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Analysis of Compounds Composition from Red Seaweed *(Kappaphycus cottonii)* Methanol Extract using GC-MS and Determination of Reducing Sugar Content

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ABSTRACT (10PT)

Red seaweed *(Kappaphycus cottonii)* contains alkanes, esters, ketones, fatty acids, and elaidic acid. The demand for red seaweed on the world market is currently increasing, so it is necessary to develop research on the compound content and sugar content of this type of seaweed. This research aims to determine the compound content of the methanol extract of *Kappaphycus cottonii* by gas chromatography-mass spectrophotometry (GC-MS) analysis and the reduced sugar content in it. The compound content in the extract was analyzed based on the similarity of compounds found in the National Institute Standards and Technology (NIST) library. Reducing sugar levels were measured with a UV-Vis spectrophotometer using the dinitrosalicylate (DNS) method at a wavelength of 512 nm. The research results showed that the methanol extract content of *Kappaphycus cottonii* was obtained using GC-MS analysis in the form of alkane, ester, ketone, fatty acid, and elaidic acid compounds. The test results for determining reducing sugar levels obtained an average level of 0.3356%. The compounds detected in the extract were 18 chemical compounds. The reduced sugar content contained in the red seaweed *Kappaphycus cottonii* is included in the low category.

1. Introduction

Seaweed is a biological resource belongs to a type of plant-like protist or often called algae. Based on the dye pigment content contained, seaweed is classified into three classes, namely green seaweed (Chlorophyceae), brown seaweed (Phaeophyceae), and red seaweed (Rhodophyceae).

According to Carpena et al., (2023) the red seaweed class (Rhodophyta) presents the highest proportion of bioactive compounds, accounting for more than 1600 individual compounds, representing 53% of the bioactive compounds reported in algae. One type of seaweed is red seaweed *(Kappaphycus cottonii)*, which produces high levels of kappa carrageenan, which is often called *Kappaphycus alvarezii*. This type of seaweed is widely cultivated in tropical areas, including Indonesia. Based on research by Das et al., (2023) the most common compounds found in this seaweed are phenols of (3.39 ± 0.41 mg/g) and tannins of (2.94 ± 0.41 mg/g). Research by Wiyanto, (2010) reported that *Kappaphycus alvarezii* has antibacterial activity as indicated by an inhibitory zone diameter of 16.60 mm against *Aeromonas hydrophilia* bacteria and 16.33 mm against *Vibrio harveyi* bacteria. This is supported by the results of research, namely that *Kappaphycus alvarezii* extract with methanol solvent has a wider inhibitory effect on bacteria compared to ethanol solvent. Research by Lantahl et al., (2017) regarding the antioxidant activity test of *Kappahycus alvarezii* using the DPPH method reported an IC50 value of 163.82 ppm, which means it has weak antioxidant activity. According to Sangha et al., (2013) seaweed is not yet commonly exploited for

pharmaceutical and/or nutraceutical purposes, but increasing demand from countries in Asia has encouraged research into the chemical content of seaweed.

The first step to utilizing red seaweed can be done by extracting it. Maceration is an extraction method that involves soaking the material in a solvent suitable for the active compound to be studied with low or no heating (Wahyudi et al., 2021). The organic solvent used for maceration is methanol. According to research by Salamah and Widyasari, (2015) methanol is a universal solvent that can attract most polar and non-polar compounds so that optimal results of active ingredients are obtained.

One sensitive and selective method that can be used to analyze compounds in the methanol extract of *Kappaphycus cottonii* is gas chromatography with a mass spectrometry detector (GC-MS). According to research by Candraningrat et al., (2021) analysis using GC-MS has various advantages, such as high sensitivity which can separate compounds that have been mixed and can analyze many compounds in low levels or concentrations.

Kappa cargenan is a type of linear polysaccharide sugar with the main unit of the galactan molecule being galactose which comes from seaweed extraction. Kappa carrageenan has an α-(1-3)-D-galactose-4-sulfate structure and is bound to β-(1-4)-3,6-anhydro-α-D-galactose and has one negative charge per disaccharide repeating unit (Bercea and Wolf, 2019). The galactose content that makes up this compound can be used as a standard in determining reducing sugar levels. The carrageenan content in seaweed is directly proportional to the amount of reduced sugar content (Zelvi et al., 2017). The demand for yeast on the world market is currently increasing, so it is necessary to develop research on reducing sugar levels in this type of seaweed.

Based on the description that has been mentioned, research on the analysis of the compound content of *Kappaphycus cottonii* extract needs to be carried out. Analysis of the compound content in *Kappaphycus cottonii* extract in this study was carried out using the gas chromatography method with a mass spectrometry detector (GC-MS). In addition, the reduced sugar content in the *Kappaphycus cottonii* extract needs to be determined so that its abundance in the extract can be determined.

2. Materials and Methods

2.1. Tools and Materials

The tools used in this research include: oven, a milling machine (Damiji Disk Mill Model FFC-23 Speed 5880 rpm power 3 Kw, China), a digital analytical scale (Adventure Ohaus, USA), an ultrasonic sonicator (Elmasonic S 100 H, Germany), magnetic stirrer (Cimarec, USA), Buchner funnel, vacuum (GAST model DOA-P504-BN specifications volts 220-240 amps: 1.9-2.2 Hz 50), rotary evaporator (Heidolph Hei-VAP Core), water bath (Memmert), a set of GC-MS tools (ISQD1702517_1), and a set of UV-Vis spectrophotometer tools (Shimadzu UV 1900). The sample used was seaweed *(Kappaphycus cottonii)* taken from Pangandaran Beach, West Java. The chemical used ini this research include: distilled water, methanol (E Merck, Germany), dimethyl sulfoxide (DMSO) (E Merck, Germany), NaOH (E Merck, Germany), potassium sodium tartrate (J. T. Baker, Italy), 3,5-dinitrosalicylic acid (CDH), and galactose standard (Sigma, Aldrich). All materials used in this research are of pro-analytical grade, unless stated otherwise.

2.2. Sample Preparation

3 kg of fresh red seaweed, *Kappaphycus cottonii,* was cleaned by washing with running water and then cut into pieces to increase the surface area of the sample. The samples were dried using an oven at 35°C. Samples that have been dried are ground by grinding to obtain samples in powder form. The powder was then sieved using a 40 mesh sieve (Warsi et al., 2023).

2.3. Sampel Extraction

A total of 35 grams of *Kappaphycus cottonii* seaweed powder was extracted using 210 mL of 80% methanol with a ratio of 1:6. The sample solution was sonified first for 1 hour, then stirred with a magnetic stirrer for 8 hours, followed by maceration for up to 24 hours. The extract was then filtered using filter paper (Whatman number 1) in a Büchner funnel. The residue was reextracted once with 80% methanol (Warsi et al., 2023).

The extract is concentrated using a rotary evaporator at a temperature of 60°- 65°C until it is solvent-free (freezes if stored in the freezer). If the extract is not thick, it can be continued with a water bath at 45°C. The dried seaweed extract was homogenized and weighed and the yield was

calculated with the formula below. The dry extract was stored in a freezer at -20°C (Warsi et al., 2023).

$$\% \text{ Yield} = \frac{\text{extract weight}}{\text{simplicia weight}} \text{ x } 100\%$$

2.4 Identification of Bioactive Compound using GC-MS

Identification was carried out to determine the profile of bioactive compounds in the red seaweed *Kappaphycus cottonii* using GC-MS (ISQD1702517_1) on the methanol extract of *Kappaphycus cottonii*. The results obtained are in the form of a chromatogram shown in a graph with several peaks. Each peak that appears represents one type of compound. Analysis by GC-MS using an Rtx-PCB capillary column (60 m x 0.25mm, thickness 0.25 mm). Helium with a purity of 99.99% is used as a carrier gas which has a flow rate of 1 mL/minute. A 1 mL extract sample was injected in spill mode using an autosampler (Shimadzu). The ion source temperature was set at 230°C. The temperature at the injector port and the interface temperature are set at 250°C and 270°C, respectively. The mass spectrophotometer was used in Electron Impact (EI) ionization mode at 70 eV with an emission current of 60mA (Das et al., 2023). The chromatogram and mass spectrum of the unknown compound were then compared with the spectrum of known compounds contained in the NIST library (Warsi et al., 2023).

2.5 Testing Reducing Sugar Levels using The DNS Method

Preparation of 20% Dimethyl Sulfoxide (DMSO) solution

A total of 10 mL of 100% absolute DMSO was pipetted and put into a 50 mL volumetric flask then distilled water was added to the mark.

Preparation of 0.5 N NaOH solution

A total of 2 grams of NaOH pellets was weighed and then dissolved in distilled water in a 100 mL volumetric flask, then diluted with distilled water to the limit mark.

Preparation of 10 mg/mL Red Seaweed Extract Solution

A total of 50.0 mg of seaweed extract was weighed and then dissolved in 5 mL of 20% DMSO.

Preparation of Dinitrosalicylate Reagent

A total of 0.25 grams of DNSA (3.5-dinitrosalicylic acid) was weighed, 7.5 g of potassium nitrate tartrate was added and dissolved in 80.0 mL of 0.5 N NaOH. The mixture was heated at 45°C until orange. After cooling, add distilled water to a volume limit of 100 mL (Warsi et al., 2023).

Preparation of 2.0 mg/mL Galactose Mother Solution

A total of 50.0 mg of galactose was weighed, dissolved in 20% DMSO in a 25.0 mL volumetric flask. The solution was then added with 20% DMSO up to the limit mark.

Blank Preparation

Pipetted 2.0 mL of 20% DMSO into a test tube and added 2.0 mL of DNS reagent and then closed. The solution was heated for 5 minutes at 95°C (Warsi et al., 2023) and cooled (Hasanah, 2015). Next, the blank solution is put into a cuvette to be used as a blank for reading standard solutions or samples with a spectrophotometer. The wavelength used is 512 nm (Mutmainnah et al., 2023).

How to find operating time

A total of 2.0 mL of the 100 µg/mL standard solution was put into a test tube and 2.0 mL of DNS reagent was added and then closed. The solution was heated for 5 minutes at 95°C (Warsi et al., 2023) and cooled (Hasanah, 2015). Next, the solution was put into a cuvette and the operating time was measured using a UV-Vis spectrophotometer. For 60 minutes, the absorbance of the solution was measured at a wavelength of 512 nm at intervals of 1 minute until the solution produced a stable absorbance. Stable absorbance can be used as operating time (Prima and Luhurningtyas, 2020).

How to find max lambda

A total of 2.0 mL of the 100 µg/mL standard solution was put into a test tube and 2.0 mL of DNS reagent was added and then closed. The solution was heated for 5 minutes at 95°C (Warsi et al., 2023) and allowed to stand according to the operating time. Next, the solution was put into a cuvette and the maximum wavelength was measured using a UV-Vis spectrophotometer at λ 400-600 nm (Prima and Luhurningtyas, 2020).

Preparation of galactose standard curve solution

A total of 0.05; 0.1; 0.15; 0.2; 0.25; and 0.3 mL of 2 mg/mL galactose stock solution were pipetted then each was put into a 5 mL volumetric flask and 20% DMSO was added to the limit. The concentrations obtained were 20, 40, 60, 80, 100, and 120 µg/mL.

Standard absorption measurement method

Take 2.0 mL of the standard solution of each concentration into a test tube and add 2.0 mL of DNS reagent and homogenize and close. The solution was heated for 5 minutes at 95°C (Warsi et al., 2023) and allowed to stand according to the operating time. The sample solution was put into a cuvette and the absorbance was measured using a UV-Vis spectrophotometer against a blank using the wavelength obtained (Warsi et al., 2023).

How to measure sample absorption

Take 2.0 mL of the extract solution then put it in a test tube and add 2.0 mL of DNS reagent and homogenize and close. The solution was heated for 5 minutes at 95°C (Warsi et al., 2023) and allowed to stand according to the operating time. The sample solution was put into a cuvette and the absorbance was measured using a UV-Vis spectrophotometer at the wavelength corresponding to that obtained for the blank (Warsi et al., 2023).

How to calculate reducing sugar levels

A linear regression equation was created for the relationship between concentration versus absorbance of the galactose standard solution with the formula below:

$$y = bx + a$$

The reducing sugar content is then calculated using the same formula below:

$$Reducing\ sugar\ content(\%) = \frac{X\ (mg/ml)x\ initial\ volume\ (mL)}{sampel\ weight\ (\mathrm{m}g)}\ x\ 100\%$$

How to analyze data

Identification of metabolite compounds was carried out by comparing the GC-MS chromatogram of the test results with the database in the library (those with area values > 0.25%, retention time, fragment m/z and spectrum area were selected). The reducing sugar content test data was obtained by calculating using the galactose standard curve equation (20-120 µg/mL). Reducing sugar levels are obtained in µg/mL units.

3. Results and Discussion

Sample identification is important to avoid errors in collecting samples and to avoid mixing samples with other seaweed. The purpose of sample identification is to determine the authenticity of the identity of the sample to be used. The results of the identification of red seaweed samples carried out by Abdul Razaq Chasani, S.Si., M.Si., Ph.D. with letter number 0448/S/Tb/IX/2023 at the Plant Systematics Laboratory, Faculty of Biology, Gadjah Mada University, Yogyakarta shows that the sample is *Kappaphycus cottonii* (Weber Bosse) Dumilag & Zucarello seaweed.

Samples that have been identified are then subjected to a sample preparation process. Several stages in sample preparation include washing, drying and pollinating the samples. The aim of washing the samples is to remove dirt attached to the thallus of the seaweed *Kappaphycus cottonii.* Next, the sample is dried so that the water content in the sample is reduced, making the storage process easier. The samples were dried using an oven at 35°C. This drying method is used to avoid damage to the compound content in the sample due to exposure to ultraviolet light from the sun.

The dried Simplicia *Kappaphycus cottonii* is ground so that it has a small surface area. This will make filtering easier because the surface area of the simplicia in contact with the filter solution is wider. *Kappaphycus cottonii* simplicia powder is sieved to ensure the powder is uniform in size. Samples were sieved using a 40 mesh sieve. Small sized particles have a large surface area so that it makes it easier for the sample to contact the solvent. This causes the solvent to easily penetrate the cell walls and attract compounds in the cells. The attracted compound will dissolve into the solvent (Asworo and Widwiastuti, 2023).

The sample that has been sieved is then extracted. Extraction aims to extract chemical compounds contained in the sample. Maceration is one of the extraction methods chosen in this research. Maceration has various advantages, including the method being easy, cheap, simple, and can prevent the destruction of thermolabile compounds (Asworo and Widwiastuti, 2023). Maceration is carried out for 24 hours. The length of maceration time affects the extract results. Maceration carried out for a short time can result in the solvent not containing all the extract compounds, while maceration carried out for too long can result in oxidation of the active extract compounds (Asworo and Widwiastuti, 2023). Mixing the sample powder with methanol solvent was carried out by sonication and stirring using a magnetic stirrer. Sonication is carried out with the aim of accelerating the contact of the sample powder with the solvent so that the separation of the sample compound into the solvent occurs quickly. This can happen because the sound waves produced can disturb the particles in the sample from moving and mixing with the solvent (Suryanto et al., 2019). Stirring using a magnetic stirrer aims to ensure that mixing between the sample and solvent can occur quickly and a more homogeneous solution is obtained (Alfita et al., 2021).

The solvent used is 80% methanol. Methanol is a universal solvent that has semi-polar properties while water is polar. The combination of these two solvents can filter compounds that are polar, semi-polar and non-polar. In research by Debebe et al., (2018) 80% methanol showed better efficiency than other solvents in extracting sugar compounds. After soaking, the solvent was filtered using a Buchner cup with a Whatman paper filter and then the filtrate was obtained. The filtrate is concentrated using a rotary evaporator by evaporating the solvent below the boiling point of the solution. The temperature used is 60-65°C. Temperatures that are too high can cause damage to compounds in the sample (Purba et al., 2019).

The samples were reconcentrated using a water bath at a temperature of 45°C-50°C to maintain the quality of the active compounds contained in the extract. Concentration is carried out again so that the remaining filter solution can be removed from the extract and does not affect the next process. The residue from the filtering is remaciated so that the remaining compounds can be removed again and dissolved in the new solvent. This event occurred because the solvent used had reached its saturation point, resulting in several compounds still remaining in the sample. The amount of solvent added is the same as the first solvent. The results of calculating the yield of red seaweed extract, *Kappaphycus cottonii,* can be seen in Table I.

Table I. *Kappaphycus cottonii* extract yield

Rumput laut merah	Replikasi	Bobot Simplisia (g)	Bobot Ekstrak (g)	Rendemen (%)	Rata-rata Rendemen ± SD (%)
Kappaphycus cottonii	1	35.15	3.4	9.67	11.76 ± 2.39
	2	35.30	3.63	10.28	
	3	35.40	5.32	15.03	

The yield obtained was greater when compared to research conducted by Lantahl et al., (2017) on *Kappaphycus alvarezii* methanol extract of 0.034%. Research conducted by Arif, (2019) on the methanol extract of *Eucheuma cottonii* obtained a yield of 10.86%. This difference in yield is influenced by the water content factor in the sample. A low yield indicates that the active compound content in the sample is also low and vice versa (Lamadjido et al., 2019). Factors that can influence the yield of an extract include the type of solvent, maceration time, maceration stirring process, temperature during the extraction process, and the ratio of solvent to sample which can affect the amount of extract (Złotek et al., 2016). The results of organoleptic observations of the *Kappaphycus cottonii* extract showed that the extract was dark purple in color, contained sugar granules, and had no odor.

Results of analysis of the compound content of *Kappaphycus cottonii* methanol extract using GC-MS. Chromatogram identification results from the GC-MS test show a similar pattern with different percentage levels. There are 19 peaks in the compounds contained in the extract with different Similarity Index (SI). Compounds were selected that had an area value > 0.25% because they have a large area. The following are the results of chemical compound identification data in the methanol extract of red seaweed *Kappaphycus cottonii* using GC-MS. The chromatogram of the identification results of the red seaweed *Kappaphycus cottonii* can be seen in Figure 1.

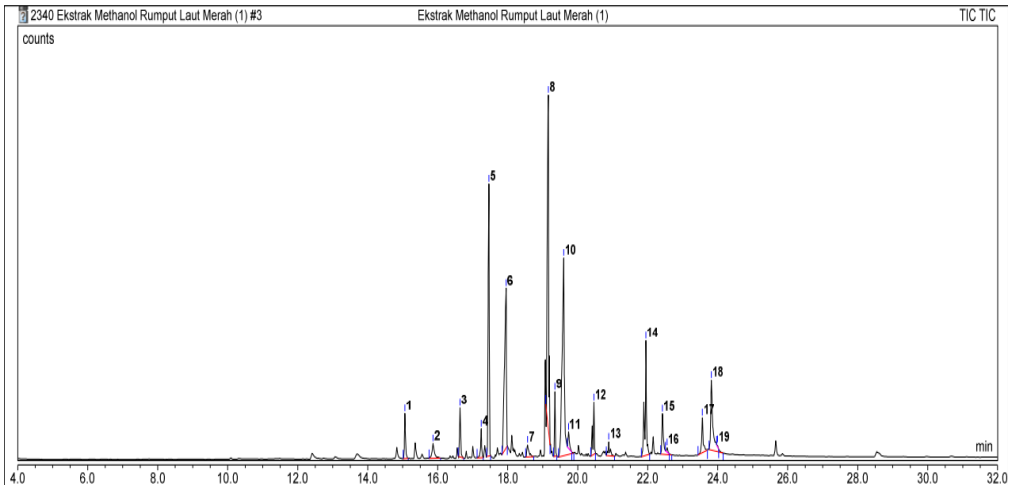

Figure 1. GC-MS chromatogram results

Based on the GC-MS test results of the methanol extract of *Kappaphycus cottonii*, it shows that the extract contains 18 types of organic compounds with different time retention (TR) values. There are four types of organic compounds that have the highest composition, characterized by high peaks at numbers 5, 6, 8, and 10. Compounds at peak numbers 5, 6, 8, and 10 respectively have retention times of 17.46 minutes, 17.96 minutes, 19.16 minutes, and 19.60 minutes. Each type of organic compound in the peak chromatogram is analyzed using mass spectroscopy. Compounds that have been analyzed are detected by determining the m/z value of the compound in the sample. The compound is then compared with the database on the GC-MS tool.

Determination of reducing sugar content can be done using titrimetric or spectrophotometric titration methods. The dinitrosalicylate (DNS) method was chosen to determine reducing sugars because the reagent is cheap, specific and non-toxic. The principle of the dinitrosalicylate method is that the reducing sugar contained in the sample oxidizes the aldehyde group to a carboxyl group and reduces 3,5-dinitrosalicylic acid by the aldehyde group to 3-amino-5-nitrosalicylic acid. The DNS solution changes color from yellow to reddish orange when it reacts with reducing sugar. The intensity of the reddish orange color produced is proportional to the amount of reducing sugar. The stronger the color intensity, the more reducing sugar there is. The standard used is galactose because the largest content in *Kappaphycus cottonii* is kappa carrageenan which is composed of galactose compounds. The solution is heated to speed up the reaction. The addition of DNS reagent aims to form a 3-amino-5-nitrosalicylic acid compound which can absorb strong light when reading with a spectrophotometer.

Preliminary tests on the UV-Vis spectrophotometer include determining the operating time. Operating time is determined to determine the time required for the analyte and reagent to react completely. Operating time is indicated by stable absorbance measurements. Operating time measurements were carried out on a standard galactose solution with a concentration of 100 mg/ml with a theoretical wavelength of 512 nm. Based on the research results, the most stable operating time was found at 7-21 minutes with an absorbance of 0.577. The results of this operating time are then used in determining the maximum wavelength, measuring standard curves and samples.

Determination of the standard maximum wavelength aims to determine the wavelength of the colored solution that has maximum absorbance. This maximum wavelength is used to measure the absorbance of the solution to be analyzed. According to the theoretical maximum wavelength of the standard galactose solution using the DNS method is 512 nm.

The maximum standard wavelength obtained was 509 nm. This is different from the maximum wavelength in theory which is caused by various factors such as differences in the equipment and conditions of the spectrophotometer used, as well as the solvent used. Next, this maximum wavelength is used in determining the standard standard curve.

Based on the results obtained, the linear regression equation states the relationship between concentration and absorption, namely Y= 0.0052x + 0.2523 with a calculated r value of 0.9945. The correlation coefficient (R) of the table at the 99% confidence level is 0.917 so it is found that calculated R > R table which shows that there is a relationship between concentration and absorption.

The results of determining the reducing sugar content showed that the average content of *Kappaphycus cottonii* extract was 0.34%. The calculation results for determining reducing sugar levels can be seen in Table II.

Table II. Calculation results of reducing sugar levels

Bobot sampel (mg)	Absorbansi	Kadar (%)	Rata-rata (%)	SD	CV (%)
50.2	0.421	0.32	0.34	0.02	0.05
50.7	0.434	0.34			
50.5	0.427	0.33			
50.8	0.446	0.37			
50.1	0.42	0.32			
50.5	0.423	0.33			

When compared with research (Zelvi et al., 2017), the reducing sugar content of Eucheuma cottonii was found to be 3.21%. The method used is the hydrolysis method with the k-carrageenase enzyme. In the hydrolysis process, the k-carrageenase enzyme plays a role in breaking down carrageenan molecules into neocarrabiodes and converting them into galactose. The high level of reducing sugar produced is because the k-carrageenase enzyme can have an influence on the concentration of sugar produced. A high enzyme concentration can cause more substrates to bind to the enzyme's active site so that more sugar is produced (Zelvi et al., 2017).

According to research conducted by Kim et al., (2018), the reducing sugar content in Gracilaria verrucosa was found to be 47.4%. The method used is acid hydrolysis with 0.1 N HCl. In the hydrolysis process, HCl acts as a catalyst which helps the process of breaking down carbohydrates into sugar.

According to research conducted by Permatasari et al., (2018), the reducing sugar content of Eucheuma cottonii is 0.72%. The method used is hydrolysis with 3% sulfuric acid and 10% inactive culture. The hydrolysis process aims to convert cellulose into reducing sugars. The use of sulfuric acid acts as a catalyst. Inactive cultures are used because they have a low degree of polymerization so that the sugar content is more easily utilized for metabolism.

In this study, lower levels of reducing sugar were obtained compared to the two studies above. This can happen because the compound content in seaweed is different due to the influence of the type of solvent and the type of seaweed.

3. Conclusion

Based on the results of research that has been carried out, it is concluded that the methanol extract of Kappaphycus cottonii contains chemical compounds such as alkanes (heptadecane), esters (tetradecanoic acid; hexadecenoic acid; hexadecanoic acid; n-hexadecanoic acid; 9-hexadecanoic acid; 9-octadecanoic acid (Z) ; methyl stearate; octadecanoic acid; hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester; 14-trimethyl), fatty acids (cis-13-octadecanoic acid; cis-11-eicosenoic acid; oleic acid, hydroxypropyl ester; glycerol palmitate; and n-propyl octadenoate), elaidic acid (2,3-dihydroxypropyl elaidate) Methanol extract Kappaphycus cottonii contains reducing sugar levels of 0.34%.

Author Contributions

This research was thought up and designed by the Firdausa Firm. Firdausa Firm did all the data analysis. The results were evaluated by Warsi. The scriptwriter is Firma Firdausa. The final manuscript was read and approved by all authors.

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Competing Interests

The authors declare no conflict of interest.

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