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THE ANTIOXIDANT ACTIVITIES OF ACID HYDROLYSIS OF κ-Carrageenan

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ABSTRACT

Carrageenans are naturally sulphated polysaccharides produced by primary metabolism in red seaweed (*Rhodophyceae*). Carrageenans are thought to be a good source of antioxidants. The antioxidant activity of commercial κ-Carrageenan and degraded κ-Carrageenan will be determined in this investigation. Hydrochloric acid was used to hydrolyzed κ-Carrageenan. A 3500 Da molecular weight dialysis tube was also used to separate the filtrate (MW 3500 Da). UV-Vis, FTIR, and GC-MS spectroscopies were used to analyze the chemical and spectral properties of commercial Carrageenan and its degradation products. The antioxidant activity of the compounds was determined using 2,2-diphenyl-2-picryl-hydrazyl (DPPH) and compared to vitamin C as a standard using UV at a wavelength of 517 nm in the concentration range of 200 - 800 ppm. The chemical structure of the degraded κ-Carrageenan was disclosed by FTIR analysis, which demonstrated a decrease in the intensity of the sulfate peak and the production of new carbonyl groups. Hexadecanoic acid ethyl ester, 1,2-benzenedicarboxylic acid, 9,19-Cyclo-9β-lanostan-3β-ol, 24-methylene-, and (E,E,E,E,E,E)-(1)-2,6,10,15,19,23-hexamethyltetracosa-1,6,10,14,18,22-hexaen-3-ol were found in the GC-MS. Analysis antioxidant activities of commercial κ-Carrageenan, degraded κ-Carrageenan with a molecular weight over 3500 Da, and κ-Carrageenan with a molecular weight below 3500 Da, respectively, have range percent inhibition from 7.83 - 13.58%, 32.64 - 42.28%, and 26.63% - 34.99%. Vitamin C, on the other hand, has a percent inhibition of 97.65% - 99.48%.

Keywords: κ-Carrageenan, Antioxidant, Percent Inhibition, Hydrolysis.

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INTRODUCTION

Many cases of degenerative diseases have been reported as the leading cause of death in the world today.[1] This disease is caused by the presence of free radicals, which impair the immune system. Because free radicals are unstable and extremely reactive, they tend to react with other molecules to maintain their stability. Free radicals have high reactivity, which means they can start a chain reaction all at once, resulting in radical chemicals that can harm vital bodily cells. The use of antioxidants can reduce the reactivity of free radicals.[2]

Antioxidants are chemicals that can prevent other molecules from oxidizing. Antioxidants work by capturing free radicals, inhibiting chain initiation, inhibiting peroxide decomposition, preventing continued hydrogen abstraction, reducing power, and binding to transition metal ion catalysts.[3] Antioxidants are classified as natural or synthetic antioxidants depending on the source. Antioxidant chemicals that occur naturally in the body and serve as a typical defense mechanism for the human body are known as natural antioxidants. Carrageenan is one of the potential antioxidant sources.[4] Depolymerization procedures also affect the antioxidant properties of κ-Carrageenan.[5]

Carrageenan is a polysaccharide compound extracted from red seaweed (*Rhodophyceae*),[6,7] that is composed of a number of galactose units with α (1,3) D-galactose and β (1,4) 3,6-anhydrogalactose bonds alternately, either containing sulfate esters or without sulfate.[8, 9] Carrageenan is present in the cell walls of seaweed or its intracellular matrix and Carrageenan is a large constituent part of the dry weight of seaweed compared to other components. Carrageenan has properties as a gelling agent, including pH, stability, viscosity, gel formation and reactivity with protein.[10] The properties of Carrageenan are widely used as a

stabilizer, thickener, gelling agent and emulsifier in the food, medicine, textile, cosmetics and other industries,[10, 11] and it is better capsule agents.[12] Carrageenan itself is classified as kappa (κ), iota (ι), and lambda (λ) based on the number of group sulfate esters and the presence of 3,6-anhydro-D-galactose.[13] The type of Carrageenan that is most in demand is κ-Carrageenan because it has a high water holding capacity. The demand for Carrageenan is growing in tandem with the growth of industries that use it as a raw material to make products.[11, 14]

κ-Carrageenan is the most abundant species in nature (constituting 60% of Carrageenan in *Chondrus crispus* and predominantly in *Euchema cottonii*).[15] This type of Carrageenan will break off in acidic solutions, but once the gel is created, it will resist degradation. In recent years, algal polysaccharides have been demonstrated to have a significant function in preventing oxidative damage to living organisms by acting as a free radical scavenger. However, research on oligosaccharides generated from algal polysaccharides (κ-Carrageenan), particularly their antioxidant activity, is limited.[16] The antioxidant activity of commercial κ-Carrageenan and the κ-Carrageenan that has been degraded from its polysaccharides (oligosaccharides) is of particular interest.

EXPERIMENTAL

Apparatus and Reagents

Digital analytical scales, glassware, magnetic stirrer, refrigerators, dialysis tube (MWCO 3500 Da), UV-Vis Spectrophotometer Cecil Aurius Series CE 2021, Gas Chromatography-Mass Spectrometry (GC-MS) GCMS-QP2010 Ultra Shimadzu and Fourier Transform Infrared Spectrophotometer (FTIR) Perkin Elmer Two ATR were among the instruments used in this study. The materials used were κ-Carrageenan obtained from Sigma Aldrich, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, vitamin C, HCl, NaOH, distilled water, cation and anion resins (Amberlite IR-120 and Amberlite IRA-400), and deionized water.

Preparation of Carrageenan Hydrolysis

κ-Carrageenan (3 g) was dissolved in 300 mL of 0.1 mol/L HCl solution and stored for 24 h after stirring. The solution was neutralized with 0.1 mol/L NaOH.[16] The solution was dialyzed five times with deionized water using a dialysis tube with a molecular weight cut of 3500 Da (MWCO 3500 Da). As in earlier investigations, a dialysis tube was employed to filter and separate molecules.[17, 18] The sample inside the dialysis tube with a molecular weight greater than 3500 Da was referred to as κ-Carrageenan MW > 3500 Da. After that, the deionized water used for dialysis was collected and the solvent was evaporated. Using cation and anion resins, the salt from deionized water was removed, and the sample was referred to as κ-Carrageenan MW < 3500 Da. The samples were dried overnight in the oven before being used for further treatment.

Antioxidant Assay

For the antioxidant activity test, the DPPH (1,1 diphenyl-2-picrylhydrazyl) compound was utilized as a source of free radicals.[19] A solution of vitamin C was used as a positive control at concentrations of 20, 40, 60 and 80 ppm. The DPPH 0.1 mM solution was prepared by dissolving 1.97 mg DPPH in 50 mL of methanol in a volumetric flask.

Preparation of Sample Test and Positive Controlled Solutions

By dissolving 0.1 g of each sample in methanol-water solution (1:1), the stock solutions of 1000 ppm from the three samples were prepared. In addition, sample solutions of 200 ppm, 400 ppm, 600 ppm, and 800 ppm were produced for each series.

As a blank absorption was carried out by piping 1 mL of DPPH and then adding 3 mL of methanol. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm after the solution was homogenized and allowed to stand for 30 minutes.[20-22] The test and comparison solution uptake was carried out by pipetting 3 mL (commercial κ- Carrageenan, κ-Carrageenan MW > 3500 Da, κ-Carrageenan MW < 3500 Da, and vitamin C) from each concentration. 1 mL of 0.1 mM DPPH was added to the test and comparison solutions, which were then left to stand for 30 minutes at 37ºC before the absorbance was measured. All of the pieces were written in triplets.

The amount of free radical inhibition percentage is calculated using the following formula:[20]

$$\text{Inhibition (\%)} = [(Ac - As)/Ac] \times 100\%$$

Where, Ac is the absorbance of a controlled negative solution (without a sample), As is the absorbance of solutions containing a sample (κ- Carrageenan, κ-Carrageenan MW > 3500 Da, κ-Carrageenan MW < 3500 Da).

Samples Characterizations

A UV-Vis spectrophotometer was used to evaluate antioxidant activity. GC-MS was used to establish the type of oligosaccharide obtained, and FTIR spectroscopy was used to determine the functional groups of κ-Carrageenan and the oligomers.

RESULTS AND DISCUSSION

Antioxidant Activity Testing in Samples

The antioxidant activity of κ-Carrageenan was assessed using DPPH as a source of free radicals.[19, 21] The DPPH test relies on a decrease in absorbance due to DPPH color change, in which DPPH acts as free radicals and reacts with antioxidants to form DPPH-H (1,1-diphenyl-2-picrylhydrazine) and antioxidant radicals. To compensate for the electron shortfall, antioxidants will transfer hydrogen atoms to DPPH radicals, resulting in more stable antioxidant radicals. The antioxidant activities of commercial κ-Carrageenan, κ-Carrageenan MW > 3500 Da, and κ-Carrageenan MW < 3500 Da were investigated at varying concentrations of 200 ppm, 400 ppm, 600 ppm, and 800 ppm. When the sample is added, the color of DPPH changes from purple to yellow. The absorbance value of DPPH free radicals decreases as the hue changes, indicating that the antioxidant content is increasing. Reduced versions of DPPH and antioxidant radicals are produced when DPPH combines with antioxidant substances.

Because antioxidants reduce DPPH radicals, the absorbance value of each sample falls with increasing concentration. The larger the antioxidant activity and the lower the absorbance, the higher the concentration of each sample containing more particles of antioxidant chemicals. Fig.-1 shows the results of a research of the absorbance value of commercial κ-Carrageenan, κ-Carrageenan MW > 3500 Da, and κ-Carrageenan MW < 3500 Da.

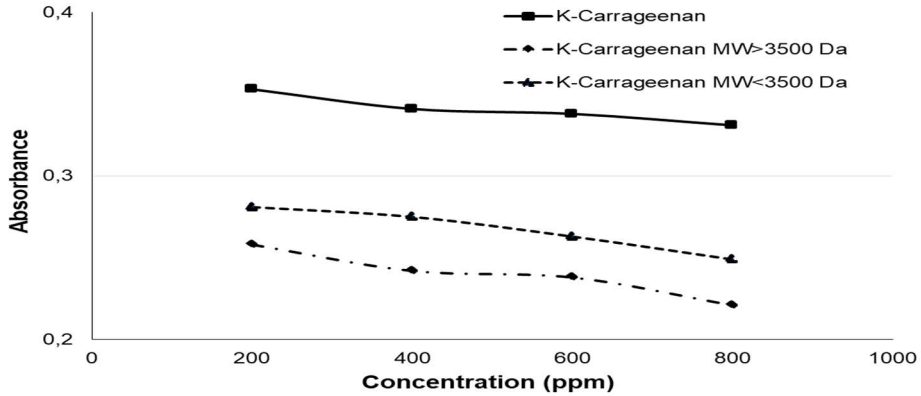

Fig.-1: The absorbance values of commercial κ-Carrageenan, κ-Carrageenan MW> 3500 Da and κ-Carrageenan MW <3500 Da

The antioxidant activity of the three samples was also determined using the findings of the percentage of free radical inhibition calculation based on the absorbance value obtained. The percent inhibition value of commercial κ-Carrageenan has the lowest compared to the κ-Carrageenan MW > 3500 Da, and κ-Carrageenan MW < 3500 Da in Fig.-2, indicating that their antioxidant activities can be seen from the percent inhibition value of commercial κ-Carrageenan is the lowest.

Measurement Antioxidant Activities of Vitamin C and κ-Carrageenan Samples

With a molecular weight of 178 g/mol and a chemical formula of $C_6H_8O_6$, ascorbic acid (vitamin C) is a white crystal with a melting point of 190-192 °C. Vitamin C is soluble in water, acetone and ethanol to a lesser extent has a low molecular weight, and is practically insoluble in chloroform, ether, and benzene. Vitamin C is utilized as a reference in this investigation because vitamin C is a highly potent natural antioxidant substance.[21] The vitamin C solutions were made comparable to the Carrageenan samples. A

UV-Vis spectrophotometer was used to evaluate antioxidant activity at a wavelength of 517 nm as the maximum absorbance of DPPH. The lower the absorbance value obtained, the higher the vitamin C concentration. The absorbance value of vitamin C, as well as the corresponding percent suppression of DPPH free radicals, may be seen in Fig.-3. The percent suppression of DPPH free radicals is shown in this graph as a function of vitamin C concentration. As the quantity of vitamin C increases, more particles can be oxidized by the existing DPPH free radicals, as seen in the figure.

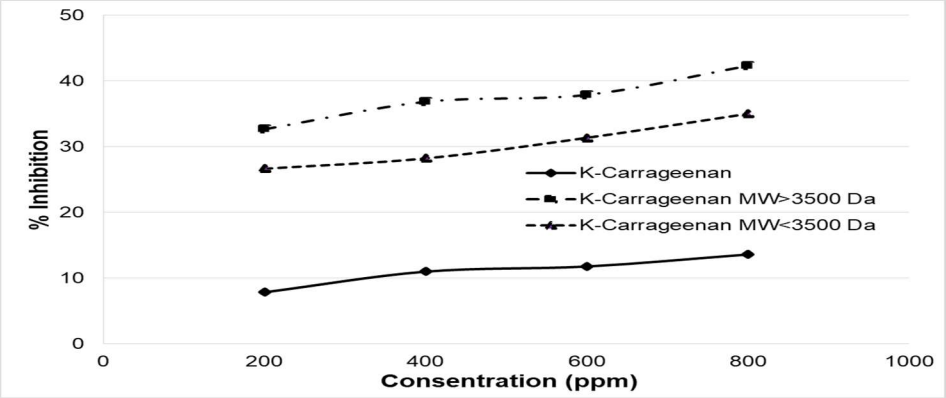

Fig.-2: Percent Inhibition of Commercial κ-Carrageenan, κ-Carrageenan MW> 3500 Da and κ-Carrageenan MW <3500 Da.

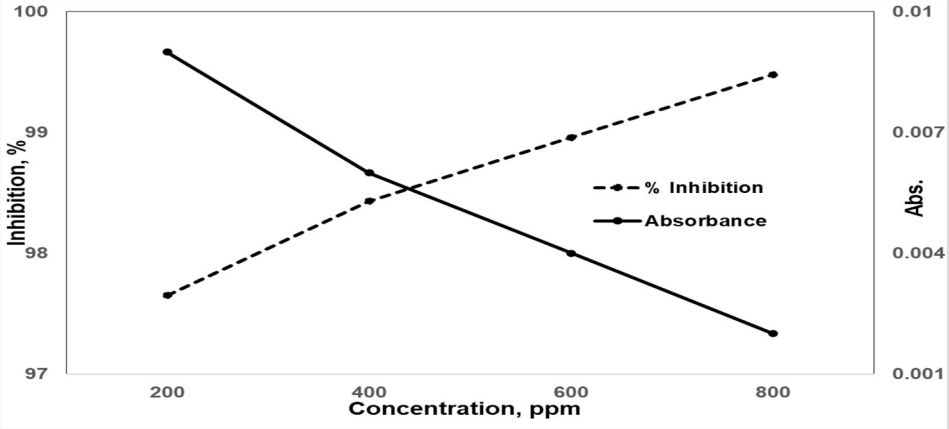

Fig.-3: The Absorbance and Percent Inhibition Value of Vitamin C

When comparing the three Carrageenan samples to vitamin C, the data obtained for the percentage of free radical scavenging by DPPH has distinct values. Table-1 shows that the three samples and vitamin C concentrations grew from the lowest to the highest. In the concentration range of 200-800 ppm, the percent inhibition of commercial κ-Carrageenan, κ-Carrageenan MW > 3500 Da, and κ-Carrageenan MW < 3500 Da was 7.83 -13.58%, 32.64 - 42.28%, and 26.63% - 34.99%, respectively. Vitamin C, on the other hand, has a very high antioxidant activity, with a percent inhibition of 97.65% - 99.48%. This is consistent with the findings that increasing the concentration increases the percentage of inhibition.[23,24] Commercial κ-Carrageenan has lower antioxidant activity than degraded κ-Carrageenan. This is consistent with findings indicating chemical modification of Carrageenan oligosaccharides can boost increase antioxidant activity in vitro.[25] This could be owing to its ability to donate hydrogen more effectively than commercial κ-Carrageenan.

FTIR Analysis of κ-Carrageenan Samples

Figure-4 shows the FTIR spectra of commercial κ-Carrageenan before and after hydrolysis. The spectra revealed an (O-H) stretching vibration from hydroxyl groups ranging between 3100 and 3600 cm^{-1}.[26] However, the degraded Carrageenan samples showed the appearance of small peaks above 3600 cm^{-1}, which could be due to resin impurities, as shown in the previous study.[27] The presence of this (O-H) group is supported by the appearance of the (C-O) alcohol group at 1070.53 cm^{-1}. A sulfate ester group was

detected in the area of 1259.43 cm^{-1}, a glycosidic bond at 1069.33 cm^{-1}, a galactose group at 970.43 cm^{-1}, a 3.6 anhydrogalactose group at 929.27 cm^{-1}, and a 3,6 anhydrogalactose-4-sulphate group at 844.17 cm^{-1} in commercial κ-Carrageenan. Cleavage of the groups from the chain may result in a decrease in the strength of this sulfate peak.[28]

Table-1: Percent Inhibition of κ-Carrageenan Samples and Vitamin C at Different Concentrations

Samples	Inhibition (%)			
	200 ppm	400 ppm	600 ppm	800 ppm
κ-Carrageenan	7.83	10.97	11.75	13.58
κ-Carrageenan BM > 3500 Da	32.64	36.81	37.86	42.28
κ-Carrageenan BM < 3500 Da	26.63	28.20	31.33	34.99
Vitamin C	97.65	98.43	98.96	99.48

Then, at a wavelength of 2945.40 cm^{-1}, 2901.04 cm^{-1}, 1462.09 cm^{-1} and 1373.36 cm^{-1}, alkanes in the (C-H) range. The range (C-H) of alkenes can be found at a wavelength of 972.16 cm^{-1}, 927.79 cm^{-1}, 844.85 cm^{-1}, 738.76 cm^{-1}, and 704.04 cm^{-1}. The presence of the (C = C) group at a wavelength of 1645.33 cm^{-1} supports the (C-H) alkene group. Several peaks are found in commercial κ-Carrageenan, κ-Carrageenan MW > 3500 Da, and κ-Carrageenan MW < 3500 Da, as shown in Fig.-4. The formation of additional carbonyl groups can be noticed in degraded κ-Carrageenan by the emergence of tiny peaks at 1740 cm^{-1}.[29]

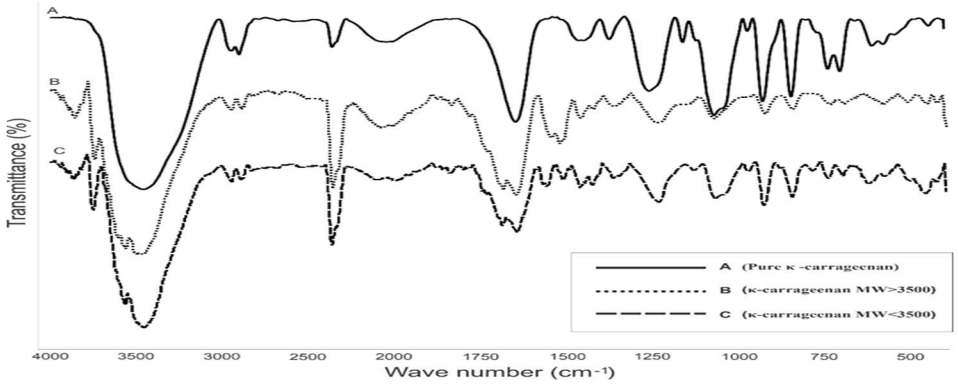

Fig.-4: FTIR Analysis Curves of Commercial κ-Carrageenan (A), κ-Carrageenan MW > 3500 Da (B), and κ-Carrageenan MW < 3500 Da (C)

GCMS Analysis

There are 15, 10 and 7 peaks identified by GC-MS analysis of commercial κ-Carrageenan, κ-Carrageenan MW > 3500 Da, and κ-Carrageenan MW < 3500 Da indicating of 12, 10, and 4 compounds, respectively (Fig.-5). Retention time, similarity index, percent area, molecular formula, and molecular weight were used to confirm their presence (Tables-2 to 4). Dodecanoic acid, 1,2,3-propanetriyl ester is the most abundant compound in commercial κ-Carrageenan (49.60%), followed by (3.beta.,24s)-Stigmast-5-en-3-ol (17.17%), 9,10-anthracenedione (7.75%), methyl commate D (5.45%), and other compounds. This chromatogram depicts a sample containing antioxidants such as hexadecanoic acid ethyl ester (0.78%) and 9,19-cyclo-9β-lanostan-3β-ol, 24-methylene- (1.11%). Hexadecanoic acid ethyl ester are compounds that are known as an antioxidant, and 9,19-cyclo-9β-lanostan-3β-ol, 24-methylene- are pentacyclic triterpenoids which are active compounds capable of capturing free radicals.[30, 31]

The GC-MS analysis of κ-Carrageenan MW > 3500 Da reveals ethyl oleate as the highest amount (21.72%), followed by hexadecanoic acid ethyl ester (19.27%), 1,2-benzenedicarboxylic acid (11.32%), (E,E,E,E,E,E)-(1)-2,6,10,15,19,23-hexamethyltetracosa-1,6,10,14,18,22-hexaen-3-ol (10,49%), and other compounds (Table 3). Hexadecanoic acid ethyl ester, 1,2-benzenedicarboxylic acid, and (E,E,E,E,E,E)-(1)-2,6,10,15,19,23-hexamethyltetracosa-1,6,10,14,18,22-hexaen-3-ol have all been found as antioxidants. Some of the compounds (E,E,E,E,E,E)-(1)-2,6,10,15,19,23-hexamethyltetracosa-1,6,10,14,18,22-hexaen-3-ol have a similar molecular weight to Squalene (410 Da), which is an antioxidant, antibacterial, anti-tumor, anti-inflammatory, and antinociceptive.[32] Meanwhile, the results of GC-MS κ-Carrageenan analysis MW < 3500 Da reveals dodecanoic acid, 1,2,3-propanetriyl ester as the major compounds (99.06%) (Table-4).

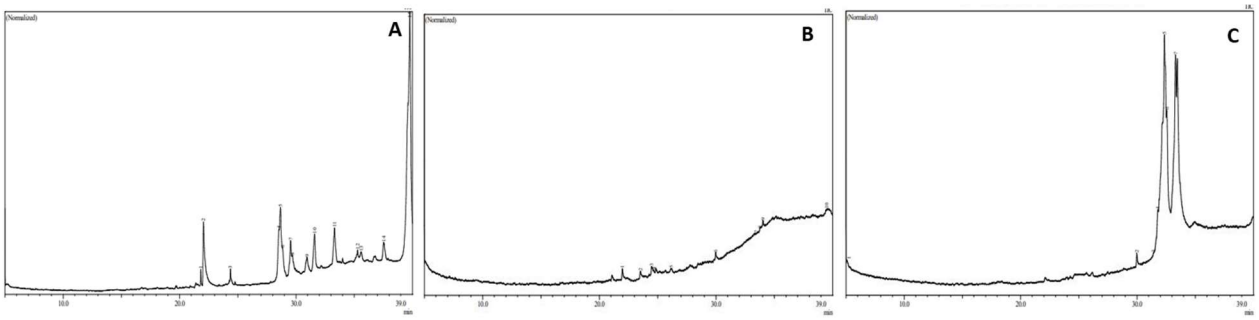

Fig.-5: GC-MS Chromatogram of Commercial κ-Carrageenan (A), κ-Carrageenan MW > 3500 Da (B), and κ-Carrageenan MW < 3500 Da (C)

Table-2: Compounds identified by GC-MS Analysis of Commercial κ-Carrageenan

Peaks	Retention Time (min)	Similarity Index	Compounds	Percent Area	Molecular Formula	Molecular Weight
1	21.820	94	Hexadecanoic acid ethyl ester	0.78	$C_{18}H_{36}O_2$	284
2	22.046	96	9,10-anthracenedione	7.75	$C_{14}H_8O_2$	208
3	24.364	91	Ethyl Oleate	1.10	$C_{20}H_{38}O_2$	310
4	28.533	84	(3.beta.,24s)-Stigmast-5-en-3-ol	4.90	$C_{29}H_{50}O$	414
5	28.663	86	(3.beta.,24s)-Stigmast-5-en-3-ol	10.31	$C_{29}H_{50}O$	414
6	28.858	65	(3.beta.,24s)-Stigmast-5-en-3-ol	1.96	$C_{29}H_{50}O$	414
7	29.535	94	1-Triacontanol	4.49	$C_{30}H_{62}O$	438
8	29.700	91	1-Triacontanol	2.15	$C_{30}H_{62}O$	438
9	30.939	86	alpha-Amyrin	2.17	$C_{30}H_{50}O$	426
10	31.596	75	Nonadecanenitrile	5.15	$C_{19}H_{37}N$	279
11	33.309	80	Methyl Commate D	5.45	$C_{31}H_{50}O_4$	486
12	35.292	91	Pentatriacontane	0.61	$C_{35}H_{72}$	492
13	35.596	89	9,19-Cyclo-9β-lanostan-3β-ol, 24-methylene-	1.11	$C_{31}H_{52}O$	440
14	37.532	83	Lup-20(29)-en-3-ol, acetate, (3beta)-	2.47	$C_{32}H_{52}O_2$	468
15	39.777	72	Dodecanoic acid, 1,2,3-propanetriyl ester	49.60	$C_{39}H_{74}O_6$	638

Table-3: Compounds identified by GC-MS Analysis of κ-Carrageenan MW > 3500 Da

Peaks	Retention Time (min)	Similarity Index	Compounds	Percent Area	Molecular Formula	Molecular Weight
1	21.979	94	Hexadecanoic acid ethyl ester	19.27	$C_{18}H_{36}O_2$	284
2	23.525	96	9-octadecenoic acid methyl ester	9.66	$C_{19}H_{36}O_2$	296
3	24.484	91	Ethyl Oleate	21.72	$C_{20}H_{36}O_2$	310
4	24.683	50	(3AS,9AS,9BR)-6,6,9AA-trimethyl-trans-perhydro naphtho[2,1-B]furan	3.44	$C_{15}H_{26}O$	222
5	26.152	79	2,6,10-trimethyl,14-ethylene-14-pentadecne	5.57	$C_{20}H_{38}$	278
6	29.970	93	1,2-Benzenedicarboxylic acid	11.32	$C_{24}H_{38}O_4$	390

7	33.450	49	5-.beta.-Card-20(22)-enolide,7-.beta.,8-epoxy-3-.beta.,11-.alpha.,14-trihydroxy-12-oxo-	3.83	$C_{23}H_{30}O_7$	418
8	33.758	44	Eicosane, 9-cyclohexyl-	9.57	$C_{26}H_{25}$	364
9	34.023	85	(E,E,E,E,E,E)-(1)-2,6,10,15,19,23-hexamethyltetracosa-1,6,10,14,18,22-hexaen-3-ol	10.49	$C_{30}H_{50}$	410
10	39.498	57	1,2,4-triazol-3-amine, 5-(1,3,5-trimethyl-4-pyrazolyl)amino-	5.15	$C_8H_{13}N_7$	207

Because there are three compounds and a high amount of content in degraded κ-Carrageenan MW > 3500 Da, the antioxidant activity is higher than the others, namely hexadecanoic acid ethyl ester (19.27%), 1,2-benzenedicarboxylic acid (11.32%), and (E,E,E,E,E,E)-(1)-2,6,10,15,19,23-hexamethyltetracosa-1,6,10,14,18,22-hexaen-3-ol (10,49%). Commercial κ-Carrageenan, on the other hand, has two antioxidant compounds, namely hexadecanoic acid ethyl ester (0.78%) and 9,19-Cyclo-9β-lanostan-3β-ol, 24-methylene- (1.11%), whereas κ-Carrageenan MW < 3500 Da contains only one antioxidant compound, namely 1,2-benzenedicarboxylic acid (0.52%). It is likely that the antioxidant activity of the three samples is low since several chemicals predominate that are not as antioxidants.

Table-4: Compounds identified by GC-MS Analysis of κ-Carrageenan MW < 3500 Da

Peaks	Retention Time (min)	Similarity Index	Compounds	Percent Area	Molecular Formula	Molecular Weight
1	5.061	87	Ethen-1-D-OL	0.19	C_2H_3DO	45
2	29.977	95	1,2-Benzenedicarboxylic acid	0.52	$C_{24}H_{38}O_4$	390
3	31.408	41	D-Mannitol, 1,1'-O-1,16-hexadecanediylbis	0.24	$C_{28}H_{58}O_{12}$	589
4	31.792	71	Dodecanoic acid, 1,2,3-propanetriyl ester	3.46	$C_{39}H_{74}O_6$	638
5	32.355	76	Dodecanoic acid, 1,2,3-propanetriyl ester	38.90	$C_{39}H_{74}O_6$	638
6	32.566	74	Dodecanoic acid, 1,2,3-propanetriyl ester	11.20	$C_{39}H_{74}O_6$	638
7	33.317	59	Dodecanoic acid, 1,2,3 propanetriyl ester	45.50	$C_{39}H_{74}O_6$	638

CONCLUSION

One of the potential antioxidant sources is κ-Carrageenan. When compared to vitamin C, commercial κ-Carrageenan, degraded κ-Carrageenan with a molecular weight above 3500 Da, and κ-Carrageenan with a molecular weight below 3500 Da demonstrate less active antioxidant. The chemical structure of the commercial κ-Carrageenan and degraded κ-Carrageenan differed, with the intensity of sulfate peak decreasing and the formation of additional carbonyl groups indicated by FTIR spectra analysis. The FTIR also revealed the presence of the potential group of antioxidants. The presence of chemicals known as sources of antioxidants in commercial κ-Carrageenan and degraded κ-Carrageenan was also discovered by GC-MS analysis, including hexadecanoic acid ethyl ester, 1,2-benzenedicarboxylic acid, (E,E,E,E,E,E)-(1)-2,6,10,15,19,23-hexamethyltetracosa-1,6,10,14,18,22-hexaen-3-ol, and 9,19-Cyclo-9β-lanostan-3β-ol, 24-methylene-.

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