenans extracted out from *H. musciformis* of Karachi coast.

MATERIALS AND METHODS

Plant *Hypnea musciformis* of class *rhodophyceae* (red algae) was collected from the coastal areas of Karachi such as Hawksbay and Cap Monz, in November to February 2005-2006. Identification of plant was confirmed by Prof. Dr. Mustafa Shameel Department of Botany, University of Karachi-75270 on request. The specimen voucher was deposited at the Herbarium of the Botanical Garden of PCSIR Laboratories Complex Karachi, Pakistan (M.A. R1130.E026) & washed with running tap water and air dried under shade on netted wooden trays. Details of crude and purified dialysed aqueous, dilute acidic (0.1N HCl), dilute basic (0.1N NaOH) extraction methods were presented in our recently published report (Arman & Qadar 2012). Commercial carrageenan was purchased from local market. All extracts and commercial sample were coated upto 300^{o}A on gold coater from Jeol model# JFC-1500 and subjected to the elemental analysis using SEM (Scanning Electron Microscope, Jeol Japan, model # JSM-6380A) coupled with EDX (Energy Dispersive X-ray Spectrometer, Jeol Japan, model # EX54175 JMU.

Fractional Extraction of Phytoalexin and Antifungal Activity

Treated tissues of chickpea were extracted with 95% alcohol (each repeated twice) concentrated below 45°C. The concentrated total alcoholic extract-1, was kept in the fridge at 4°C for three to four days. A yellow precipitate was collected and the supernatant was partitioned between pet ether. Pooled pet ether extract, concentrated and designated as Ext-2, the aqueous phase was extracted with chloroform-ethyl acetate 7:3 v/v concentrated and collected as Ext-3, leaving behind a residual aqueous Extract as Ext-4. All the extracts and precipitate collected were evaluated for their antifungal activity.

Pathogens i.e. *Macrophomina phaseoli*, *Fusarium moniliforme*, *Curvilarialunats*, *Aspergillus niger* and *Rhizoctonia solani* were procured from Karachi University, Botany Department. Culture were monitored on Subouraud glucose agar and were subculture on Subouraud glucose broth for testing. Inocula were prepared using 72 hours old broth culture. The antifungal activity was done by agar cup plate method (118). Various extracts 1-4 and precipitate-5, were soluble in water, therefore methanol 95% was used as a solvent. Each sample as a solution of 100ug/ 0.1 Ml (1:1 v/v) was used to evaluate their antimicrobial activity. Each cavity received 0.15ug of the test substance respectively. Test was run in triplicate. Controls were run simultaneously using 95% methanol in same quantity. All plates were incubated at 27-29°C for 72 hours and tabulated. Each experiment was run thrice to confirm the result. Extract with high and low activity were analyzed by the HPLC method developed earlier.

RESULTS

Elemental analysis of isolated Carrageenans from *Hypnea musciformis*, red algae was performed using Scanning Electron Microscopy technique equipped with Energy Dispersive X-ray Spectrometer. In modern age, this technique considered as more reliable and accurate method for both qualitative and quantitative analysis.

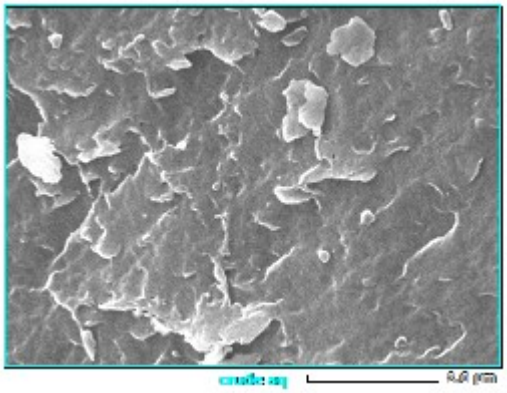

Fig. 1(A): SEM image of crude aqueous extract of *H. musciformis.*

Fraction extraction and antimicrobial activity of phytoalexin induced in chickpea tissues

Phytoalexin are plant antibiotics which have been shown to contribute to disease resistance and accumulated

rapidly in response to pathogenic interaction, their role in disease resistance has been deduced from the antifungal activity.

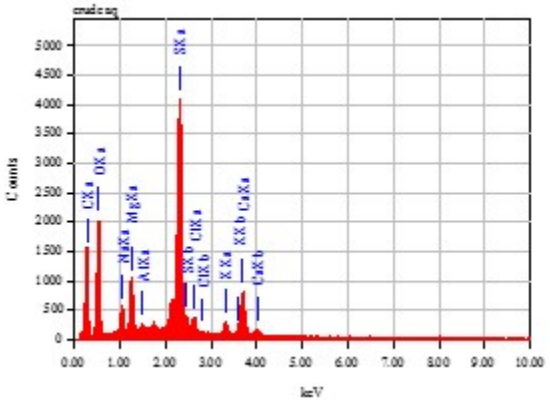

Fig. 1(B): EDX spectrum of crude aqueous extract of *H. musciformis*

Fig. 2(A): SEM image of crude basic extract of *H. musciformis*

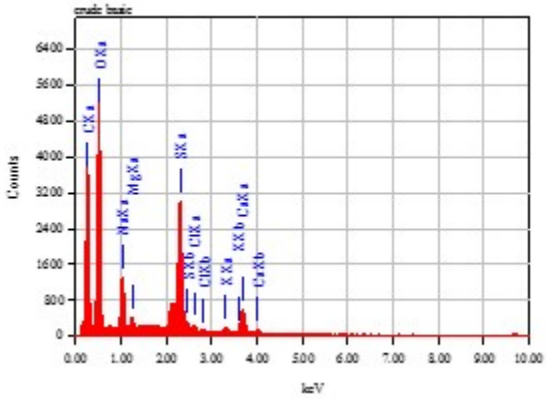

Fig. 2(B): EDX spectrum of crude basic extract of *H. musciformis.*

On the basis of preliminary results, HMWCEP (High Molecular weight Crude Elicitor Preparation) of *H. musciformis* (red algae) were used for large scale elicitation of chickpea tissues and production of Phytoalexin. Elicited tissues were extracted with 95% ethanol. Total alcoholic extract and various functional extracts 1-4 and yellow precipitate as described in fig. 10 were evaluated for their antifungal activity. It is evident from the data collected in table 3 that growth of *M. phaseoli* and *F. monliforme* were inhibited by the application of all the extracts, 1-5 under test. The chloroform-ethyl acetate extract exhibited maximum activity and found to be the most active fraction, low levels of activity shown by the total alcoholic extract and yellow precipitate.

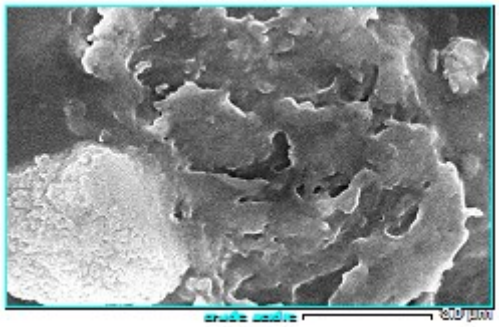

Fig. 3(A): SEM image of crude acid extract of *H. musciformis.*

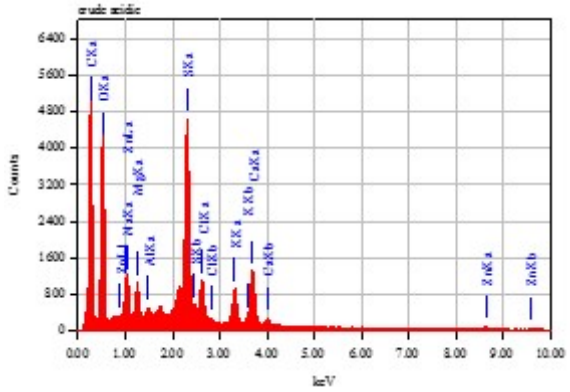

Fig. 3(B): EDX spectrum of crude acid extract of *H. musciformis.*

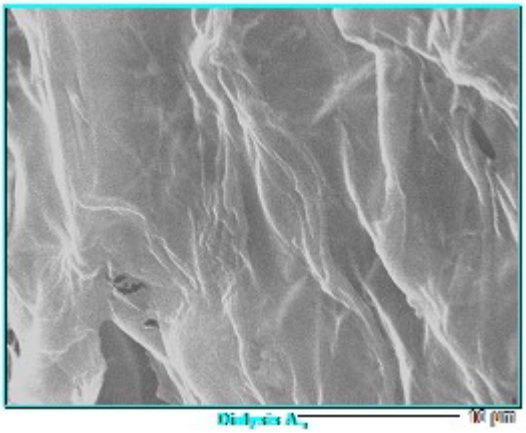

Fig. 4(A): SEM image of dialyzed aqueous extract of *H. musciformis.*

DISCUSSION

Algal foods including carrageenans are rich in mineral elements along with protein, carbohydrates and vitamins etc. Carrageenans are the phycocolloids extensively used

in food and pharmaceutical industries, considering its so much industrial importance and also for their characterization it is essential to determine the elemental composition of these polysaccharides.

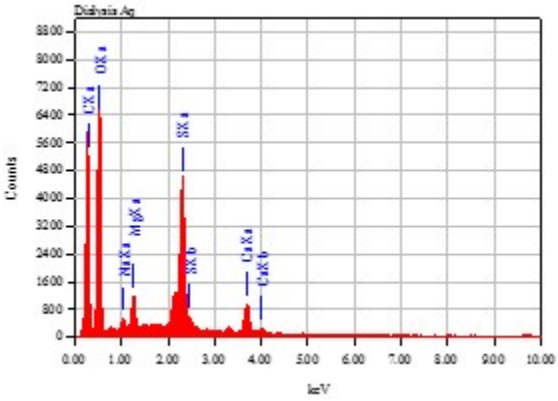

Fig. 4(B): EDX spectrum of dialyzed aqueous extract of *H. musciformis.*

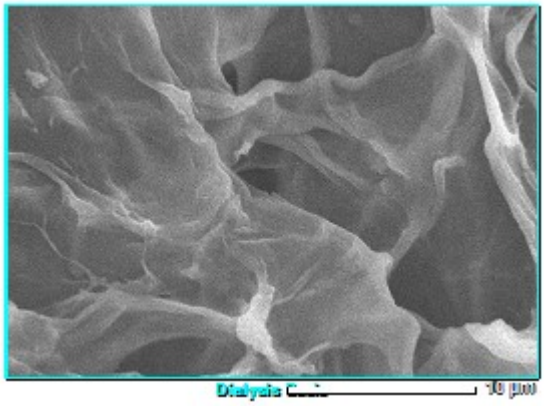

Fig. 5(A): SEM image of dialyzed basic extract of *H. musciformis.*

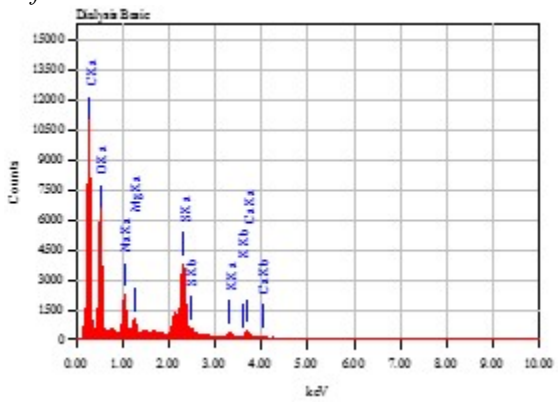

Fig. 5(B): EDX spectrum of dialyzed basic extract of *H. musciformis.*

The characterization of plant material, aiming safety and nutritional worth, is important to attain a phyto therapeutic product meeting quality criteria (Correia *et al.,* 2018). Analytical methodologies, based on different principles, have been adopted for evaluation of plant drugs to establish their effective use in different pharmaceutical preparations. Scanning electron microscopy (SEM) is a technique, which utilizes the beam of electron for visual inspection of geometric characteristic such as size, shape and surface morphology. It provides high magnification range and is therefore appreciably applied for elaborated morphological studies (Mohammad and Abdullah, 2019). Energy dispersive X-ray spectroscopy (EDS) is usually used in association with SEM and provides qualitative and quantitative evaluation of different elements found in the sample. It presents a plot of ionization energy (keV) vs. counts and higher counts reflect greater quantity of element in the specimen (Scimeca, 2018).

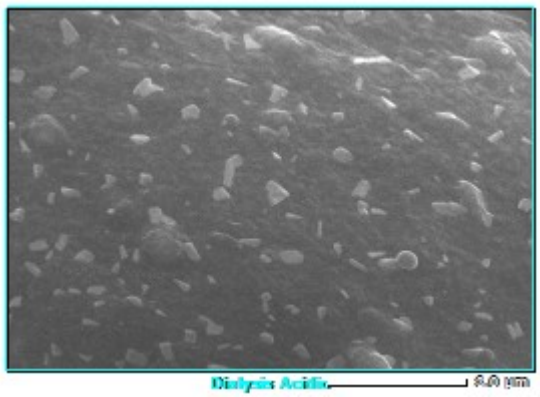

Fig. 6(A): SEM image of dialyzed acidic extract of *H. musciformis.*

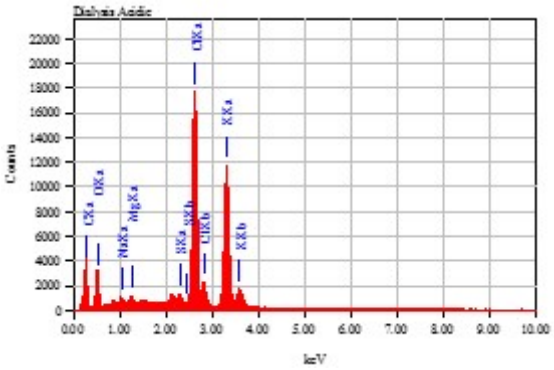

Fig. 6(B): EDX spectrum of dialyzed acidic extract of *H. musciformis.*

The present study is a continuation of the research work carried out by our group on polysaccharides extracted from various seaweeds that were collected from different coastal areas of Karachi (Bi & Iqbal 1998; Bi *et al*., 2006, 2007, 2008, 2010 and Arman & Qadar 2012). The work presented here has done probably for the first time to test the carrageen an polysaccharides isolated from *H. musciformis* for their elemental composition.

The major mineral elements detected in various crude and dialyzed extracts of carrageenan are shown in table 1 and figs. 1 to 6. The data shows the mean concentrations of ten elements such as C, O, Na, Mg, Al, S, Cl, K, Ca and Zn in these extracts.

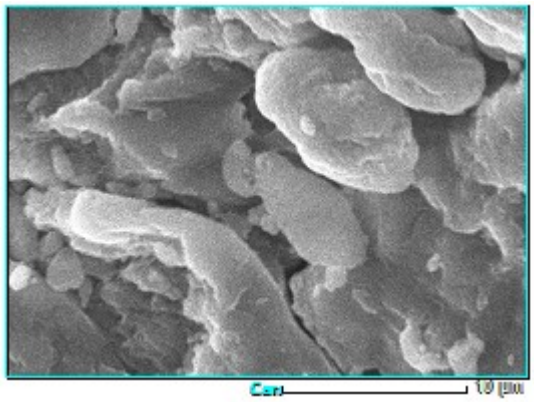

Fig.7 (A): SEM image of commercial carrageenan

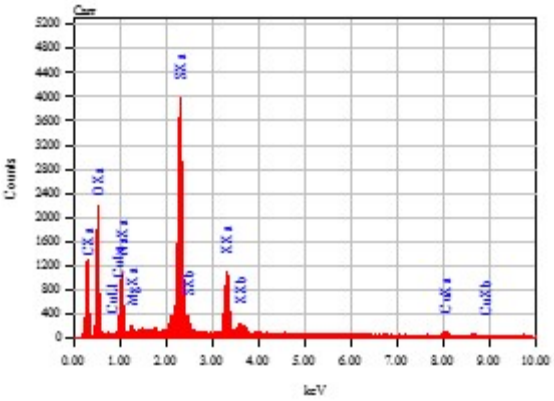

Fig.7 (B): EDX spectrum of commercial carrageenan

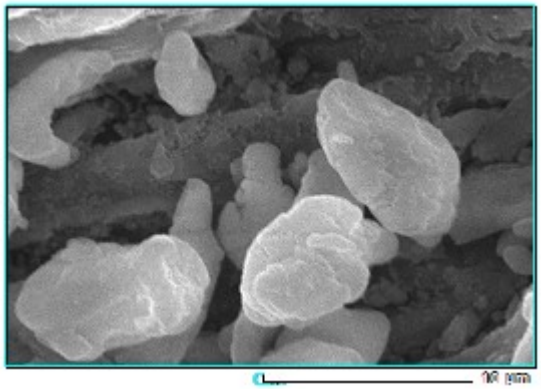

Fig. 8 (A): SEM image of commercial carrageenan

C and O were present in all extracts. Among other elements Na, Mg, S and Cl were also found in each extract. Similarly K was found in all except one (dialyzed aqueous) extract. Ca was present in all crudes and only one dialyzed (basic) extract. Al was detected in crude aqueous and acidic extracts. Zn was present in crude acidic and in crude and dialyzed basic extracts. Number of elements found in the crude extracts was greater than that in the dialyzed extracts.

Quantitative estimation of these elements showed that both C and O concentrations were significantly higher (except O in dialyzed acidic extract 17.0%) than other elements found in these extracts. Highest concentrations of C and O were found in dialyzed basic (52.46%) and crude basic (43.46%) extracts respectively. These C and O represent the organic nature of these carrageen an polysaccharides. Organic compounds are generally considered as non toxic or minimally toxic in the living systems (Devi *et al*., 2011). The higher C and O concentrations show the presence of various nutritive constituents in the plant extracts such as carbohydrates, proteins, fats, fibers and vitamins etc. (Mohammadzai *et al*., 2011).

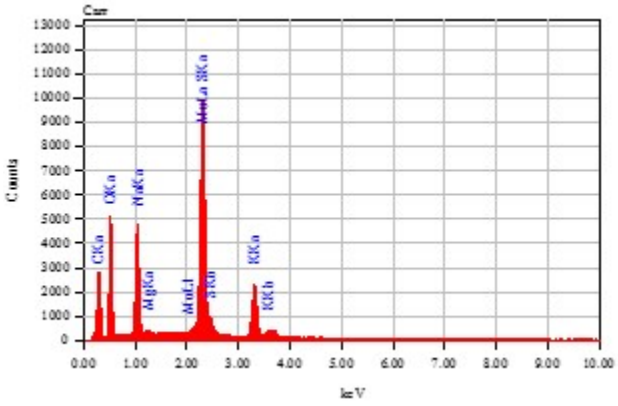

Fig. 8 (B): EDX spectrum of commercial carrageen an

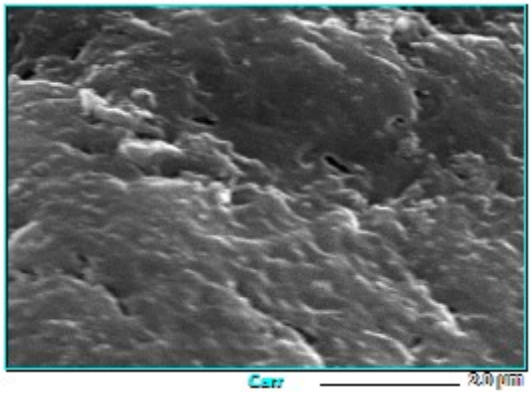

Fig. 9 (A): SEM image of commercial carrageen an

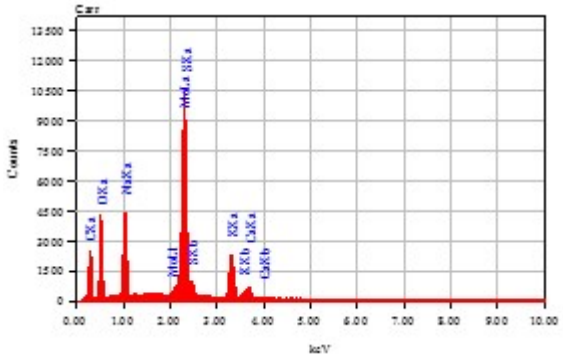

Fig. 9 (B): EDX spectrum of commercial carrageenan

Red algae are generally rich in Na, Mg, K and Ca, marine algae exhibit high contents of ash mainly due to the presence of these cations (Jayasankar & Paliwal 2002). It

Table 1: Mean concentrations of major elements in various crude and purified dialyzed extracts (Carrageenans) obtained from *H. musciformis* red algae.

Elements	Extracts (Mass % of Elements)					
	Crude Aqueous	Crude Basic	Crude Acidic	Dialyzed Aqueous	Dialyzed Basic	Dialyzed Acidic
C	36.73	38.24	42.52	41.57	52.46	30.34
O	29.24	43.46	30.80	42.88	35.27	17.00
S	17.92	9.04	9.87	9.44	5.95	0.74
Ca	6.72	3.43	5.46	ND	1.10	ND
Mg	3.35	0.60	1.37	1.73	1.03	0.49
Na	2.17	4.05	1.49	0.63	3.59	0.65
Cl	1.77	0.59	2.63	3.74	ND	25.31
K	1.77	0.58	2.92	ND	0.59	25.46
Al	0.33	ND	0.33	ND	ND	ND
Zn	ND	ND	2.60	ND	ND	ND

ND: Not Detected

Table 2: Mean concentrations of major elements in Commercial Carrageenan.

Elements	Mass % of elements		
	Scan-1	Scan-2	Scan-3
O	38.99	37.21	34.89
C	34.12	31.05	31.28
S	13.60	12.56	13.66
Na	5.92	10.12	9.89
K	5.18	4.41	4.55
Cu	1.66	ND	ND
Mg	0.53	0.30	ND
Ca	ND	ND	1.20
Mo	ND	4.35	4.53

Table 3: Antimicrobial Activity of Induced Metabolites of Chickpea Tissues Induced by Treatment of *H. musciformis* (red algae) Elicitors

Name of Cultures	T. alc. Ext.1	Pet ether Ext.2	Chlor-ethyl acetate Ext.3	Residual aq. Ext.4	Yellow ppt.
Fusarium monitiforme	++	+	+++	+	+
Macropho-mina phaseoli	+	++	+	+	+
Curvularia lunata	-	++	+++	+	++
Aspergillus niger	+	++	++	-	-
Rhizoctonia solani	-	+	-	++	-

– shows no activity (0 mm zone of inhibition)
+ shows low activity (1-5 mm zone of inhibition)
++ shows medium activity (6-10 mm zone of inhibition)
+++shows maximum activity (> 10 mm zone of inhibition)

is also evident from the present data that concentrations of these elements are high in most of the extracts. In crude and dialyzed basic extracts higher amounts of Na (4.05 and 3.59%) were observed. It is quite possible that during alkali (0.1N NaOH) extraction of the plant *H. musciformis* some alkali remains intact with the extract and therefore increases the Na content and also the yield of these extracts as compared to the aqueous and acidic extracts (Arman & Qadar 2012). During dialysis which is a purification process some Na could be removed and so its concentration was decreased in dialyzed basic extract.

Highest concentration of Mg (3.35%) was observed in crude aqueous extract. Almost equal quantities (1.03-1.73%) were found in other three extracts such that crude acidic and dialyzed aqueous and basic extracts. It is reported in literature that carrageenans are highly sulphated galactans (polysaccharides) extracted from red seaweeds (Murano et al., 1997). The present analysis also shows that except one extract (dialyzed acidic 0.74%), the remaining extracts contained higher S contents (5.95-17.92%). The highest value was observed in the crude aqueous extract.

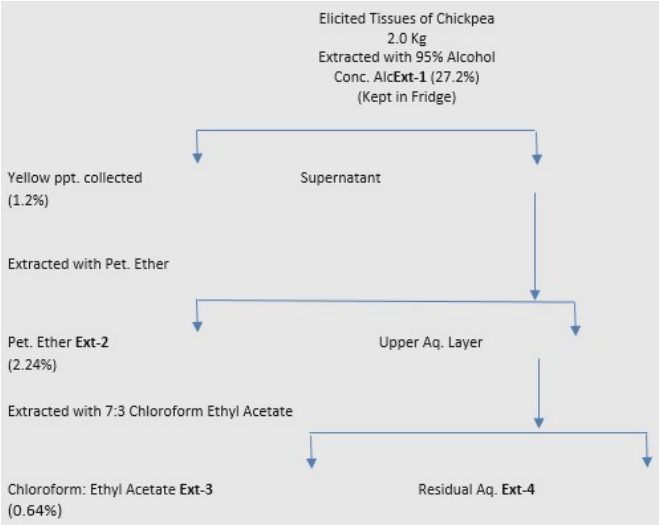

Fig. 10: Fraction Extraction

Cl and K concentrations were nearly same in the four extracts such as all crudes and one dialyzed (acidic) extract. However these elements showed exceptionally high values 25.31% and 25.46% in dialyzed acidic extracts respectively. These concentrations were the highest among all the elements found in these extracts except C and O (only one extract i.e dialyzed acidic showed less O = 17.0%). The results also showed that the concentrations of these major elements such as Na, Mg, S, Cl and K were very low (< 1%) in few extracts like crude basic and some dialyzed extracts.

Literature search shows that Ca has found in the largest concentration and widest range in *rhodophyta* (Mohammadi & Asbchin 2011). High Ca concentrations ranging from 3.43-6.72% were also found in all the investigated crude extracts while present in low concentration (1.10%) in dialyzed basic extract. The presence of Al in algal extracts generally shows the impurities coming from seawater into seaweeds. Seawater of Karachi coast is also contaminated with industrial and domestic wastes (Qari and Siddiqui 2010). In the present work Al was present in small amounts such as 0.33% in only two crude extracts (aqueous and acidic) while other extracts were devoid of it. Zn was found in large quantity (2.60%) in crude acidic extract.

In general the declining concentrations of these mineral elements in various crude extracts are summarized as C> O>S>Ca>Na>Mg>K >Cl> Zn >Al and that for the dialyzed extracts are in the order of C>O>CL>K>S> Na>Mg> Ca >Zn. The trend shows that C was present in the highest concentration followed by O among all the crude and dialyzed extracts while Al and Zn were in the lowest concentrations in these extracts. Data also shows that except C, Cl and K, the other elements were found in higher concentrations in crudes than in the purified dialyzed extracts.

Commercial carrageenan procured from market was also analyzed; its elemental composition is presented in table 2 and in figs. 7-9 (Scans 1-3). Nine elements were detected in varying quantities. C & O were found in the highest concentrations. The ranges of concentration of C, O, Na, Mg, S and K in these scans were 31.05-34.12%, 34.89-38.99%, 5.92-10.12%, 0.30-0.53%, 12.56-13.66%, 4.41-5.18% respectively. Ca (1.20%) was detected only in scan -3 while Mg was absent in this scan. The concentrations of these elements were more or less similar to that observed in the investigated crude and purified extracts. Two additional elements Mo and Cu were also found in the commercial sample. Mo concentrations (4.35% and 4.53%) were very high as shown in scan-2 & 3. Cu (1.66 %) was detected only in scan 1. According to IUFoST Scientific Information Bulletin (SIB 2011) some metals including Pb, Cd, Hg, Cu, Se and Mo can be particularly harmful to animals and men, mostly they arise indirectly in food stuffs from the environment or sea water, so due to the presence of large quantity of Mo, this commercial carrageenan is not safe to use in food and pharmaceutical products.

Elemental composition of *H. musciformis*, red algae of Karachi coast is reported recently (Rizvi *et al*., 2001; Qari & Siddiqui 2010). Comparing the concentrations of elements found in the present investigated extracts with that recorded for this algal plant which is a raw material used to obtain these carrageenans, indicates that concentrations of most of the elements were significantly higher in the present extracts than that found in the whole plant, it may be because these polysaccharide extracts concentrate minerals from its parental algae during extraction. This is quite similar to the fact reported earlier that seaweeds concentrate minerals and trace elements from seawater and the mineral concentration in seaweeds is very high as compared to the concentration in the surrounding water (Devi *et al*., 2011). Seasonal variations

in the yield, chemical and elemental compositions have also been reported in *H. musciformis* and various other marine macro algae (Aziza *et al*., 2008; Rizvi *et al*., 2001; Qari & Siddiqui 2005 & 2010).

Major and trace elements found in plant extracts including algal polysaccharides are vital for the metabolic reactions in human and animal health such as enzymatic regulation of lipid, carbohydrate and protein metabolism. Na, Mg, Cl and K are responsible for the maintenance of body fluid balance (Nisizawa *et al*., 1987; Insel *et al*., 2007). Ca is involved in many physiological processes such as lactation and hormone release etc. it also plays an important role in the construction of cell wall, bones, teeth and other structural constituents (Soon *et al*., 2007). Zn has got some role in saving the enzymes and its deficiency is characterized by recurrent infections (Devi *et al*., 2011).

Seaweeds are a potential source of plant minerals due to their high levels and their mineral contents are often higher than that of the land plants (Ortega-Calvo *et al*., 1993). Bioaccumulation of the major elements like Na, Mg, Ca, K and Zn in the present carrageenan extracts increases their nutritional values. These elements were in the same or in less quantity than reported previously for some medicinal plants which also shows that these extracts are safe for human use (Muruganantham *et al*., 2009). Seaweed products intake in human beings is also thought to restore fitness (Bougle *et al*., 1996). A recent study showed a co-relation between the elemental contents and the biological activities of crude extracts of selected medicinal plants, the significant biological activities of some of these plants may be due to the presence of balance amount of C and O in combination with other elements (Khan et al 2006).

Homogeneous pure fractions of elicitor molecules with known structure are highly desirable for targeting receptor molecules and to understand the recognition mechanism of plant-pathogen or plant-elicitor molecules interactions.

In these experiments seaweed elicitors were used against chickpea tissues, but studies can be extended to examine the effects of these polysaccharides on some other cash crops such as tomato, potato, beans and cotton growth to establish the activity spectrum of these preparations.

It is our interest to study the systemic induction of Phytoalexins using the cell cultures of selected host, inoculated by respective fungal pathogen or pathogen derived elicitor molecules as well as by the treatment of elicitor molecules derived from various algal plants side by side, under identical conditions to record the possible differences in the induced resistance responses, especially biosynthesis of Phytoalexins.

Repeated experiments revealed that polysaccharides obtained from *H. musciformis* (red algae) were the most active elicitors and partially characterized as complex mixture of carrageenans. Complete analysis and full characterization of these polysaccharides is very much desirable and considering to make progress in this field.

CONCLUSION

The results presented in this study revealed variability in mineral elements concentration between crude and purified polysaccharide extracts. The presence of major elements like Na, Mg, Ca, K, and Zn in high quantities and non detection of toxic elements such as Cd, Hg and Pb show the safer and more versatile utilization of these carrageenan extracts in food and pharmaceutical industries. This study can also help in the production of desired quality products by using these polysaccharides. On the basis of studies, it was concluded that treated tissues of chickpea responded differentially to the various polysaccharides preparations of algae, and produced a positive and definite resistance response in term of induced browning and Phytoalexin production. The extent of response was different for various samples, could be due to compositional and structural differences of these preparations. It was also observed that maximum elicitor activity was produced by the sugars obtained from red algae plant.

ACKNOWLEDGEMENTS

This work was done in the in-house R & D project of PCSIR Laboratories Complex Karachi. Authors are thankful to the Centralize Science Laboratories, University of Karachi, Karachi, Pakistan for providing instrumental facilities to carry out this work.

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