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Antidiabetic and antioxidant activities of *Gracilaria verrucosa* extract: In vitro and in vivo assessment

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Abstract: The increasing prevalence of diabetes mellitus and limitations of conventional treatments have intensified the search for natural remedies. This study evaluated the antidiabetic and antioxidant activities of red seaweed *Gracilaria verrucosa* extracts through *in vitro* and *in vivo* assessments. Phytochemical screening revealed alkaloids, triterpenoids, saponins, and phenolics. Three extraction methods using ethanol, distilled water, and warm distilled water were employed, with warm distilled water yielding the highest extraction (7.56%). Gas Chromatography-Mass Spectrometry analysis identified n-hexadecanoic acid as the predominant compound in ethanolic (37.87%), distilled water (12.33%), and warm distilled water extracts (11.42%). For antidiabetic activity, α-amylase inhibitory assay using 3,5-dinitrosalicylic acid method demonstrated that warm distilled water extract exhibited the strongest inhibition (IC_{50} = 45,117.16 ppm). Antioxidant evaluation using 2,2-diphenyl-1-picrylhydrazyl radical scavenging showed ethanolic extract possessed the most potent activity (IC_{50} = 21,730.07 ppm). *In vivo* studies utilized alloxan-induced diabetic male mice (*Mus musculus* L.) treated orally with *G. verrucosa* extracts (300 mg/kg BW) for 14 days. Results demonstrated that both ethanolic and warm distilled water extracts significantly reduced blood glucose levels in diabetic mice (initial fasting blood glucose >126 mg/dL) to below 100 mg/dL. These findings suggest *G. verrucosa* extract has considerable potential as a natural therapeutic agent for diabetes mellitus management.

Keywords: *Antidiabetes, Antioxidant, Gracilaria verrucosa extract, Phytochemical profiles.*

1. Introduction

Diabetes mellitus (DM) is characterized by the dysregulation of carbohydrate metabolism, leading to elevated blood glucose levels (hyperglycemia) [1, 2]. Hyperglycemia triggers the generation of free radicals, causing oxidative stress within cells [3]. This oxidative stress hampers insulin sensitivity and reduces insulin production. In the long term, DM significantly raises the risk of complications, including the blockage of heart arteries, strokes, renal failure, and even mortality. Though commonly prescribed, synthetic medications like thiazolidinediones, biguanides, and sulphonylureas are suspected to have numerous adverse effects. Consequently, scientists have recently shifted their focus to natural remedies. Seaweed has emerged as a particularly intriguing natural source, capturing the attention of researchers in the quest for alternative treatments [4].

A previous study demonstrated that the green seaweed *Caulerpa serrulata* exhibited moderate antioxidant activity against free radicals [5, 6]. Red seaweeds *Portieria hornemannii* and *Spyridia fusiformis* were identified as potential antidiabetic agents due to their strong inhibitory effects on α-amylase and α-glucosidase enzymes, crucial in carbohydrate digestion. Another seaweed species, *G. verrucosa*, also shows promise as an antidiabetic agent. This seaweed contains various compounds such as polyphenols, alkaloids, terpenoids, pigments, polyunsaturated fatty acids (PUFAs), and

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polysaccharides, all of which have been reported to possess both antidiabetic and antioxidant properties [7].

However, research on the potential of *G. verrucosa* as an antidiabetic agent remains limited, especially in Indonesia, where the seaweed is primarily used as a raw material for agar-agar production. Therefore, this study aimed to assess the antidiabetic and antioxidant activity of *G. verrucosa* extract, both in vitro and in vivo. The findings suggest that *G. verrucosa* extract holds promise as a nutraceutical and alternative therapy for diabetes mellitus (DM) treatment in the future.

2. Materials and Methods

2.1. Materials

Materials needed in this research were red algae (*G. verrucosa*) collected from Surabaya (East Java, Indonesia), ethanol, distilled water, Folin-Ciocalteu reagent (Merck), HCl (J.T. Baker), H_2SO_4 (J.T. Baker), chloroform (Merck), NaOH (Merck), methanol, Whatmann no 1 filter paper, Eppendorf tube (AXYGEN), 96-well microplate (IWAKI), phosphate buffer, 3,5-dinitro salicylic acid (DNS) (Sigma Aldrich), starch, Na_2CO_3 (Merck), DPPH (Sigma Aldrich), ascorbic acid (Merck), ultra-pure water, acarbose, male mice (*Mus musculus* L.) Wistar strain, alloxan tetrahydrate (Sigma Aldrich), sucrose, and sterile syringe (One Med).

2.2. Gracilaria verrucosa Preparation

Red algae *G. verrucosa* was washed with distilled water to remove impurities and debris. After that, the sample was dried in a vacuum oven at 40°C for 4 days. The sample was stored in a closed container until further analysis.

2.3. Phytochemical Screenings of G. verrucosa

2.3.1. Alkaloids Test

About 2 g of sample was added with 5 mL of ethanol, and the filtrate was pipetted into the reaction tube. The solution was added with 5 mL of HCl 2 N and then divided into two new reaction tubes. The first tube served as blank. This tube was added with 3-5 drops of HCl 2 N. About three drops of Dragendorff reagent were added to the second tube. The formation of orange precipitate indicated alkaloids presence in the second tube [8].

2.3.2. Triterpenoids and Steroids Test

A total of 2 g of sample was added with 5 mL of ethanol, and then the filtrate was pipetted into the reaction tube. The filtrate was dissolved in 0.5 mL of chloroform. About 0.5 mL of acetic acid anhydrate and 3-5 drops of concentrated H_2SO_4 were added to the reaction tube. The purple or brownish ring formation indicated the presence of triterpenoids, while the formation of blue-green ring8 indicated the presence of steroids [8].

2.3.3. Flavonoids Test

2 g of sample was added with 5 mL ethyl acetate, then boiled for 3 minutes. About 4 mL of filtrate was reacted with 1 mL of 10% (w/v) NaOH. The formation of a yellow solution indicated the presence of flavonoids [8].

2.3.4. Saponins Test

A total of 2 g of sample was added with 5 mL of distilled water, then pipetted into a reaction tube. The filtrate was homogenized for 10 seconds, then left to stand for 10 seconds. If no foam formed, about 1-3 drops of HCl 2 N was added. The formation of 1-10 cm foam that did not disappear for 1 min indicated the presence of saponins [9].

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2.3.5. Phenolics Test

The test was carried out using the Folin-Ciocalteu method described by Bursal and Köksal [10]. A total of 2 g of sample extract was added with 5 mL of ethanol, and then the filtrate was pipetted into a new reaction tube. About 1 mL of filtrate was mixed with 1 mL Folin-Ciocalteu reagent and then incubated at room temperature for 15 min. After that, the mixture was added with 100 µL Na_2CO_3 and then incubated at 42°C. The formation of the blue complex indicated the presence of phenolic compounds [10].

2.4. Bioactive Compound Extraction of G. verrucosa

This research step was carried out using a modified extraction method described by Savitri, et al. [11]. First, the sample was pulverized using a food processor. Then, about 50 g of the sample was added into a beaker glass for each solvent. A total of 500 mL of solvent (ethanol, distilled water, and warm distilled water) was added to the beaker glass. Maceration with ethanol was carried out for 3 days (26°C); 1 day for distilled water (26°C), and warm distilled water (60°C). The solution obtained from the maceration process was filtered for ethanol solvent using Whatmann no 1 filter paper. The filtrate was evaporated using a rotary evaporator at 40°C, and the rotation speed used was 60 rpm to produce concentrated extract. The concentrated extract was dried in a vacuum oven at 40°C until the remaining solvent was vaporized. For distilled and warm distilled water, the solution obtained from the maceration process was centrifuged at 5000 rpm for 5 min to remove solid residue. The supernatant was then filtered using Whatman no 1 filter paper and dried with a vacuum oven at 40°C to produce a concentrated extract. The crude extract was weighed, placed in an Eppendorf tube (wrapped in aluminum foil), and stored in a refrigerator at 4°C.

2.5. GC-MS Analysis of Phytochemical Constituents

GC-MS analysis was performed using a Thermo Scientific single quadrupole mass spectrometer coupled with a Thermo Scientific gas chromatograph and fitted with Ultra ALLOY+- 5 capillary columns (30 m column length x 0.25 mm column i.d. x 0.25 µm film thickness). Helium was used as carrier gas at a 1.0 ml/min flow rate. The injection port was maintained at 250°C, and the split ratio was 40:1. Oven temperature programming was done from 50 to 280°C, at 10°C/min, and it was kept at 280°C for 5 min. The interface temperature was kept at 250°C. The ionization mode was electron impact ionization, and the scanning range was from 40 amu to 400 amu. Mass spectra were obtained at 0.5-sec intervals. The total retention time of GC-MS analysis was approximately 30 min [12].

2.6. α-Amylase Inhibitory Activity Assay in Vitro

This assay was carried out using 3,5-dinitrosalicylic acid (DNS) method [8]. A total of 250 µL of sample extract with varied concentrations (1000, 2000, 4000, and 8000 ppm) was pipetted into the Eppendorf tube. About 250 µL of phosphate buffer pH 6.9 containing 3.692 U/mL α-amylase solution was added into the Eppendorf tube. The solution was incubated for 30 minutes at room temperature (± 25°C). After that, a total of 250 µL of 1% (w/v) starch solution diluted in phosphate buffer pH 6.9 was added and then incubated for 30 minutes at room temperature (± 25°C). The enzyme reaction was terminated with the addition of 500 µL of DNS. The Eppendorf tube was placed in boiling water for 10 min and then cooled at room temperature (± 25°C). Absorbance measurement was carried out using a microplate reader at 540 nm wavelength. The blank was a mixture of distilled water, α-amylase solution, starch solution, and DNS. The standard used in this assay was acarbose. The inhibition percentage of α-amylase was calculated using this equation:

$$\% \text{ inhibition} = \frac{\text{Abs of blank} - \text{Abs of sample}}{\text{Abs of blank}} \text{x } 100\%$$

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The IC_{50} value was determined using a linear regression graph between % inhibition and concentration of sample extract. The IC_{50} value described the concentration of sample extract that could inhibit 50% of α-amylase activity.

2.7. Antioxidant Activity Assay

This assay used the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity method described by Almurdani, et al. [13]. About 50 µL sample extract of different concentrations (1000, 2000, 4000 and 8000 ppm) dissolved in methanol was pipetted into the Eppendorf tube. After that, 50 µL of DPPH (50 ppm) was added into the Eppendorf tube. Then, the mixture was incubated in dark conditions for 30 min. The blank used was a mixture of DPPH and methanol without sample extract. Absorbance measurement was carried out using a microplate reader at 517 nm wavelength. The standard used in this assay was ascorbic acid. The DPPH scavenging activity of antioxidant was calculated using this equation:

$$\% \text{ inhibition} = \frac{\text{Abs of blank} - \text{Abs of sample}}{\text{Abs of blank}} \text{x } 100\%$$

The IC_{50} value was determined using a linear regression graph between % inhibition and concentration of sample extract. The IC_{50} value described the concentration of sample extract that could scavenge DPPH by 50%.

2.8. Antidiabetic Assay in Vivo

This study was done using 12-week-old male mice (*Mus musculus* L.) Wistar strain weighing approximately 25-30 g as animal model. Mice were placed in standard cages at room temperature (± 25°C), relative humidity (± 60%), and given 12 hours of light/dark cycle. The mice had free access (*ad libitum*) to regular pellets and water. The acclimatization of mice was carried out for 3 days.
The total number of mice needed for this study was determined using Federer's equation:

$$(k-1)(n-1) > 15$$

Description:
k = number of groups
n = number of mice in groups

A total of 18 male mice were divided into 6 groups (Table 1). The groups were blank, positive control, negative control, mice ethanolic extract (MEE), mice distilled water extract (MDE), and mice warm distilled water extract (MWD). All mice were given different marks as identity on their backs.

Table 1.
The treatment of mice groups.

Group*	Mice Mode	Treatment
Blank	Normal	Mice were given water and normal pellet (*ad libitum*)
Negative control	Diabetes	Mice were given ultra pure water, water, and regular pellet (*ad libitum*)
Positive control	Diabetes	Mice were given acarbose, water, and a regular pellet (*ad libitum*)
MEE	Diabetes	Mice were given ethanol extract of *G. verrucosa*, water, and regular pellet (*ad libitum*)
MDE	Diabetes	Mice were given distilled water extract of *G. verrucosa*, water, and regular pellet (*ad libitum*)
MWD	Diabetes	Mice were given warm distilled water extract of *G. verrucosa*, water, and regular pellet (*ad libitum*)

Note: *Mice ethanolic extract (MEE), mice distilled water extract (MDE), and mice warm distilled water extract (MWD).

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2.9. Diabetes Induction in Animal Model

The induction of diabetes was carried out for 4 days. During diabetes induction, fasting blood glucose (FBG) level measurement was done on the 0 and 4th day. The measurement was carried out after mice fasted for 8 h (mice were given water only). The blood of mice was taken from the capillary vein in its tail via an intravenous route and dropped on glucometer strips to measure the initial FBG level. After that, 0.5 mL of 100 mg/kg BW alloxan tetrahydrate diluted in sterile ultra-pure water was injected via intraperitoneal route to induce diabetes in mice. The injection was only done once. After the alloxan tetrahydrate injection, the mice were given a 10% sucrose drink. During diabetes induction, the mice were given regular pellets and water. On the 4th day of induction, mice with FBG levels> 126 mg/dL were considered diabetes and used for further study.

2.10. Diabetes Treatment

Mice used as positive control were given 0.5 mL of acarbose (150 mg/kg BW) orally. Mice in MEE, MDE, and MWD groups were given 0.5 mL of *G. verrucosa* extract (300 mg/kg BW) according to each designed treatment. Acarbose and *G. verrucosa* extract were given to mice twice daily for 14 days at 9 a.m. and 2 p.m. The measurement of FBG level was carried out on the 0th, 3rd, 7th, 10th, 12th, and 14th day after mice fasted for 8 hours (mice were given water only). FBG measurement was done before the administration of acarbose or G. verrucosa extract on that particular day. During the diabetes treatment period, regular pellets and water were given to mice.

2.11. Statistical Analysis

The data obtained from α-amylase inhibitory activity, antioxidant activity, and antidiabetic assays were analyzed statistically using the one-way ANOVA method (SPSS software). Data were considered significantly different if $p < 0.05$.

3. Results

3.1. Phytochemicals Screening of G. verrucosa

Phytochemical screening revealed the presence of secondary metabolites such as alkaloids, triterpenoids, saponins, and phenolics in *G. verrucosa*. Steroids and flavonoids were not detected in the sample. Phytochemical screening results are summarized in Table 2.

Table 2.
Qualitative phytochemical screening result of *G. verrucosa*.

Compound	Test	Indicator	Result
Alkaloid	Dragendorff's	Orange precipitate	+
Triterpenoid	Liebermann-Buchard	Brownish-red complex	+
Steroid	Liebermann-Buchard	Blue-green complex	-
Saponin	Foam	Foam does not disappear for 1 min	+
Flavonoid	NaOH	Yellow solution	-
Phenolic	Folin-Ciocalteu	Blue solution	+

3.2. Bioactive Compound Extraction of G. verrucosa

Extraction of *G. verrucosa* using ethanol as solvent gave a 0.70% yield (Table 3). The yield obtained from distilled water was 3.57%. Warm distilled water produced the highest yield out of three solvents (7.56%).

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Table 3.
Yield percentage of *G. verrucosa* extracts.

Solvent	Yield (%)
Ethanol	0.70
Distilled water	3.57
Warm distilled water	7.56

3.3. GC-MS Analysis of Phytochemical Constituents

GC-MS chromatogram of 50 peaks of the compounds detected in ethanolic extract was shown in Figure 1. Phytochemical compounds that showed antidiabetic and antioxidant activity in ethanolic extract are presented in Table 4.

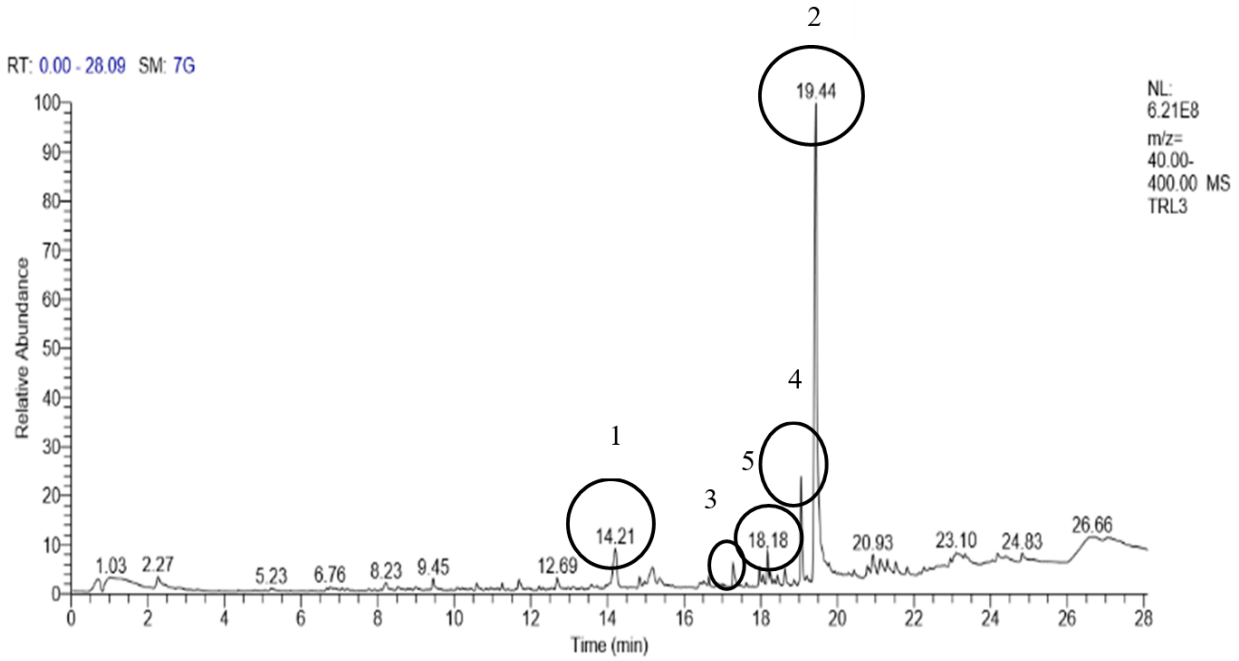

Figure 1.
GC-MS chromatogram of ethanolic extract of *G. verrucosa*.

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Table 4.
Phytochemical compounds with antidiabetic and antioxidant activity in ethanolic extract of *G. verrucose.*

No.	Name of the compound	Peak area (%)	Mol. Formula	Nature of compound	Biological Activity	References
1	n-Hexadecanoic acid	37.87	$C_{16}H_{32}O_2$	Palmitic acid	Antioxidant, nematicide, pesticide, antiandrogenic, antifouling	Uma Maheswari and Reena [14]
2	Hexadecanoic acid, methyl ester	5.3	$C_{17}H_{34}O_2$	Palmitic acid ester	Antioxidant, nematicide, pesticide, antiandrogenic, flavor	Uma Maheswari and Reena [14]
3	D-Allose	4.53	$C_6H_{12}O_6$	Monosaccharide	Antioxidant, immunosuppresive, anticancer	Onyeaghala and Hyacinth [15]
4	Neophytadiene	2.28	$C_{20}H_{38}$	Sesquiterpenoid	Antioxidant, antipyretic, analgesic, anti-inflammatory, antimicrobial	Patra, et al. [16]
5	Tetradecanoic acid	1.58	$C_{14}H_{28}O_2$	Fatty acid	Antioxidant, flavoring agent, nematicide, lubricant	Patra, et al. [16]

GC-MS chromatogram of 30 peaks of the compounds detected in distilled water extract was shown in Figure 2. Phytochemical compounds that showed antidiabetic and antioxidant activity in distilled water extract are presented in Table 5.

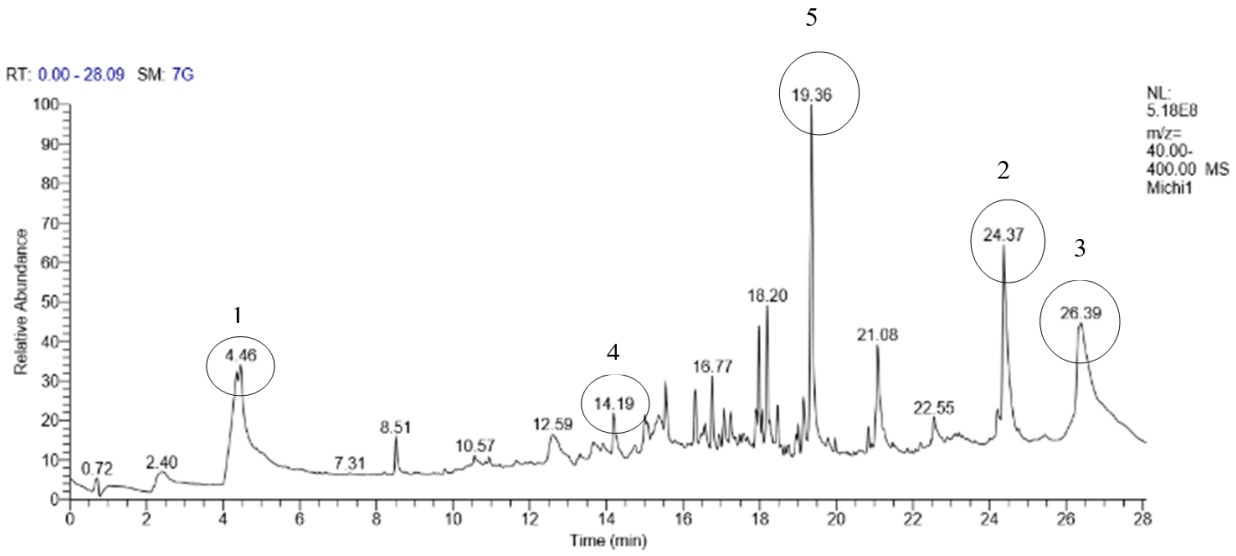

Figure 2.
GC-MS chromatogram of distilled water extract of *G. verrucosa.*

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Table 5.
Phytochemical compounds with antidiabetic and antioxidant activity in distilled water extract of *G. verrucose*.

No.	Name of the compound	Peak area (%)	Molecule Formula	Nature of compound	Biological Activity	References
1	n-Hexadecanoic acid	12.33	$C_{16}H_{32}O_2$	Palmitic acid	Antioxidant, nematicide, pesticide, antiandrogenic, antifouling	Uma Maheswari and Reena [14]
2	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	12.07	$C_{19}H_{38}O_4$	Fatty acid ester	Antioxidant, antimicrobial, hemolytic, pesticide, flavor	Priya Rani, et al. [17]
3	1-Heptatriacotanol	8.57	$C_{37}H_{76}O$	Fatty alcohol	Antioxidant, antimicrobial, antiinflammatory, anticancer	Sreejith, et al. [18]
4	Cholestan-3-ol, 2-methylene-, (3á,5à)-	2.14	$C_{28}H_{48}O$	Steroid	Antioxidant, antimicrobial, anti-inflammatory, anticancer, diuretic, antiarthritic, antiasthma	Al-Marzoqi, et al. [19]
5	Furyl hydroxymethyl ketone	1.38	$C_6H_6O_3$	Glycoside	Antidiabetic, antioxidant, anticancer, antiallergenic,	Al-Marzoqi, et al. [19]

GC-MS chromatogram of 20 peaks of the compounds detected in warm distilled water extract was shown in Figure 3. GC-MS analysis of phytochemical compounds that showed antidiabetic and antioxidant activity in warm distilled water extract are presented in Table 6.

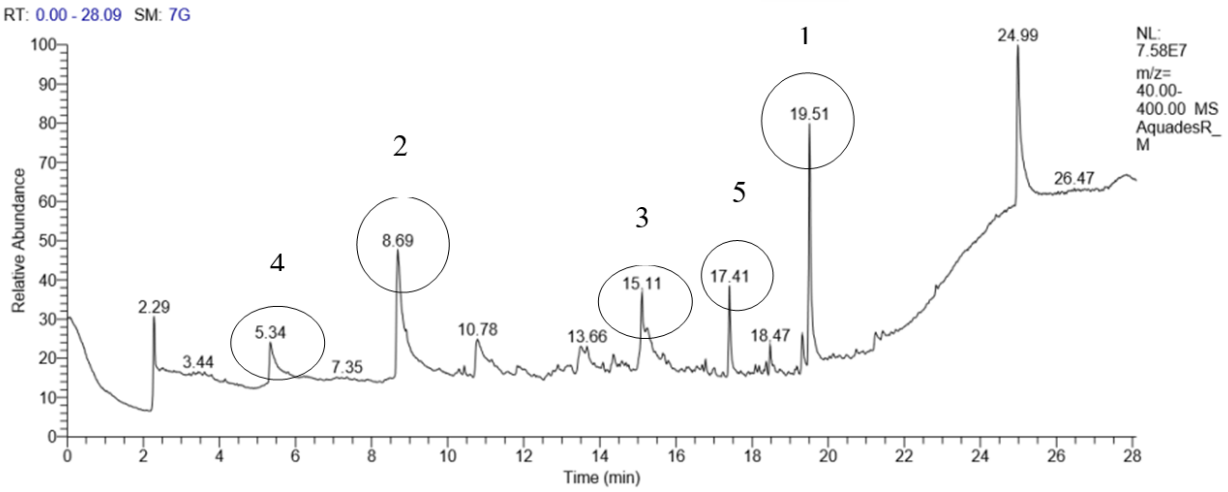

Figure 3.
GC-MS chromatogram of water distilled water extract of *G. verrucose*.

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Table 6.
Phytochemical compounds with antidiabetic and antioxidant activity in water distilled water extract of *G. verrucose*.

No.	Name of the compound	Peak area (%)	Molecule Formula	Nature of compound	Biological Activity	References
1	n-Hexadecanoic acid	11.42	$C_{16}H_{32}O_2$	Palmitic acid	Antioxidant, nematicide, pesticide, antiandrogenic, antifouling	Uma Maheswari and Reena [14]
2	Furyl hydroxymethyl ketone	11.35	$C_6H_6O_3$	Glycoside	Antioxidant, anticancer, antiallergenic, antidiabetic	Al-Marzoqi, et al. [19]
3	2-Myristynoyl pantetheine	10.14	$C_{25}H_{44}N_2O_5S$	Organosulfur	Used to treat lipid abnormalities and diabetes mellitus	Rahul, et al. [12]
4	Tetraacetyl-d-xylonic nitrile	5.71	$C_{14}H_{17}NO_9$		Antioxidant, antitumor, antiviral	Hameed, et al. [20]
5	Tetradecanoic acid	3.97	$C_{14}H_{28}O_2$	Fatty acid	Antioxidant, flavoring agent, nematicide, lubricant	Patra, et al. [16]

3.4. α-Amylase Inhibitory Activity in Vitro

α-amylase inhibitory activity of *G. verrucosa* extracts are presented in Figure 4. All *G. verrucosa* extracts showed concentration-dependent inhibitory activity against α-amylase as the increase of extract concentration significantly increased percentage inhibition ($p < 0.05$). Ethanolic extract inhibitory activity against α-amylase significantly differed from distilled water and warm distilled water extract at concentrations 1000 and 2000 ppm. Warm distilled water extract showed the highest inhibition activity against α-amylase out of three extracts of *G. verrucosa* with IC_{50} value = 45117.16 ± 19979.90 ppm (Table 7). All three extracts of *G. verrucosa* were categorized as weak inhibitors of α-amylase with IC_{50} value > 200 ppm. There was no significant difference between IC_{50} values of *G. verrucosa* extracts ($p > 0.05$).

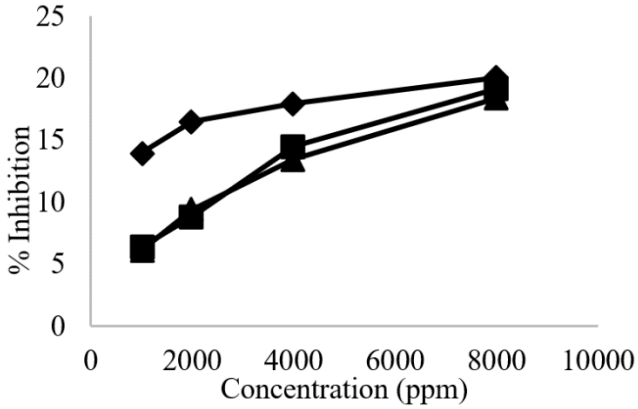

Figure 4.
α-amylase inhibitory activity of ethanolic (◆), distilled water (▲) and warm distilled water (■) extract of *G. verrucosa* at various concentrations (1000, 2000, 4000, and 8000 ppm).

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Table 7.
The IC_{50} values (α-amylase inhibitory activity) of *G. verrucosa* extracts.

Extract	IC_{50} value (ppm)
Ethanolic	60615.89 ± 41408.51[a(*)]
Distilled water	50770.99 ± 18371.44[a]
Warm distilled water	45117.16 ± 19979.90[a]

Note: (*) The values are expressed as means ± SEM of triplicate tests. Means down vertical columns with the same letter were not significantly different ($p > 0.05$).

3.5. Antioxidant Activity

All *G. verrucosa* extracts showed concentration-dependent scavenging activity against DPPH as the percentage inhibition significantly increased along with the increase of extract concentration ($p < 0.05$) (Figure 5). DPPH scavenging activity of ethanolic extract was significantly different from distilled water and warm distilled water extract at a concentration of 2000 ppm. Meanwhile, the DPPH scavenging activity of warm distilled water extract significantly differed from ethanolic and warm distilled water extract at a concentration of 8000 ppm. Ethanolic extract showed the most vigorous scavenging activity against DPPH out of three extracts with IC_{50} value = 21730.07 ± 8706.02 ppm (Table 8). All three extracts of *G. verrucosa* were categorized as weak antioxidants with IC_{50} value > 200 ppm. There was no significant difference between IC_{50} values of *G. verrucosa* extracts ($p > 0.05$).

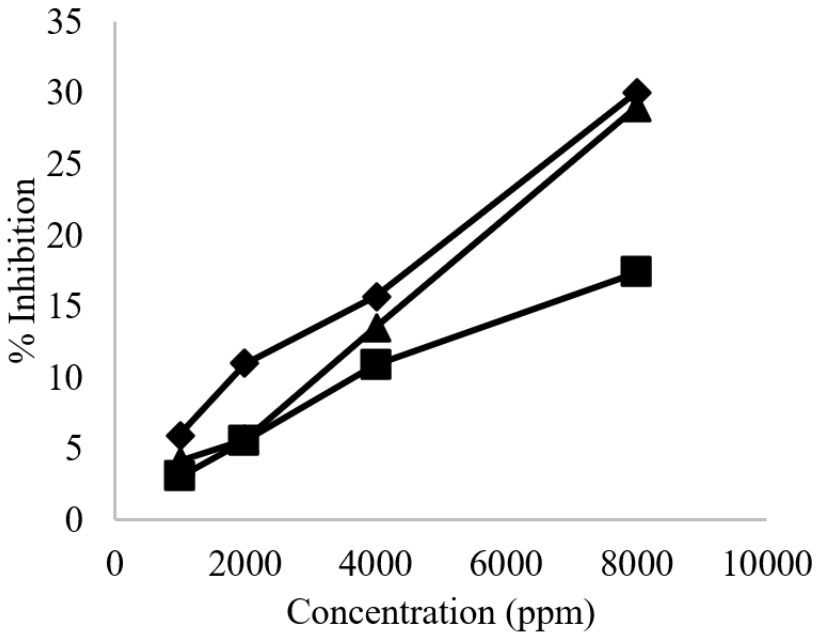

Figure 5.
DPPH scavenging activity of ethanolic (◆), distilled water (▲), and warm distilled water (■) extract of *G. verrucosa* at various concentrations (1000, 2000, 4000, and 8000 ppm).

Table 8.
The IC_{50} values (DPPH scavenging activity) of *G. verrucosa* extracts.

Extract	IC_{50} value (ppm)
Ethanolic	21730.07 ± 8706.02[a(*)]
Distilled water	26025.36 ± 10799.00[a]
Warm distilled water	37159.86 ± 16486.04[a]

Note: (*) The values are expressed as means ± SEM of triplicate tests. Means down the vertical column with the same letter was not significantly different ($p > 0.05$).

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3.6. Antidiabetic Activity in Vivo

The bar chart in Figure 6 shows the alterations in the FBG level of mice during the *in vivo* study. Before treatment, mice were induced with alloxan to increase FBG level until above 126 mg/dL (except the blank group). During *in vivo* study, the FBG level of mice in the blank group was stable below 100 mg/dL. After DM induction, the FBG level of mice in negative control, positive control, and all extract groups increased until above 126 mg/dL. The result proved that alloxan had successfully induced diabetes in mice. FBG levels of mice in the negative control group were stable above 126 mg/dL after DM induction until the end of treatment. FBG level of mice in the positive control group treated with acarbose decreased from 136 ± 11.79 to 113 ± 10.44 mg/dL. Treatment with 300 mg/kg BW ethanolic extract reduced the FBG level of the MEE group from 141 ± 12 to 80.67 ± 5.51 mg/dL, while FBG level of mice in the MDE group showed a reduction of FBG level from 143 ± 14.11 to 104 ± 12 mg/dL. Mice treated with 300 mg/kg BW warm distilled water extract showed a decline of FBG level from 150 ± 1.73 to 82 ± 2 mg/dL. After treatment, the FBG level of mice in the MEE and MWD groups showed significant differences ($p < 0.05$) compared to the negative control group (Figure 7). FBG levels of mice in the positive control and MDE groups were not significantly different ($p > 0.05$) from the negative control group. FBG levels of mice in positive, MEE, MDE, and MWD groups were not significantly different.

The bar chart in Figure 8 shows the alterations in mice body weight during *in vivo* study. The body weight of mice in the blank group increased from 26.33 ± 4.93 (before DM induction) to 31.67 ± 3.79 g (after treatment). Mice in the negative control group showed a constant decline in body weight during *in vivo* study, from 34.33 ± 5.51 to 30 ± 4.58 g, while mice in the positive control group showed a body weight increase from 29 ± 2.65 to 31 ± 2.65 g. Mice in the MEE group improved body weight after being treated with 300 mg/kg BW ethanolic extract (from 28 ± 0.00 to 31 ± 1 g). The body weight of mice in the MDE group increased from 34 ± 6.08 g to 34.67 ± 6.35 g. Mice treated with 300 mg/kg BW warm distilled water extract (MWD group) also increased body weight from 27 ± 2.65 to 29 ± 3.61 g. However, the body weight of mice in the positive control and all extract groups showed no significant differences when compared to the body weight of mice in the negative control group ($p > 0.05$) (Figure 9).

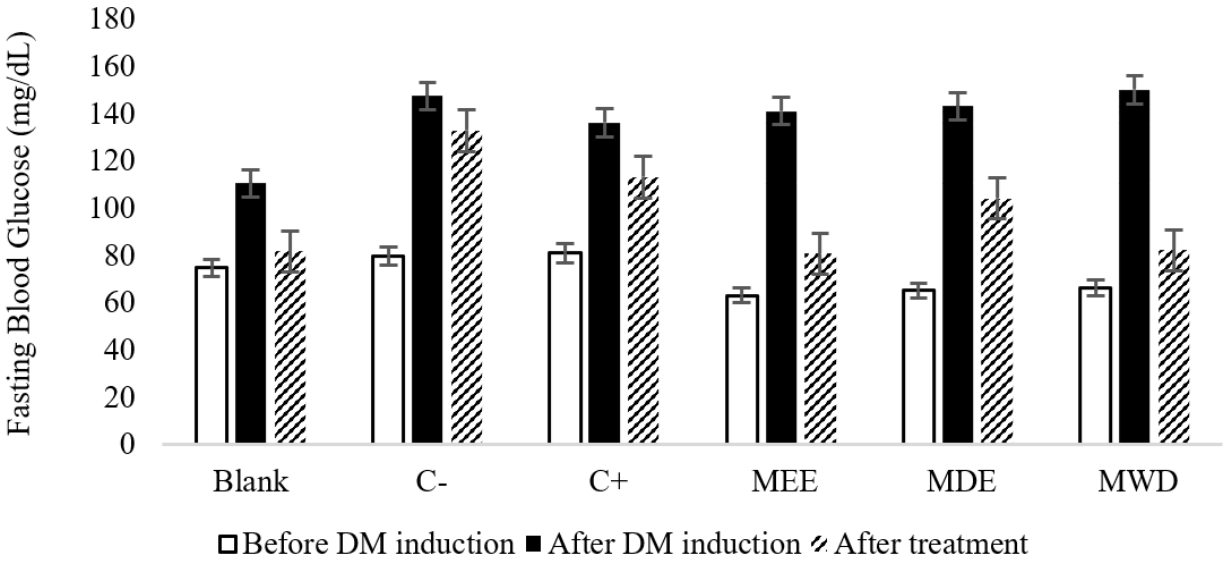

Figure 6.
Alterations of FBG level before DM induction, after DM induction and after treatment with *G. verrucosa* extracts and acarbose for 14 days. Blank, negative control (C-), positive control (C+), MEE (Mice ethanol extract), MDE (mice distilled water

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extract) and MWD (Mice warm distilled water extract). Values are means ± S.E.M for three mice in each group.

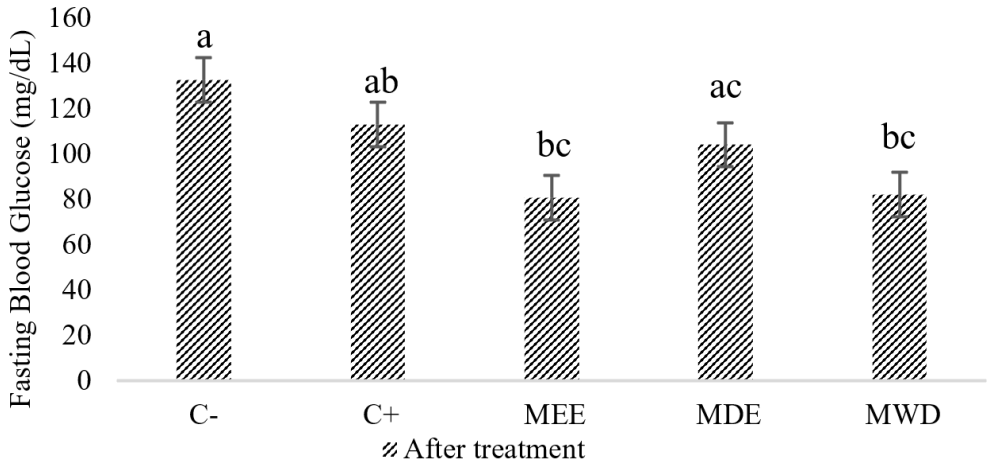

Figure 7.
FBG level difference between groups after treatment with *G. verrucosa* extracts and acarbose for 14 days. Negative control (C-), positive control (C+), MEE (Mice ethanol extract), MDE (Mice distilled water extract) and MWD (Mice warm distilled water extract). Values are means ± S.E.M for three mice in each group. Means not sharing the same letter were significantly different ($p < 0.05$).

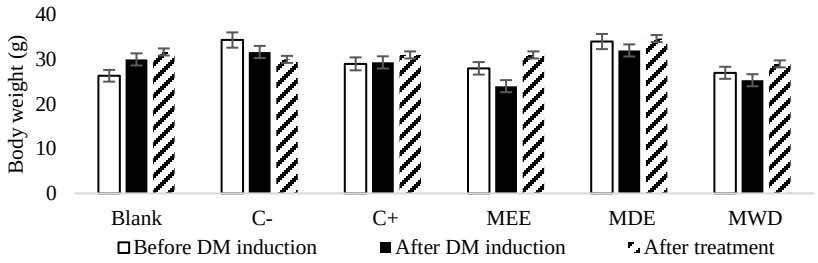

Figure 8.
Alterations of mice body weight before DM induction, after DM induction and after treatment with *G. verrucosa* extracts and acarbose for 14 days. Blank, negative control (C-), positive control (C+), MEE (Mice ethanol extract), MDE (Mice distilled water extract) and MWD (Mice warm distilled water extract). Values are means ± S.E.M for three mice in each group.

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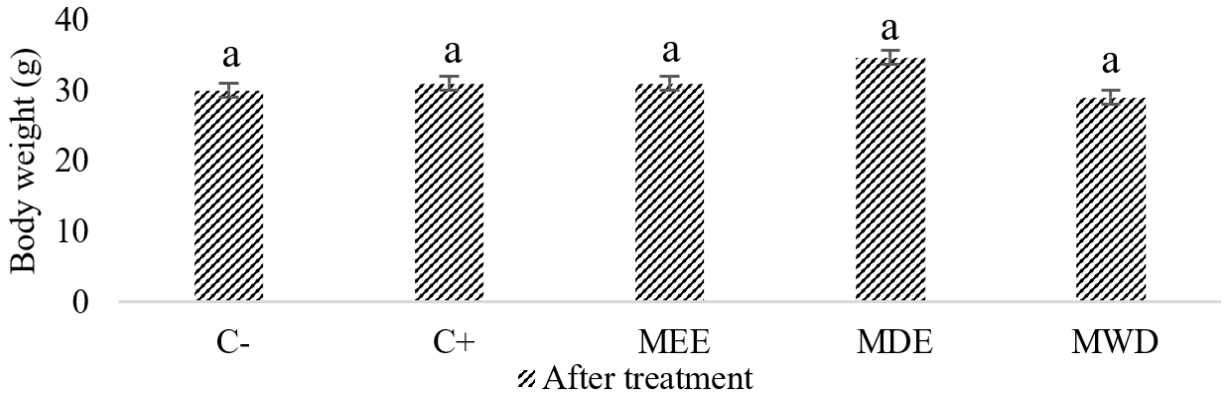

Figure 9.
Mice body weight difference between groups after treatment with *G. verrucosa* extracts and acarbose for 14 days. Negative control (C-), positive control (C+), MEE (Mice ethanol extract), MDE (Mice distilled water extract) and MWD (Mice warm distilled water extract). Values are means ± S.E.M for three mice in each group. It was significantly different after treatment ($p > 0.05$).

4. Discussion

Alkaloids, triterpenoids, saponins, and phenolics were found in *G. verrucosa* phytochemical screenings. Antioxidant and antidiabetic properties of these bioactive substances were widely recognized [21-23]. Alkaloids in red algae *Hypnea cervicornis* have been found to have hepatoprotective, antioxidant, and α-amylase inhibiting properties [24]. Isoprene triterpenoids are bioactive chemicals. Lanostane terpenoids in *Poria cocos* reduced postprandial blood glucose and improved insulin sensitivity in diabetic rats [25]. Saponins strongly scavenged hydroxyl and superoxide free radicals. Saponins delayed stomach-to-small-intestine glucose transfer [26]. Seaweed's antioxidant and antidiabetic effects come from phenols. Bromophenols from red seaweed include bis (2,3-dibromo-4,5-dihydroxybenzyl) ether and 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol. Sharifuddin et al. (2015) found *Symphyocladia latiuscula* significantly reduced α-glucosidase activity with IC_{50} values of 0.03 and 11.0 μM [27].

Solvent type and extraction temperature affected *G. verrucosa* bioactive component extraction yield. Polarity affected solvent component solubility and extraction yield. Ethanol and distilled water were employed for extraction since they were safer than methanol and acetone. Semi-polar ethanol has a polarity index of 5.2, while distilled water has a polarity index 9 [28]. The highest yield was warm distilled water, followed by ethanol and distilled water. Proteins, carbohydrates, and phenolics had better solubility in distilled water than ethanol, which increased extraction yield Do, et al. [29]. Tan, et al. [30] studied extraction temperature and found that mild heating increased solvent diffusivity into cells and bioactive chemical solubility in a solvent, boosting recovery. Increasing the extraction temperature to 65°C may decrease heat-sensitive phenolic content.

GC-MS analysis performed that phytochemical component profiles in all *G. verrucosa* were primarily fatty acids, glycosides, hydrocarbons, and alcohols. *G. verrucosa* extracts contained steroids, alkaloids, triterpenoids, carotenes, aldehydes, quinolones, organosulfur, acetate compounds, amino acid derivatives, silicon-based polymers, and monosaccharides. *G. verrucosa* extract bioactive components have many biological actions, including antidiabetic and antioxidant. Fatty acids, glycosides, triterpenoids, and steroids were among the 14 antioxidants in *G. verrucosa* extracts. Desulphosinigrin, 17-Octadecynoic acid, and Tetradecanoic acid are antioxidant bioactive chemicals. Bioactive substances like (2-Aziridinylethyl) amine from alkaloid groups and Furyl hydroxymethyl ketone from glycoside groups have antidiabetic action. About four *G. verrucosa* extract components were anti-diabetic. N-hexadecanoic acid has the most significant peak area % in all *G. verrucosa* preparations. Palmitic acid is an essential fatty acid in palm oil found in animals, plants, and microbes. Long-chain saturated fatty acid

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having C16 backbone. N-hexadecanoic acid is antioxidant, nematicide, insecticide, antiandrogenic, and antifouling [14].

GC-MS matched the phytochemical screening of *G. verrucosa* alkaloids, triterpenoids, and saponins. Ethanolic extract included alkaloids (2-Aziridinylethyl), amine, and Deoxyspergualin. Astaxanthin and Betulinaldehyde were found in the ethanolic extract. All *G. verrucosa* extracts contained glycosides such as paromomycin and 5-hydroxymethylfurfural, while ethanolic extract contained desulphosinigrin and warm distilled water extract had Digitoxin.

Phytochemical screening found no steroids in *G. verrucosa*. Previous study identified steroid compounds from *G. verrucosa*, including (24R)-5α-stigmast-9-(11)-en-3β-D-glucopyranoside and (Z)-9-hexadecanoic GC-MS found Ethyl iso-allocholate in all *G. verrucosa* extracts and cholestan-3-ol, 2-methylene-, (3á,5à)-, and Estra-1,3,5(10)-trien-17á-ol in distilled water extract despite negative phytochemical screening. Brown algae *Padina pavonica* contained antioxidant and hepatoprotective steroids [31].

Phytochemical screening and GC-MS analysis showed G. verrucosa lacks flavonoids. It contradicts with findings by Kurniawati and Adam [32]. They found quercetin-7-methyl-ether in *G. verrucosa*. This chemical was derived from flavonoids, which showed cardiovascular, anti-cancer, and anti-inflammatory properties. Steroids and flavonoids were unstable in the sample and easily oxidized when exposed to heat, light, or oxygen, which may have contributed to their unfavorable phytochemical screening results [30]. Phenolics were found in phytochemical screening but not GC-MS. Red seaweed has lower phenolic content than brown seaweed [33], and elevated GC-MS analysis temperatures may have lowered *G. verrucosa*'s phenolic content [30].

The α-amylase inhibitory activity assay revealed that heated distilled water extract was the most effective, followed by distilled water and ethanolic extract. Polysaccharides and dietary fibers dissolved better in distilled water than in ethanol. These gel-forming chemicals delayed carbohydrate digestion and glucose absorption into the bloodstream and hindered enzyme-substrate interaction by binding to enzyme sites and changing enzyme shape [34]. These compounds were also more soluble in warm distilled water after mild heat extraction [30]. According to Lantah, et al. [35] all *G. verrucosa* extracts showed weak inhibition of α-amylase with IC_{50} values > 200 ppm [35]. Another study also showed that natural extracts with significant α-amylase inhibition were not recommended as they may lead to the buildup of undigested carbohydrates, which can be used as a substrate for bacterial fermentation in the colon [8]. As previously observed by GC-MS, *G. verrucosa* ethanolic and distilled water extracts included MUFAs such as oleic acid. According to Sharifuddin, et al. [27] MUFAs in seaweed reduce the incidence of DM by boosting GLUT4 translocation to membrane cells and showing cytoprotective action on pancreatic β-cells [27].

Ethanolic extract scavenged DPPH the most, followed by distilled water and warm distilled water extract. Lower polarity in ethanol allowed it to dissolve more fatty acid compounds than distilled water. Along with phenolic components, fatty acids are antioxidants in seaweed. Red seaweed Laminaria japonica essential oil had 80.45% DPPH scavenging efficacy at 500 µg/mL. Hexadecanoic acid, tetradecanoid acid, and (9Z)-hexadec-9-enoic acid in *Laminaria japonica* neutralized DPPH by donating electrons or hydrogen atoms to stop radical chain reaction[16]. All *G. verrucosa* extracts were weak antioxidants. It was likely due to crude extracts in the assay. Crude extracts contained salts, minerals, and other elements that could reduce antioxidant activity of *G. verrucosa* [36].

Alloxan, an oxygenated pyrimidine derivative, resembles glucose. DM can be induced in mice. Alloxan can decrease glucose-mediated insulin secretion and selectively cause pancreatic β-cell necrosis [37]. Alloxan is converted to dialuric acid and re-oxidized. This cycle produces ROS and superoxide radicals that damage cell biomolecules [38, 39]. In alloxan-induced animals, tiny amounts of insulin were insufficient to regulate FBG, resulting in FBG levels > 126 mg/dL [40]. Ethanolic and warm distilled water extract (300 mg/kg BW) effectively reduced FBG levels in diabetic mice to below 100 mg/dL. Ethanolic and warm distilled water extracts lowered FBG in mice better than acarbose (150

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mg/kg BW) and the same dose. *G. verrucosa* bioactive substances reduced FBG levels by inhibiting α-amylase and attacking free radicals in cells. Tetraacetyl-d-xylonic nitrile, furyl hydroxymethyl ketone, 2-myristynoyl pantetheine, tetradecanoic acid, and n-hexadecanoic acid are antioxidants and anti-diabetic [14]. Lower FBG levels may result from better insulin secretion and increased utilization of peripheral glucose [41]. Diabetic mice lost weight following alloxan induction. It may be related to insulin insufficiency, which prevents glucose from entering the cell and uses fat and protein for energy. Acarbose and all G. verrucosa extracts improved alloxan-induced diabetic mice's body weight. Insulin secretion and glucose homeostasis improved [42].

5. Conclusion

This study demonstrates that Gracilaria verrucosa extracts possess significant antidiabetic and antioxidant activities. Phytochemical screening confirmed the presence of bioactive compounds (alkaloids, triterpenoids, saponins, and phenolics), with GC-MS analysis identifying n-hexadecanoic acid as the predominant compound across all extracts. Warm distilled water extraction yielded the highest recovery (7.56%), while ethanolic extract exhibited superior antioxidant activity (IC_{50} = 21,730.07 ppm) and warm distilled water extract showed the strongest α-amylase inhibition (IC_{50} = 45,117.16 ppm). Critically, in vivo testing revealed that both ethanolic and warm distilled water extracts (300 mg/kg BW) significantly reduced fasting blood glucose levels in alloxan-induced diabetic mice to normoglycemic levels (<100 mg/dL), comparable to acarbose. These findings position G. verrucosa as a promising natural candidate for diabetes management, warranting further investigation into its bioactive isolates and clinical potential.

Transparency:

The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.

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