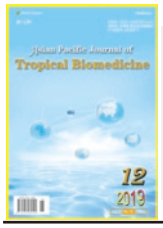

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Antidiabetic and antioxidant activities of red seaweed *Laurencia dendroidea*

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ABSTRACT

Objective: To investigate antidiabetic and antioxidant activities of the extract and fractions from Vietnamese red seaweed *Laurencia dendroidea*.

Methods: The seaweed *Laurencia dendroidea* was extracted by using microwave-assisted extraction method in 80% methanol. The seaweed extract was then fractionated using different solvents (*n*-hexane, chloroform, ethyl acetate, butanol and water). These obtained fractions were evaluated for α-glucosidase inhibitory and antioxidant activities. Antioxidant activities were tested using DPPH, nitric oxide radical scavenging and metal chelating assays. The enzyme inhibition mode was determined using Lineweaver-Burk plot. For acidic and thermal stabilities, the ethyl acetate fraction was treated at pH 2.0 and 100 ℃, respectively. The residual inhibitory activity of the fraction was calculated based on the initial inhibitory activity. For *in vivo* antidiabetic activity, mice were divided into four groups, including normal control, diabetic control, diabetic mice treated with ethyl acetate fraction and diabetic mice treated with gliclazide. Blood glucose level of treated mice during acute and prolonged treatments was measured. To evaluate the toxicity of the ethyl acetate fraction, the body weight changes and activities of liver function enzymes (aspartate transaminase, alanine transaminase and gamma-glutamyl transferase) were carried out.

Results: The extract of *Laurencia dendroidea* showed strong α-glucosidase inhibitory and DPPH radical scavenging activities. Methanolic concentrations affected both α-glucosidase inhibitory and antioxidant activities. A 80% aqueous methanol was the suitable solvent for extraction of enzyme inhibitors and antioxidants. Among solvent fractions, ethyl acetate fraction had the highest inhibitory activities against α-glucosidase with a mixed type of inhibition and the strongest antioxidant activities, and was stable under acidic and thermal conditions. The ethyl acetate fraction treated diabetic mice significantly reduced blood glucose level compared with the diabetic control group (13.16 mmol/L *vs*. 22.75 mmol/L after 3 hours of treatment). Oral administration of ethyl acetate fraction did not exhibit toxicity at a dose of 100 mg/kg body weight as determined by body weight changes and liver biochemical parameters.

Conclusions: *Laurencia dendroidea* could be a potential source for production of antidiabetic and antioxidative agents.

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1. Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both[1]. Several types of DM have been identified, in which type 2 diabetes is the most common, accounting for 90%–95% of the cases[2]. The number of people (age 18-99 years) with diabetes was 451 million in 2015 and is estimated to reach 693 million in 2045. The global cost for diabetes treatment in 2015 was $673 billion USD and that cost would be estimated $802 billion by 2040[3].

Mammalian α-glucosidases located in the brush-border surface membrane of intestinal cells, are key enzymes catalyzing the final step in the digestive process of carbohydrates into glucose. Hence, inhibiting these enzymes can slow down the elevation of blood sugar after a carbohydrate meal[4]. In addition to diabetes, α-glucosidase inhibitors are also known as a possible treatment for various diseases such as cancer, hepatitis B, 45 human immunodeficiency virus and heart disease[5]. Some commercial α-glucosidase inhibitors used in the treatment of patients with type 2 diabetes are acarbose, miglitol and voglibose[6]. However, these α-glucosidase inhibitors show several drawbacks such as diarrhea, abdominal cramping and liver toxicity[6,7]. To prevent negative effects of these drugs and also to find out more drug candidates for treating type 2 diabetes, it is necessary to investigate novel α-glucosidase inhibitors. Recently, many α-glucosidase inhibitors have been isolated from natural resources including medicinal plants and microorganisms[8,9].

Oxidation is essential for living organisms. Reactive oxygen species (ROS) are generated during oxidation process[10]. Excessive accumulation of ROS may lead to development of various diseases, including cellular aging, mutagenesis, carcinogenesis, hepatopathies, diabetes and neurodegeneration[11]. Therefore, internal antioxidant defense systems play important roles in reducing negative effects of ROS. External antioxidants are also used to protect human from diseases caused by ROS[12]. Natural antioxidants have received considerable attention due to its safety and effective properties.

Seaweeds are known as a potential source of bioactive compounds, particularly α-glucosidase inhibitors and antioxidants[13,14]. Several compounds having α-glucosidase inhibitory and antioxidant activities have been identified from different seaweed species, such as unsaturated fatty acids, water-soluble polysaccharides, polyphenols, and pigments[15]. Bioactive compounds from seaweeds have been proved to have antidiabetic and antioxidant activities in both *in vitro* and animal models[15,16]. Vietnam has an abundance of seaweed resources with nearly 1 000 species[17]. Seaweeds have been harvested and utilized by Vietnamese people for a long time as food source, animal feeds and traditional medicines. Some studies have already dealt with evaluation of antioxidant and antibacterial activities of crude seaweed extracts[18,19].

To our best knowledge, the antidiabetic and antioxidant properties of Vietnamese red seaweed extracts have not been studied. The objective of this study was to investigate antidiabetic and antioxidative activities of seaweed *Laurencia dendroidea* (*L. dendroidea*) harvested from different coastal areas of Vietnam.

2. Materials and methods

2.1. Reagents

p-Nitrophenyl-α-*D*-glucopyranoside (*p*-NPG), *p*-nitrophenyl phosphate, α-glucosidase from *Saccharomyces cerevisiae* (E.C. 3.2.1.20), gliclazide, rat intestinal acetone powder, Folin-Ciocalteu reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrozine and sodium nitroprusside were purchased from Sigma Aldrich. Protein tyrosine phosphatase 1B (PTP1B, human recombinant) was purchased from Biomol International LP (Plymouth Meeting, PA, USA). All other reagents were of analytical grade and acquired from commercial sources.

2.2. Seaweed samples

Nineteen red seaweed species were harvested during March to July, 2017 at the coast of Khanh Hoa, Quang Ngai and Kien Giang provinces, Vietnam. The samples were authenticated in place by seaweed experts (Prof. Nguyen Huu Dai, Institute of Oceanography, Nha Trang city, Vietnam and MSc. Do Anh Duy, Research Institute for Marine Fisheries, Hai Phong city, Vietnam). The voucher specimen (RIMF-018) of *L. dendroidea* was deposited at the Research Department of Marine Conservation, Research Institute for Marine Fisheries, Hai Phong city, Vietnam. Seaweed samples were individually rinsed to remove impurities and air-dried in the shade. Dried seaweed samples were cut into small pieces, vacuum packaged in PA bags and stored at -40 ℃ until analysis.

2.3. Seaweed extraction and fractionation

To screen the enzymatic inhibitory and antioxidant activities, 50 g of each seaweed sample was extracted with 1 L 80% aqueous methanol for 2 h. The mixture was filtered using Whatman No. 1 to obtain the crude extract. The residue was re-extracted under the same conditions and the filtrates were mixed together. The solvent was removed using a rotary evaporator (IKA RV 10 control, Staufen, Germany) under vacuum pressure at 40 ℃. The extracts were used to evaluate α-glucosidase inhibitory and antioxidant activities.

The seaweed *L. dendroidea* which showed potential α-glucosidase and antioxidant activities was then used to investigate the effect of solvent concentration on activities. Different methanolic concentrations in water of 0%, 20%, 40%, 60%, 80% and 100% were used to extract under the same procedure as mentioned above. The enzymatic inhibitory and antioxidant activities of the extracts were evaluated to find out the suitable methanolic concentration. After identification of suitable methanolic concentration, seaweed was extracted using different methods, including conventional extraction, microwave-assisted extraction, ultrasound-assisted extraction and head reflux extraction.

The crude seaweed extract obtained under suitable conditions was then suspended in water. The mixture was then fractionated with *n*-hexane, chloroform, ethyl acetate and butanol, successively. The α-glucosidase and antioxidant activities of all fractions were evaluated.

2.4. Determination of total phenolic content

The total phenolic content was determined using the method of Kumar *et al*[20]. Gallic acid was used as the external standard and the results were expressed as milligram gallic acid equivalents per gram of dry extract (mg GAE/g dry extract).

2.5. Determination of antioxidant activity

The DPPH radical scavenging activity of the sample was measured according to the method of Shimada *et al*[21]. The nitric oxide radical scavenging activity of the sample was measured by the method of Bor *et al*[22]. The metal chelating activity of the sample was determined following the method of Dinis *et al*[23]. The antioxidant activities were expressed by IC_{50} values (μg/mL).

2.6. Determination of α-glucosidase inhibitory activity

The yeast α-glucosidase inhibitory activity was determined according to the method of Kim *et al*[24]. Briefly, a reaction mixture containing 2 200 μL of 0.01 mol/L phosphate buffer (pH 7.0), 100 μL of 1 U/mL α-glucosidase in 0.01 mol/L phosphate buffer (pH 7.0) and 100 μL of sample was incubated for 5 min at 37 ℃. Then, 100 μL of 3 mmol/L *p*-NPG used as a substrate in 0.01 mol/L phosphate buffer (pH 7.0) was added to the mixture. The mixture was further incubated at 37 ℃ for 30 min. The reaction was stopped by adding 1 500 μL of 0.1 mol/L Na_2CO_3. The absorbance of the mixture was recorded at 405 nm by using a spectrophotometer (Cary 50, Varian, USA).

The rat intestinal sucrase and maltase (mammalian α-glucosidases) inhibitory activities were determined following the method of Shihabudeen *et al*[25] with some modifications. A 100 mg of rat intestinal acetone powder was homogenized in 3 000 μL of 0.9% NaCl and sonicated for 30 s at 4 ℃. The mixture was centrifuged at 12 000 *g* for 30 min and the enzyme supernatant was used for the assay. Fifty μL of sample was added to 100 μL of supernatant and incubated at 37 ℃ for 5 min. Then, 50 μL of 5 mmol/L *p*-NPG in 0.01 mol/L phosphate buffer (pH 7.0) was added. The mixture was further incubated at 37 ℃ for 15 min. Absorbance was measured at 405 nm using an ELISA reader (Bio-Rad Laboratories Inc.).

PTP1B inhibitory activity was measured using the method of Nguyen *et al*[26]. A total volume of 50 μL including 40 μL PTP1B enzyme in a buffer containing 50 mmol/L citrate (pH 6.0), 0.1 mol/L NaCl, 1 mmol/L EDTA and 1 mmol/L dithiothreitol with or without samples was added to 96-well plate. The mixture was incubated at 37 ℃ for 10 min. Then, 50 μL of 2 mmol/L *p*-nitrophenyl phosphate in a buffer containing 50 mmol/L citrate (pH 6.0) was added to the mixture and further incubated at 37 ℃ for 20 min. The reaction was terminated with the addition of 10 mol/L NaOH and the absorbance was measured at 405 nm using an ELISA reader (Bio-Rad Laboratories Inc.).

The enzymatic inhibitory activity (%) was calculated as follows: Inhibition (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_1 was the absorbance of the sample. The enzymatic inhibitory activity was expressed by IC_{50} values (μg/mL), which was defined as the concentration of sample that inhibited 50% of enzyme activity. Acarbose and ursolic acid were used as the positive controls for the α-glucosidase and PTP1B assays, respectively.

2.7. Characterization of α-glucosidase inhibitory kinetic

The mode of yeast α-glucosidase inhibition of seaweed extract was determined by analyzing the double reciprocal (1/v *versus* 1/[S]) plot using Lineweaver-Burk plot[24].

2.8. Acidic and thermal stability of α-glucosidase inhibitors

The stability of α-glucosidase inhibitors in the ethyl acetate fraction under acidic and thermal conditions was performed according to the method of Kim *et al*[27].

For acidic stability, the ethyl acetate fraction was treated at pH 2.0 for different times of 20, 40, 60, 80, 100 and 120 min. The fraction was then used for the inhibition assay against α-glucosidase as described previously. The residual inhibitory activity of the fraction at pH 2.0 was calculated based on the initial inhibitory activity.

For thermal stability, the ethyl acetate fraction was incubated at 100 ℃ for different times of 20, 40, 60, 80, 100 and 120 min. The inhibitory activity of the fraction was determined as described previously. The residual inhibitory activity of the fraction at 100 ℃ was calculated based on the initial inhibitory activity.

2.9. In vivo antidiabetic assay

2.9.1. Experimental design

Swiss albino mice (54 mice) of either gender weighing (20 ± 2) g were brought up in clean polypropylene cages at temperature of 22–25 ℃ and relative humidity of 40%–70%. Mice were fed with standard pellet diet and water *ad libitum*. The animal experiments were performed at Animal Care Division, Quality Control Department, Institute of Vaccines and Medical Biologicals (IVAC), Vietnam. The animal studies were approved by the Animal Ethical Committee of IVAC (No: 159/QĐ-VXSPYT/ Dt. 29.08.2017) and carried out following the guideline of pharmacological practices approved by Ministry of Health Portal of Vietnam.

2.9.2. Estimation of fasting blood glucose

Diabetic mice were induced by a single intraperitoneal injection of alloxan at a dose of 150 mg/kg body weight. Diabetic mice were confirmed by the determination of fasting blood glucose concentration after 72 hours of injection. Mice with a blood glucose level above 14 mmol/L were used for the experiment[28]. Mice were divided into four groups of 9 mice each, including normal control

mice, diabetic control mice, diabetic mice treated orally with 100 mg/kg body weight ethyl acetate fraction and diabetic mice treated with 100 mg/kg body weight gliclazide. The dose of ethyl acetate fraction (100 mg/kg body weight) was chosen based on our pre-tested results. For acute treatment, blood glucose level of these mice was measured using a glucometer (On Call Plus, ACON Laboratories, Inc. USA) before treatment and after treatment of 0.5, 1 and 3 h. For prolonged treatment (after 10 days of treatment), blood glucose level of untreated and treated groups was measured as mentioned above.

2.9.3. Measurement of body weight and liver enzymes

In order to evaluate the toxicity of the ethyl acetate fraction from seaweed, body weight changes and activities of liver function enzymes of normal mice control and normal mice treated with the fraction were examined. The body weight changes during 7 days of treatment were recorded using an automatic electronic balance. For measurement of biochemical parameters, after 10 days of treatment, the blood samples were collected. The activities of aspartate transaminase (AST), alanine transaminase (ALT) and gamma-glutamyl transferase (GGT) were measured by an auto analyzer (COBAS 8000, ROCHE, Germany) using the Roche kits.

2.10. Statistical analysis

Statistical analysis was performed using the SPSS for Windows (version 16.0, SPSS Inc., Chicago, IL, USA). Mean values were compared using the one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Screening α-glucosidase inhibitory and antioxidant activities of seaweed samples

Nineteen Vietnamese red seaweed species collected from different coastal areas were evaluated for α-glucosidase inhibitory and antioxidant activities. Among the screened seaweed species, *L. dendroidea* had the highest α-glucosidase inhibitory activity (IC_{50} value of 8.14 μg/mL) and antioxidant activity evaluated by DPPH radical scavenging assay (IC_{50} value of 312.09 μg/mL) (Supplementary Table). As a result, this species was used for further investigations in the present study.

3.2. Effects of extraction conditions

The results indicated that methanolic concentration strongly affected both α-glucosidase inhibitory and antioxidant activities of the *L. dendroidea* extract. These activities increased with increased methanol concentration ranging from 0% to 80%, but tended to decline at concentration of 100% (Table 1). A similar pattern of change was observed for phenolic content. Thus,

Table 1. Effect of aqueous methanolic concentrations on α-glucosidase inhibitory and antioxidant activities of *Laurencia dendroidea* extract (mean ± SD, $n = 3$).

Methanolic concentration (%)	Total phenolic content (mg GAE/g dry extract)	α-Glucosidase inhibitory activity (IC_{50}, μg/mL)	DPPH radical scavenging activity (IC_{50}, μg/mL)	Metal chelating activity (IC_{50}, μg/mL)	Nitric oxide radical scavenging activity (IC_{50}, μg/mL)
0	5.63 ± 0.28^{d}	22.25 ± 0.54^{a}	450.12 ± 6.34^{a}	278.05 ± 4.25^{b}	188.07 ± 1.18^{a}
20	6.52 ± 0.30^{c}	18.87 ± 0.91^{b}	425.48 ± 4.49^{b}	251.11 ± 3.07^{c}	167.82 ± 2.44^{b}
40	6.88 ± 0.13^{c}	16.34 ± 0.77^{c}	380.37 ± 4.03^{c}	248.62 ± 6.44^{c}	158.00 ± 3.45^{c}
60	7.55 ± 0.30^{b}	15.22 ± 0.49^{c}	359.77 ± 3.33^{d}	232.05 ± 3.91^{d}	154.83 ± 2.36^{c}
80	7.99 ± 0.07^{a}	10.82 ± 2.17^{d}	302.67 ± 4.28^{e}	198.30 ± 4.15^{e}	153.47 ± 4.99^{c}
100	4.07 ± 0.15^{e}	18.28 ± 1.31^{b}	420.81 ± 5.69^{b}	310.63 ± 3.72^{a}	192.08 ± 4.30^{a}

Different letters in the same column indicated significant difference ($P<0.05$).

Table 2. Effect of extraction methods on α-glucosidase inhibitory and antioxidant activities of *Laurencia dendroidea* extract (mean ± SD, $n = 3$).

Extraction method	Total phenolic content (mg GAE/g dry extract)	α-Glucosidase inhibitory activity (IC_{50}, μg/mL)	DPPH radical scavenging activity (IC_{50}, μg/mL)	Metal chelating activity (IC_{50}, μg/mL)	Nitric oxide radical scavenging activity (IC_{50}, μg/mL)
CE	7.42 ± 0.15^{c}	10.97 ± 1.03^{a}	301.20 ± 5.64^{a}	196.45 ± 4.41^{a}	155.37 ± 3.14^{a}
MAE	11.95 ± 1.52^{a}	5.17 ± 0.93^{c}	262.55 ± 3.88^{c}	140.03 ± 2.31^{b}	116.47 ± 4.63^{c}
UAE	9.21 ± 1.09^{b}	7.51 ± 0.21^{b}	271.79 ± 3.59^{b}	139.90 ± 5.18^{b}	124.06 ± 5.26^{b}
HRE	9.54 ± 0.98^{b}	7.69 ± 0.17^{b}	267.81 ± 5.28^{bc}	142.82 ± 3.41^{b}	127.51 ± 5.12^{b}

CE: conventional extraction, MAE: microwave assisted extraction, UAE: ultrasound assisted extraction, HRE: heat reflux extraction. Different letters in the same column indicated significant difference ($P<0.05$).

Table 3. α-Glucosidase inhibitory and antioxidant activities of solvent-partitioned fractions of *Laurencia dendroidea* extract (mean ± SD, $n = 3$).

Fraction	Extraction yield (%)	Total phenolic content (mg GAE/g dry extract)	Yeast α-glucosidase inhibitory activity (IC_{50}, μg/mL)	DPPH radical scavenging activity (IC_{50}, μg/mL)	Metal chelating activity (IC_{50}, μg/mL)	Nitric oxide radical scavenging activity (IC_{50}, μg/mL)
n-Hexane	45.58 ± 2.03^{a}	8.93 ± 0.77^{b}	218.40 ± 4.50^{c}	700.79 ± 4.07^{a}	643.87 ± 7.25^{a}	623.63 ± 8.24^{a}
Chloroform	23.37 ± 1.62^{b}	6.42 ± 0.58^{c}	287.97 ± 6.02^{a}	678.24 ± 3.45^{b}	644.39 ± 4.62^{a}	612.88 ± 5.17^{a}
Ethyl acetate	17.41 ± 1.03^{c}	27.15 ± 0.77^{a}	2.71 ± 0.59^{e}	75.48 ± 2.07^{e}	64.82 ± 3.17^{d}	57.96 ± 2.34^{e}
Butanol	5.33 ± 1.21^{e}	7.86 ± 0.19^{d}	98.42 ± 1.54^{d}	508.48 ± 3.64^{c}	399.52 ± 4.36^{b}	294.38 ± 5.43^{b}
Water	9.43 ± 0.38^{d}	3.89 ± 0.59^{d}	264.76 ± 6.28^{b}	474.39 ± 4.18^{d}	347.70 ± 5.81^{c}	254.11 ± 5.48^{c}

Different letters in the same column indicated significant difference ($P<0.05$).

80% aqueous methanol was selected for extraction of enzyme inhibitors and antioxidants. Extraction methods also affected α -glucosidase inhibitory and antioxidant activities as depicted in Table 2. Accordingly, microwave-assisted extraction was found to be the most effective method. Ultrasound-assisted extraction and heat reflux extraction showed similar effect, and were better than conventional extraction method. Therefore, the microwave-assisted extraction method was used to recover crude seaweed extract for solvent-fractionating step.

3.3. Fractionation of L. dendroidea extract

The crude extract of *L. dendroidea* was fractionated to obtain *n*-hexane, chloroform, ethyl acetate, butanol and water fractions. All fractions exhibited α -glucosidase inhibitory and antioxidant activities (Table 3), in which the ethyl acetate fraction showed the highest (P<0.05) activities and total phenolic content. However, the extraction yield of this fraction (17.41%) was lower compared to *n*-hexane (45.58%) and chloroform (23.37%) fractions.

3.4. Mammalian α -glucosidase and PTP1B inhibitory activities

Inhibition of mammalian α -glucosidases and PTP1B of the ethyl acetate fraction was evaluated and compared with acarbose and ursolic acid as the positive controls. The fraction inhibited strongly all tested enzymes, with IC_{50} values of 3.71, 14.17, 23.31 and 1.15 μg/mL against yeast α -glucosidase, rat intestinal sucrase, rat intestinal maltase and PTP1B, respectively (Table 4), which were significantly (P<0.05) lower than those of positive controls.

3.5. Kinetics of enzyme inhibition

To find out the inhibition mechanism of the ethyl acetate fraction against α -glucosidase, Lineweaver-Burk plot was used. Data derived from enzyme assay containing different concentrations of *p*-NPG in the absence or presence of different inhibitor concentrations, were plotted. The ethyl acetate fraction showed a mixed type of inhibition (Figure 1) with the K_i value of 2.01 μg/mL.

Table 4. Yeast, rat intestinal α -glucosidase and protein-tyrosine phosphatase 1B inhibitory activities of the ethyl acetate fraction from *Laurencia dendroidea* (mean ± SD, n = 3).

Enzyme	α -Glucosidase inhibitory activity (IC_{50}, μg/mL)		
	Ethyl acetate fraction	Acarbose	Ursolic acid
Yeast α -glucosidase	3.71 ± 0.59[b]	173.57 ± 3.28[a]	-
Rat intestinal sucrase	14.17 ± 1.33[b]	51.40 ± 5.35[a]	-
Rat intestinal maltase	23.31 ± 0.60[b]	33.58 ± 1.65[a]	-
Protein-tyrosine phosphatase 1B	1.15 ± 0.04[b]	-	8.78 ± 1.02[a]

"-": Not determined. Different letters in the same row indicated significant difference (P<0.05).

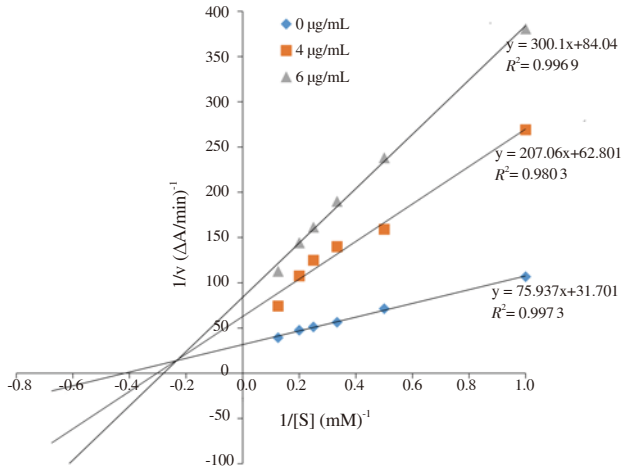

Figure 1. Lineweaver-Burk plot of the ethyl acetate fraction from *Laurencia dendroidea* against yeast α -glucosidase at different concentrations of *p*-NPG (Mean ± SD, n = 3).

3.6. Stability under acidic and thermal conditions

To investigate whether the α -glucosidase inhibitory activity of the ethyl acetate fraction from seaweed *L. dendroidea* was stable when being ingested into the body stomach, an acidic condition (pH 2.0) was used. The inhibitory activity of the fraction against yeast α -glucosidase was rather stable at pH 2.0 for up to 60 min and the inhibitory activity remained approximately 90.15% (Figure 2A). When the treatment time increased from 60 to 120 min, the inhibitory activity decreased rapidly, remaining 55.59% after 120 min of treatment. Similar results were also observed for thermal treatment, in which the activity remained 87.89% after 120 min at 100 ℃ (Figure 2B). The decreased α -glucosidase inhibitory activity of the fraction was in accordance with the decrease in phenolic content (Figure 2A & 2B).

3.7. In vivo antidiabetic effects of ethyl acetate fraction

3.7.1. Blood glucose level

For acute study, the results demonstrated that ethyl acetate fraction (dose of 100 mg/kg body weight) significantly suppressed glucose level in diabetic mice (Figure 3A). After 1 hour of oral administration, the blood glucose level decreased from 22.04 to 13.16 mmol/L and remained unchanged until 3 hours after treatment. The decreased rate was similar to the mice treated with gliclazide (dose of 100 mg/kg body weight). The glucose level of untreated diabetic control mice was stable throughout the treatment time.

For prolonged study, blood glucose levels of the ethyl acetate fraction treated group were also significantly (P<0.001) lower than the diabetic group after 10 days of treatment (Figure 3B). The percentage reduction in blood glucose level was 41.19% in mice treated with 100 mg/kg body weight of ethyl acetate fraction while 37.37% in mice treated with 100 mg/kg body weight of gliclazide.

3.7.2. Body weight changes and liver enzymes

The initial and body weight changes of mice in the experimental

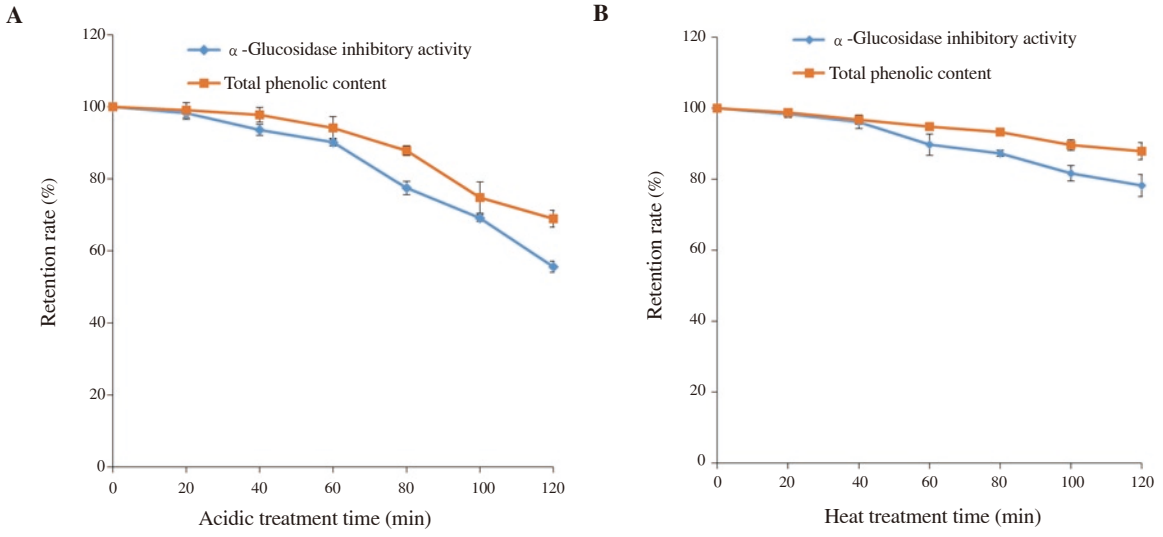

Figure 2. Stability of the α -glucosidase inhibitors in the ethyl acetate fraction from *Laurencia dendroidea* under acidic (A) and thermal (B) conditions (Mean ± SD, $n = 3$).

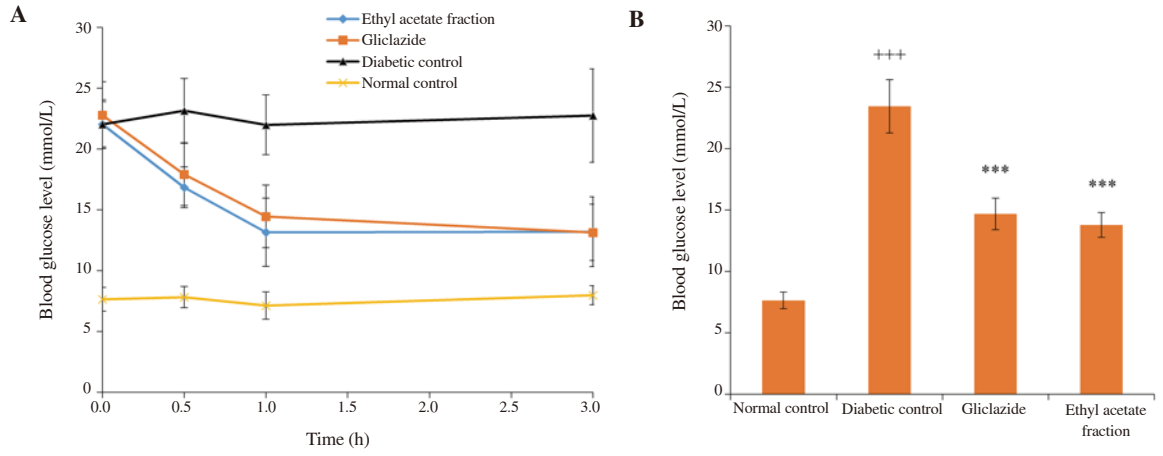

Figure 3. Antidiabetic effect of the ethyl acetate fraction from *Laurencia dendroidea* on blood glucose level of alloxan-induced diabetic mice during acute (A) and prolonged (B) treatment ((Mean ± SD, $n = 9$). $^{+++}$: $P<0.001$ *vs.* Normal control group, ***: $P<0.001$ *vs.* Diabetic control group.

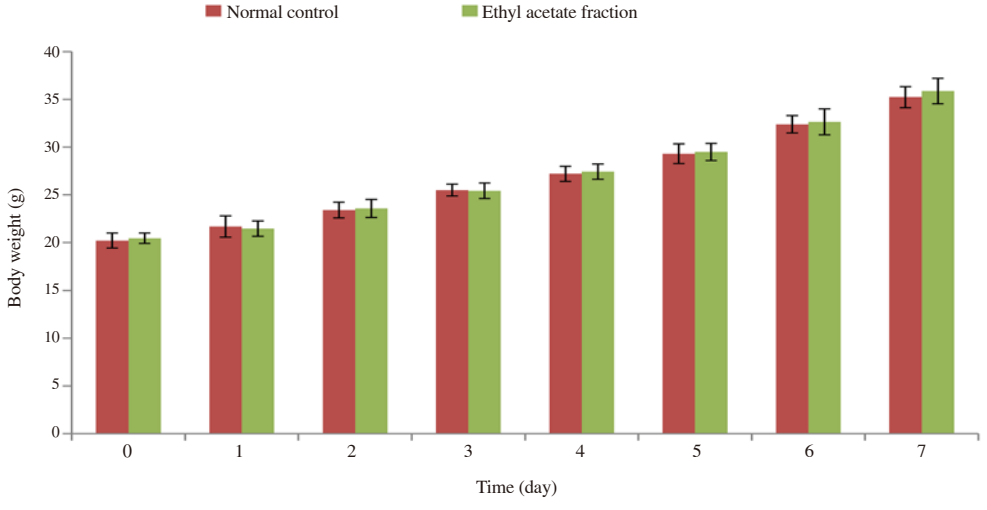

Figure 4. Effect of ethyl acetate fraction from *Laurencia dendroidea* on changes in body weight of nomal mice (Mean ± SD, $n = 9$).

groups during treatment are shown in Figure 4. The body weight of both untreated and ethyl acetate fraction treated normal mice groups increased gradually throughout the 7 days of treatment. However, no significant difference was observed between the two groups. AST, ALT and GGT were evaluated in normal control mice and normal treated group after 10 days of oral administration. No significant differences (P>0.05) in AST, ALT and GGT activities were found between two groups. The AST, ALT and GGT activities of nomal control mice and ethyl acetate fraction treated mice were 141.40, 53.15, 0.67 and 142.73, 55.25, 0.83 U/L, respectively.

4. Discussion

Use of antioxidants and carbohydrate hydrolyzing enzyme inhibitors (*e.g.* α-glucosidase and α-amylase inhibitors) is thought to be one of the most effective approaches for diabetes treatment and related diseases[29]. Seaweeds are considered as a potential resource for extraction of α-glucosidase inhibitors and antioxidants[13,14]. It is the first time that nineteen red seaweed species collected from different coastal areas of Vietnam have been screened for α-glucosidase inhibitory and antioxidant activities. Among the screened seaweed species, the seaweed *L. dendroidea* showed the highest activities. Therefore, it was used for further examinations of extraction conditions, enzyme inhibition mode, stability and *in vivo* experiments.

Both α-glucosidase inhibitory and antioxidant activities of the *L. dendroidea* extract increased with increased methanol concentration in the range of 0% to 80%, but tended to decline at concentration of 100%. The results were in accordance with previous studies[24,30]. A similar pattern of change in phenolic content was also observed. Thus, enzyme inhibitors and antioxidants of *L. dendroidea* may be determined to be phenolic compounds. Regarding the extraction method, microwave-assisted extraction improved significantly activities and total phenolic content compared to conventional extraction and was more sufficient compared to ultrasound-assisted extraction and heat reflux extraction. Microwave-assisted extraction has been proved as a sufficient method for extraction of various bioactive compounds from natural resources[31]. The mechanisms of microwave-assisted extraction are that (i) microwaves penetrate to material and induce the molecular oscillation, thus heat is generated throughout the volume of the material and (ii) the interaction between microwave and solvent results in the rupture of membrane cells, leading to quick release of intracellular extractant into the solvent[32].

The crude extract obtained by microwave-assisted extraction method with suitable methanol concentration (80%) was further fractionated into *n*-hexane, chloroform, ethyl acetate, butanol and water. The α-glucosidase inhibitory and antioxidant activities of the ethyl acetate fraction were significantly higher than other fractions. Besides, higher extraction yields were obtained in *n*-hexane fraction (45.58%) and chloroform fraction (23.37%), which may be because lipids, chlorophyll, refined oil, sterols and resin were well dissolved in *n*-hexane and chloroform[33], but these fractions had low activities. Ethyl acetate was typically used to fractionate polyphenols. These compounds are known as the main compounds responsible for a number of biological properties[34]. Many phenolic compounds having α-glucosidase inhibitory and antioxidant activities from different seaweeds have been reported[15]. Phytochemical analysis of the ethyl acetate fraction from *L. dendroidea* showed that phenolics, flavonoids, alkaloids, anthraquinones and fatty acids are presented (data not shown). Although yeast α-glucosidase is extensively used for preliminary screening α-glucosidase inhibitory activity of certain extracts and/or isolated compounds, the results may not be the same with those obtained in mammals. Use of PTP1B inhibitors is a pathway to improve insulin sensitivity in different cell types and to control diabetes[35]. Therefore, mammalian α-glucosidases and PTP1B were also tested in this study. It can be seen that the fraction inhibited all tested enzymes, with IC_{50} values significantly lower than those of acarbose and ursolic acid used as the positive controls. Furthermore, the inhibitory activity against yeast α-glucosidase of ethyl acetate fraction was significantly stronger than that of rat intestinal sucrase and maltase. The same results have been demonstrated by other authors. The *Saccharomyces cerevisiae* α-glucosidase inhibitory activity of bioactive compounds isolated from seaweeds such as bromophenols from *Symphyocladia latiuscula*[36], *Polyopes lancifolia*[24] and *Grateloupia elliptica*[37], and extracts of *Ecklonia stolonifera*[16] was significantly higher than mammalian α-glucosidases. The differences in inhibitory activity between enzymes would be due to the differences in enzyme structure and/or inhibitory mechanism.

A mixed type of inhibition against yeast α-glucosidase of ethyl acetate fraction from *L. dendroidea* extract was found. This finding is thought to be resulted from components in seaweeds. A bromophenol, bis(2,3-dibromo-4,5-dihydroxybenzyl) ether, purified from *Polyopes lancifolia* inhibited yeast α-glucosidase with a mixed type of inhibition[24]. A mixed inhibition mode against yeast α-glucosidase was also found for phlorotannins isolated from different edible brown seaweeds[38,39]. This mixed-type inhibition can be explained by different ways. Firstly, the binding of inhibitors in the ethyl acetate fraction to α-glucosidase results in conformational changes, which leads to declined α-glucosidase activity. Secondly, irreversible modifications of α-glucosidase caused by inhibitors in the fraction lead to decreased enzyme activity. Finally, the later intermediate in the reaction can interact with inhibitors but not with the initial enzyme-substrate complex[40].

To investigate the stability of the α-glucosidase inhibitory activity of the ethyl acetate fraction from seaweed *L. dendroidea* when being ingested into the body stomach, an acidic condition (pH 2.0) was used. On the other hand, in reality, drugs and functional foods normally undergone through high temperature processing. Therefore, the fraction was heated at 100 ℃ for 120 min. The results revealed that α-glucosidase inhibitory activity of the ethyl acetate fraction from *L. dendroidea* was stable under both acidic and thermal conditions. Thus, α-glucosidase inhibitors in the seaweed *L. dendroidea* would be stable when they were digested in stomach and underwent food processing.

The obtained results in *in vitro* model indicated the ethyl acetate

fraction of seaweed *L. dendroidea* was a potential antidiabetic agent. However, further studies need to be conducted *in vivo*, because, if the structure of seaweed compounds is degraded in the animal body by stomach acid or digestive enzymes, their inhibitory activity could be decreased. Evaluation of *in vivo* antidiabetic activity of the ethyl acetate fraction was carried out in alloxan-induced diabetic mice. The diabetic mice treated with the ethyl acetate fraction had significantly lower glucose level compared to the diabetic control group in both acute and prolonged studies, which was comparable with a commercial antidiabetic drug (gliclazide). The reduction in blood glucose level in treated mice is due to the inhibition of carbohydrate hydrolysing enzymes. As obtained by *in vitro* studies, the fraction strongly inhibited both yeast and mammalian α-glucosidases. The seaweed fraction may also have acted through other mechanisms by either stimulating the pancreatic secretion of insulin from the cells of islets of langerhans or its release from bound insulin[41,42]. This mechanism was confirmed by the inhibitory ability of the fraction against PTP1B as a key negative regulator of the insulin signaling pathways[35]. Moreover, DM is induced by oxidative stress in case of overaccumulation of ROS[43]. The obtained results indicated the ethyl acetate fraction from seaweed had strong antioxidant activities as evaluated by different assays. Thus, the antidiabetic activity of the ethyl acetate fraction can also be arisen from its antioxidant properties. Antidiabetic activities of different seaweed species have been reported in previous studies. Bromophenols of red seaweeds[15] and phlorotannins of brown seaweeds[16] are key components responsible for antidiabetic activities both *in vitro* and *in vivo* models.

Regarding the acute oral toxicity study, oral administration of the ethyl acetate fraction at a dose of 100 mg/kg body weight did not produce any signs of toxicity, no death occurred (data not shown). Mice treated with the seaweed fraction grew normally as indicated by a gradual increase in body weight compared to untreated group. GGT, ALT and AST, as enzymatic markers of liver function, are usually used to signal liver damage in clinics. The results demonstrated no significant differences in the levels of GGT, ALT and AST were found between treated and untreated mice. Thus, the ethyl acetate from seaweed *L. dendroidea* did not exhibit any toxicities.

In conclusion, the ethyl acetate fraction from the red seaweed *L. dendroidea* had strong *in vitro* α-glucosidase inhibitory and antioxidant activities. *In vivo* results revealed that the ethyl acetate fraction can significantly suppress the glucose level of diabetic mice without any signs of toxicity in mice. Therefore, the ethyl acetate fraction from *L. dendroidea* could be a potential candidate for development of new drugs and functional foods for prevention and treatment of diabetic and oxidative stress related diseases. Further clinical experiments should be carried out in the future studies.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

NT. Han designed experiments for both *in vitro* and *in vivo* models. DAD contributed to seaweed sample collection and identification. NT. Han, DAD and NVM performed the *in vitro* experiments. NT. Han, NT. Huyen, NTLP and TTVA carried out the *in vivo* experiments. NT. Han, NVM and SMK wrote and finalized the manuscript. SMK also served as the project's supervisor.

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Supplemented table. Total phenolic content, antioxidant and enzymatic inhibitory activities of seaweed extracts

No.	Seaweed species	Total phenolic content (mg GAE/g dry extract)	α-Glucosidase inhibitory activity (IC_{50}, μg/mL)	DPPH radical scavenging activity (IC_{50}, μg/mL)
1.	*Actinotrichia fragilis*	1.27 ± 0.05	52.73 ± 3.68	1250 ± 17.52
2.	*Amphiroa ancepes*	4.45 ± 0.64	12.34 ± 0.88	871.68 ± 7.90
3.	*Amansia rhodantha*	3.84 ± 0.55	10.62 ± 0.32	792.54 ± 4.97
4.	*Asparagopsis taxiformis*	2.30 ± 0.31	10.25 ± 1.32	981.77 ± 6.00
5.	*Claudea batanensis*	1.27 ± 0.65	21.85 ± 0.98	1454.26 ± 8.73
6.	*Gelidiella acerosa*	4.18 ± 0.79	17.98 ± 2.31	661.14 ± 2.09
7.	*Gracilaria asiatica*	1.25 ± 0.26	48.58 ± 0.63	1114.27 ± 9.88
8.	*Gracilaria arcuata*	4.36 ± 0.51	26.83 ± 1.36	679.49 ± 8.87
9.	*Gracilaria eucheumatoides*	2.60 ± 0.08	79.24 ± 5.15	1281.76 ± 7.08
10.	*Gracilaria firma*	2.49 ± 0.29	47.72 ± 3.15	992.18 ± 3.85
11.	*Gracilaria salicornia*	4.30 ± 0.37	54.76 ± 2.57	569.11 ± 5.53
12.	*Halymenia dilatata*	5.76 ± 0.19	23.71 ± 1.11	491.62 ± 1.08
13.	*Helminthocladia australis*	7.59 ± 0.57	23.11 ± 1.72	422.81 ± 7.37
14.	*Laurencia dendroidea*	7.49 ± 0.57	8.14 ± 0.02	312.09 ± 4.19
15.	*Laurencia intermedia*	7.80 ± 0.40	17.67 ± 2.33	332.72 ± 5.91
16.	*Mastophora rosea*	3.44 ± 0.27	29.52 ± 1.10	725.81 ± 9.13
17.	*Titanophora weberae*	0.33 ± 0.19	18.57 ± 1.85	1478.43 ± 14.01
18.	*Tricleocarpa cylindrical*	5.92 ± 0.05	39.62 ± 2.24	414.79 ± 3.21
19.	*Tricleocarpa fragilis*	5.66 ± 0.32	37.35 ± 3.72	394.38 ± 9.71