



Ameliorative effects of phlorotannin-rich fraction of *Sargassum tenerrimum* in high-fat diet and low dose streptozotocin-induced metabolic changes and oxidative stress in diabetic rats

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ABSTRACT

Introduction: Type 2 diabetes mellitus (T2D) is a progressing polygenic disease demanding a multitargeted treatment strategy. *Sargassum tenerrimum* (ST) is a marine brown alga with potentially bioactive chemicals that could be used as innovative biotherapeutics for diabetes treatment. The current research examined the potential of the phlorotannin-rich fraction from *S. tenerrimum* (PST) to mitigate diabetes in Wistar albino rats induced with high-fat diet (HFD) and streptozotocin (STZ) administration.

Methods: Diabetic rats were given PST (200 and 400 mg/kg) or metformin (250 mg/kg) orally three weeks, and followed by the measurements of insulin, glycemic factors, biological markers of oxidative stress, tumor necrosis factor-alpha (TNF- α), as well as hepatic and pancreatic histopathological changes.

Results: PST treatment significantly decreased fasting blood glucose, insulin resistance, lipid profile, hepatic profile, and TNF- α levels and improved serum insulin and glucose tolerance in diabetic rats. In the skeletal muscles of diabetic rats, PST led to a significant rise in antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) reductase, and a decrease in lipid peroxidation. Furthermore, PST treatment significantly reduced pancreatic-cell damage and hepatic fatty accumulation. PST was more efficacious at 400 mg/kg exhibiting a dose-dependent effect.

Conclusion: PST improves glucolipid metabolism in HFD and STZ-induced diabetic rats, probably by reducing hyperglycemia, insulin resistance, dyslipidemia, oxidative stress, inflammation, and damage to pancreatic and hepatic architectures. The findings suggest that PST has a curative impact on diabetes mellitus type 2 and represents a new subject of study for the treatment of diabetes naturally.

Implication for health policy/practice/research/medical education:

Phlorotannin-rich fraction of *Sargassum tenerrimum*, a brown marine alga, attenuates hyperglycaemia, insulin resistance, dyslipidemia, and oxidative stress in high-fat diet and streptozotocin-induced diabetic rats. Phlorotannin extracts of *S. tenerrimum* have the potential to be viable marine drug candidates in the development of anti-diabetic therapeutics.

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Introduction

Diabetes is a chronic metabolic ailment that affects individuals of all ages. As per the statistics by the International Diabetes Federation, 537 million people were diagnosed with diabetes in 2021, and it is anticipated that by 2045, the number will rise to 783 million globally (1). Among several types, type 2 diabetes mellitus (T2D)

is the most prevalent form of diabetes, accounting for over 90-95% of all cases. It is characterised by alterations in insulin resistance, dysfunction of pancreatic beta-cells, and fluctuations in lipid metabolism (2). One of the leading causes of T2D is insulin insufficiency and/or resistance, which leads to deregulated glucose metabolism and diminished glycolysis enzyme activity. These

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abnormalities result in reduced peripheral glucose usage and increased hepatic glucose synthesis (3). The most frequent risk factor for the emergence of T2D is obesity, which can result in increased triglyceride levels in the blood, hypertension, and insulin resistance (4). Another critical factor in the development and progression of diabetes is oxidative stress. According to reports, forming free radicals contributes to the development of more than 50% of diseases. If ignored, increasing oxidative stress from hyperglycemia activates the polyol pathway, which results in inflammation and can cause serious consequences (5). Growing evidence indicates that under the influence of the risk factors mentioned above, inflammatory pathways are the primary, common pathogenetic mediators in the natural course of diabetes (6).

Dietary interventions for T2D can be used alone, particularly at the beginning and during mild hyperglycemia, or in conjunction with oral hypoglycemic medications like biguanides, peroxisome proliferator-activated receptor- γ agonists, sulfonylureas, α -glucosidase inhibitors, or insulin (7). There is a scarcity of effective treatments free of side effects. Therefore, it is essential to develop innovative preventative measures and improved therapies to battle T2D and its complications. As a result, there has recently been a surge in interest in complementary therapies and the therapeutic application of natural diabetes remedies, particularly those derived from the sea (8).

The value of marine seaweeds as a source of novel bioactive molecules has grown exponentially over the past decade, considering the plethora of various bioactive molecules present, such as polysaccharides, amino-acid derivatives, polyphenols, terpenoids, and steroids. Brown seaweeds contain phlorotannins, a class of polyphenols with a unique structural composition that sets them apart from other marine algae and terrestrial flora (9). Phlorotannins are essential for an alga's physiological integrity and serve a number of critical support functions, such as chemical defence, preventing oxidative damage caused by fluctuations in nutrient levels and UV rays, and serving as crucial cell wall components (10). They also exhibit a wide range of bioactivities, such as potent antioxidant defences and anticancer and anti-inflammatory effects (11-13). In addition, several investigations also highlighted the significance of phlorotannins' bioactive potential as an anti-diabetic remedy (8,14).

Sargassum tenerrimum (ST) is a brown marine algae found across the tropics and is predominantly distributed on the shores of India, Pakistan, Japan, China, and other countries of East Asia (15). Its preliminary phytochemical evaluation revealed the presence of bioactive secondary metabolites like steroids, alkaloids, glycosides, phenolic groups, saponins, tannins, flavonoids, terpenoids, carbohydrates, reducing sugars, and xanthoproteins. ST shows anticancer, anti-inflammatory, antioxidant, antifungal, antibacterial, antiviral, and anti-predatory

activities evident from the preclinical reports (15-18). Studies have been conducted to extract and identify phlorotannins from *Sargassum* species; however, studies on ST are lacking (10,19). Hence, this study aimed to extract and identify rich phlorotannin fractions from ST using the ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) technique and evaluate its anti-diabetic potential in T2D rats.

Materials and Methods

ST sampling and chemicals

Fresh algae ST was collected in November 2021 from the Mandapam region (09 17.417 0N; 079 08.558 0E), located on the southeast coast of India. The algae sample was transferred in sealed containers after being cleaned with fresh water to remove debris, epiphytes, and adherent sand particles. *S. tenerrimum* J. Agardh was identified on the herbarium sheets, which were submitted to the Center for Medicinal Plants Research in Homoeopathy in Emerald, Tamil Nadu, and the accession number 003/2021 was given. In subsequent work, it was washed in tap water to remove any salt residue, dried, crushed into a powder that could pass through a 40-mesh filter, and stored at room temperature.

Phloroglucinol, 2,4-dimethoxybenzaldehyde (DMBA), and metformin were obtained from Sigma-Aldrich chemicals Pvt. Ltd., India. Ketamine and Xylazine were procured from Delphis Pharmaceuticals, Gujarat, India and DE Mega formulations Private Limited, Gujarat, India, respectively. Analytical grade quality chemicals and reagents were used for the remainder of the study.

Extraction and fractionation of ST ethanolic extract

The extraction conditions were modified slightly from those proposed by Li et al (10). One kilogram of powdered and shade-dried seaweed was extracted twice with 600 mL of n-hexane over 24 hours at room temperature to clear the lipid pigments. The residue was allowed to draw out any remaining traces of n-hexane for two hours. Then, the residue (100 g) was extracted twice with 30% ethanol (2 \times 500 mL) at room temperature for 3 hours under constant agitation in a rotary shaker (Remi Scientific Instruments, India) with a speed of 125 rpm. A rotary flash evaporator condensed the combined filtrate in a vacuum at 40°C to around 100 mL. Later, the combined extract was partitioned 03 times with ethyl acetate (1:1, v/v) and 1-butanol (1:1, v/v) to fractionate polar and non-polar compounds. The fractions were concentrated in a rotary flash evaporator, producing ethyl acetate, 1-butanol fractions, and aqueous residue. Before further examination at 4°C, all the fractions were freeze-dried.

Estimation of total phlorotannin content (TPC) of crude extract and fractions

TPC in the crude extract and fractions obtained was assessed using the DMBA colorimetric method, as

reported earlier by Amarante et al with slight modifications (20). Equal parts of DMBA (2 g/100 mL solution) and hydrochloric acid (6%, v/v), prepared in glacial acetic acid, were combined to create the working reagent, which was stored at room temperature. Then, 250 µL of working reagent was mixed with 50 µL of each extract/fraction and left at room temperature for an hour. Absorbance was measured at 515 nm using a UV-visible spectrometer (Perkin Elmer, USA). The TPC was expressed as mg phloroglucinol equivalent/g dry weight extract. TPC was found to be highest in the ethyl acetate fraction.

Characterization of phlorotannins by UPLC-MS/MS technique

The phlorotannins in the ethyl acetate fraction of ST were identified using the UPLC-MS/MS technique. MS analysis of phlorotannin-rich fraction, i.e., ethyl acetate fraction, was carried out on Ultra High-Performance Liquid Chromatographic separation (Thermo Dionex UltiMate 3000 RS Pump, Autosampler) coupled with a Thermo Orbitrap Q Exactive (Multimode source – HESI) mass spectrometer. Acquity UPLC (BEH C18, 100 mm × 2.1 mm 1.7 µm) column was used with a flow rate of 0.5 mL/min. The mobile phases used were 0.1% formic acid in water (Phase A) and 0.1% formic acid in acetonitrile (Phase B). The scan mode monitoring (SCAN) experiment was performed on the mass spectrometer operated in both positive and negative ion modes with a scan range of -100 to 1500 m/z, 70000 resolution, a capillary voltage of 3 kV, and 440°C temperature. Based on mass and published literature, compounds were identified.

Experimental animals

Male Wistar albino rats (150–200 g) were obtained from the in-house animal facility of DAC Regional Research Institute for Homeopathy, Kolkata (India). Animals were placed in polycarbonate cages (3/cage) with corncob bedding (Sparconn Life sciences, Karnataka) under controlled environment (Temperature: 23°C ± 2°C; Relative humidity: 55% ± 5%, and 12 hour light: dark cycle). Experimental rats were given standard laboratory rat feed (VRK Nutritional solutions, India) and water *ad libitum*. The study was executed following the Committee for Control and Supervision of Experimentation on Animals (CCSEA) guidelines.

In vivo anti-diabetic activity

The ethyl acetate fraction of ST was found to be phlorotannin-rich in the study. As a result, it was further evaluated for its anti-diabetic potential in high-fat diet (HFD) and streptozotocin (STZ)-induced diabetic rats.

Induction of diabetes

A total of 50 rats were used in the study. Ten rats were randomly chosen for the non-diabetic control (NDC) group after a one-week acclimatization period. During

the study period, they were fed a typical pelleted diet (3.6% fat, 18% protein, and 53% carbohydrate, as a proportion of total kcal). The remaining 40 animals were fed HFD for three weeks, which contained 58% fat, 25% protein, and 17% carbohydrate. HFD's preparation and formulation (Table 1) have previously been discussed (21). Weekly, HFD was prepared and kept at 4 ± 1°C in airtight containers. After 3 weeks, the animals received STZ at 35 mg/kg dose via intraperitoneal route to induce diabetes. Blood was drawn from the animals by the retro-orbital plexus and analysed for fasting blood glucose levels three days after STZ injection. Animals having fasting blood sugar levels greater than 200 mg/dL were employed for further anti-diabetic action evaluation (4).

Treatment protocol

Group 1, i.e. NDC included 10 rats, which were administered 0.5% sodium carboxy methyl cellulose (Na CMC) (2 mL/kg/d, p.o.). Forty diabetic rats were randomly assigned to four groups (n=10) and given the following treatments: Rats in group 2, i.e. diabetic control (DC) received 0.5% Na CMC (2 mL/kg/d, p.o.). Rats in group 3 (positive control) were given metformin (250 mg/kg/d, p.o.). Phlorotannin-rich fraction from *Sargassum tenerrimum* (PST) was administered to groups 4 and 5 diabetic rats at 200 and 400 mg/kg/d, p.o., respectively. The rats received treatments for 3 weeks, during which standard laboratory rat feed was given.

Sampling

After 21 days of treatment, the animals were weighed while fasting overnight and blood samples were taken by retro-orbital puncture. The anaesthesia for the procedure was administered through an intra-peritoneal injection of a ketamine-xylazine mixture (ketamine 100 mg/kg and xylazine 10 mg/kg). Blood samples were centrifuged for 15 minutes at 3000 rpm to obtain serum. The clear, clean serum was put into plastic tubes with labels and stored in the freezer at -4°C for further use. Animals were euthanized under carbon dioxide asphyxiation and the liver and pancreas were separated and cleaned using normal saline. Sections of the liver and pancreas were kept

Table 1. High fat diet (HFD) composition

Ingredients	Weight (g/100 g)
Powdered normal pellet feed	36.5
Casein	25
Lard	31
Cholesterol	1
Vitamin and mineral mix	6
Sodium cholate	0.5
DL-Methionine	0.3
Sodium chloride	0.1
Yeast powder	0.1

in a 10% buffered formalin solution for histopathological examination.

Determination of fasting blood glucose, serum insulin, and insulin resistance

Fasting blood sugar levels were assessed weekly using an Accu-Check glucometer (Roche Diagnostics Pvt. Ltd, India), while serum fasting insulin levels were determined using rat-specific ELISA kits (BT Labs, China) in accordance with the manufacturer's instructions. The insulin-sensitizing potential was characterized by the homeostasis model assessment of insulin resistance (HOMA-IR) (22).

$$\text{HOMA-IR} = (\text{Fasting Glucose (mM)} \times \text{Fasting Insulin } (\mu\text{U/mL})) / 22.5$$

Renal and lipid profiles and liver function tests

The serum levels of creatinine, urea, as well as triglycerides, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were determined using commercially available kits (Transasia Bio-Medicals Pvt. Ltd, India) in accordance with the manufacturer instructions using fully automated biochemistry analyzer (ERBA EM 360, Transasia Pvt. Ltd, India).

Oral glucose tolerance test (OGTT)

OGTT was conducted in control and treated rats on the last day of the experimental period using the methodology of Wang et al (23). The rats were deprived of food for 12 hours before oral glucose administration at 2 g/kg body weight. After glucose loading, blood samples were taken at 0, 30, 60, 90, and 120 minutes. Blood glucose levels were then measured at these intervals using a glucometer.

Measurement of oxidative stress markers in gastrocnemius muscle

The gastrocnemius muscle samples from all experimental rats were homogenized in ice-cold 0.067 M phosphate buffer using a tissue homogenizer (Dynaken, Malaysia). After homogenisation, the samples were centrifuged at 4000 rpm for 10 minutes at 4°C. The subsequent supernatants were kept at -20°C and the levels of superoxide dismutase (SOD) (24), catalase (CAT) activity (25), reduced glutathione (GSH)(26), and malonaldehyde (MDA) (27) were estimated as described previously.

Serum pro-inflammatory marker

Rat-specific ELISA kit (Elabscience, USA) was used to measure tumor necrosis factor-alpha (TNF-α), a serum pro-inflammatory marker tightly linked to the progression of diabetes. The serum was obtained from the blood collected, and further analysis was carried out

using an ELISA reader (iMark™, BIO-RAD, USA) as per manufacturer's protocol.

Histopathological analysis

The pancreas and liver tissues were fixed in 10% formalin, cut into sections and embedded in paraffin wax. Specimen slides with 5 μm thick tissue sections were prepared using a semi-automatic rotatory microtome (M/s Biocraft, India). Hematoxylin and eosin stains were used to stain these slides, and a light microscope (BX 43, Olympus, India) was used to take photomicrographs.

Statistical analysis

G3 (v.3.1) software was used to calculate the sample size. Ten rats per group were obtained with an alpha error of 0.05 and a power of 82%, and the same number was employed for the anti-diabetic activity. The data were presented as mean ± standard deviation (n = 10 or n=6). Data from the groups were analysed using one-way analysis of variance followed by Tukey's *t* test. The value of *P* < 0.05 was considered statistically significant in the results. SPSS software (version 26) was employed to carry out statistical analysis.

Results

Estimation of TPC of ethanolic extract and fractions of ST

The yield of the crude ethanolic extract of ST was about 21.4% w/w. The yields of the aqueous, ethyl acetate, and 1-butanol fractions produced from the ethanolic extract following solvent partitioning were 28.4%, 19.9%, and 5.1%, respectively (Table 2). The ethyl acetate fraction's increased purity, confirmed by its TPC (60.1 ± 1.9 mg PGE/g extract), was higher than that of the crude extract. At the same time, aqueous (7.2 ± 3.3 mg PGE/g extract) and 1-butanol (1.8 ± 0.9 mg PGE/g extract) portions were poor in TPC. Therefore, in the current investigation, the phlorotannin-rich fraction of the ethyl acetate was further examined for its anti-diabetic activity in Wistar albino rats.

Tentative assignment of compounds from phlorotannin rich fraction using UPLC-MS technique

As shown in Table 3 and Figure 1, 8 compounds present in the phlorotannin-rich fraction, i.e., ethyl acetate fraction

Table 2. Extraction mass yield and total phlorotannin content of *Sargassum tenerrimum* ethanolic crude extract and fractions

Extract/Fraction	Yield (%)	Total phlorotannin content (mg PGE/g extract)
Ethanolic crude extract	21.4	16 ± 2.3
1-Butanol	5.1	1.8 ± 0.9
Ethyl acetate	19.9	60.1 ± 1.9
Aqueous	28.4	7.2 ± 3.3

Yield is expressed as %w/w of dry algae (for ethanolic crude extract) or ethanolic crude extract (for fractions). PGE: Phloroglucinol equivalents.

Table 3. Tentative peak assignment of the compounds identified by UPLC-MS/MS found in the phlorotannin-rich fraction of *Sargassum tenerrimum*

Peak	Retention time (min)	Mass (m/z)	Tentative assignment	Reference(s)
1	1.322	247	Dibenzodioxine-1,3,6,8, tetraol	(19)
3	7.340	387	Phlorethohydroxycarmalol	(10)
6	8.250	521	Phlorotannin derivative	(19)
8	9.36	265	Fuhalol	(19)
13	12.121	287	Phlorotannin derivative	(19)
14	12.748	371	Eckol	(10)
18	13.733	389	Trifuhalol	(10)
19	14.068	371	Eckol	(10)

of ST, were identified. The compounds were tentatively identified as phlorotannins and their derivatives. The compounds identified were dibenzodioxins-1,3,6,8, tetraol, phlorethohydroxycarmalol, fuhalol, trifuhalol, and eckol in the ethyl acetate fraction of ST. The chemical structures of dibenzodioxins-1,3,6,8, tetraol, fuhalol, and eckol are presented in Figure 2.

Effect of PST on body weight

Before treatment initiation (Day 1 body weights) the body weights of the diabetic rats were considerably ($P < 0.001$) higher than the NDC rats. At the end of the experiment, the diabetic rats showed a 23.10% increase in body weight compared to the NDC rats ($P < 0.001$). Treatment with PST (200 mg/kg & 400 mg/kg) and metformin (250 mg/kg) resulted in a significant reduction in body weights by 6.54%, 7.47%, and 8.94%, respectively, compared to the

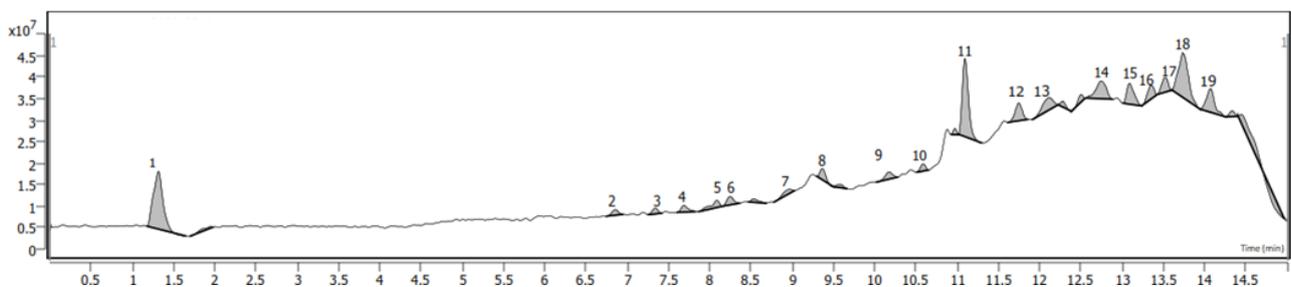
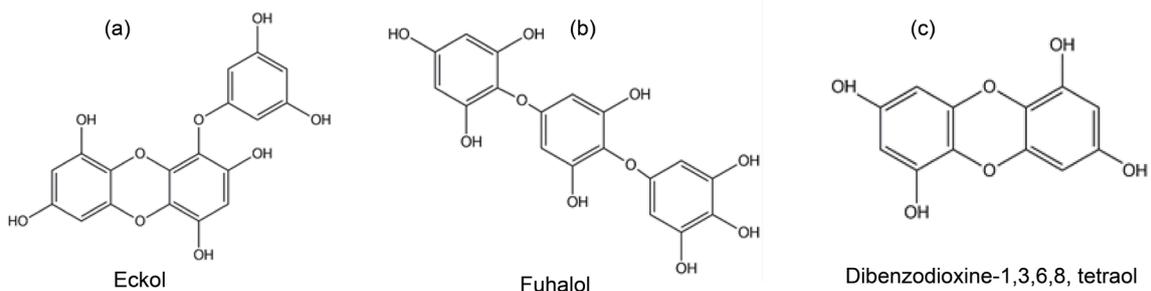
diabetic rats (Figure 3).

Effect of PST on fasting blood glucose values

All animals, excluding the NDC group, showed increased fasting blood glucose levels after receiving HFD and STZ treatment. Hyperglycemia is one of the important signs of diabetes induction. Compared to the DC group, PST administration reduced blood glucose levels from the 14th day to the completion of the study, with the impact being maximum at 400 mg/kg and similar to metformin (Figure 4). From the seventh day of treatment, the diabetic rats receiving metformin significantly improved their blood glucose levels.

Effect of PST on serum insulin and HOMA-IR values

In DC group rats, serum insulin content decreased and HOMA-IR values increased at the completion of the

**Figure 1.** UPLC-MS/MS analysis of the *Sargassum tenerrimum* phlorotannin-rich fraction. The peaks denoted by numbers relate to the identified compounds in Table 3.**Figure 2.** Chemical structures of (a) Eckol, (b) Fuhalol, and (c) Dibenzodioxine-1,3,6,8, tetraol.

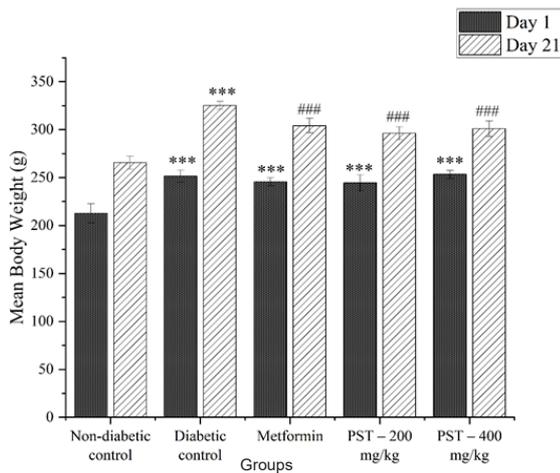


Figure 3. Effect of the phlorotannin-rich fraction of *Sargassum tenerrimum* on mean body weight in high-fat diet and streptozotocin-induced diabetic rats (n = 10). PST: Phlorotannin-rich fraction of *S. tenerrimum*. *** $P < 0.001$ vs. non-diabetic control group; ### $P < 0.001$ vs. diabetic control group.

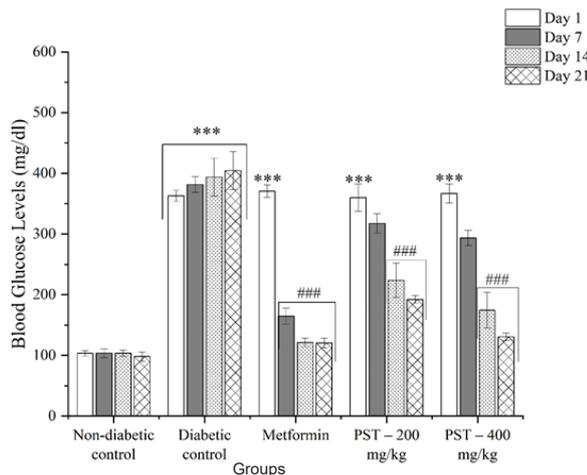


Figure 4. Effect of the phlorotannin-rich fraction of *Sargassum tenerrimum* on mean body weight in high-fat diet and streptozotocin-induced diabetic rats (n = 10). PST: Phlorotannin-rich fraction of *S. tenerrimum*. *** $P < 0.001$ vs. non-diabetic control group; ### $P < 0.001$ vs. diabetic control group.

study. In PST and metformin-treated rats substantial elevation in insulin levels and a reduction in HOMA-IR values was observed after 21 days of treatment ($P < 0.001$). No significant changes in insulin levels were observed between PST 400 mg/kg and metformin. In contrast to the DC group, all treatment groups' HOMA-IR values dramatically dropped, demonstrating the insulin-sensitizing effects of PST and metformin (Figure 5).

Effect of PST on lipid, renal, and hepatic biochemical parameters

Our findings demonstrated that rats in the DC group had elevated levels of triglycerides, TC, and LDL-C, along

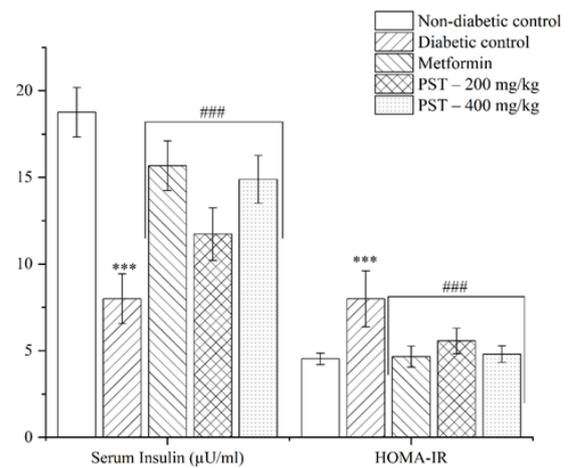


Figure 5. Effect of a phlorotannin-rich fraction of *Sargassum tenerrimum* on serum insulin and HOMA-IR in high-fat diet and streptozotocin-induced diabetic rats (n=06). PST: Phlorotannin-rich fraction of *S. tenerrimum*. *** $P < 0.001$ vs. non-diabetic control group; ### $P < 0.001$ vs. diabetic control group.

with reduced levels of HDL-C. PST treatment enhanced insulin secretion resulting in the lowering of serum levels of triglycerides and cholesterol. PST treatment significantly reduced LDL-C, triglyceride, and cholesterol levels ($P < 0.001$) and increased HDL-C ($P < 0.001$) levels compared to the DC group. Along with hepatic biomarkers, SGPT, SGOT, and ALP, the DC group's urea and creatinine levels were elevated. PST treatment significantly reduced the elevated hepatic enzymes and renal markers, urea, and creatinine levels by the completion of the study. In a similar vein, diabetic rats in the metformin-treated group demonstrated improved lipid, renal, and hepatic biomarkers ($P < 0.001$) (Table 4). The effects of PST at 400 mg/kg dose were comparable to metformin and were superior to the 200 mg/kg dose of PST.

Effect of PST on glucose tolerance

To assess the insulin-sensitizing potential of PST, OGTT was conducted on the last day of the study. In NDC group rats, the administration of Glucose (2 g/kg) did not cause any significant changes in blood glucose levels for 120 minutes in non-diabetic rats. However, in DC rats, there was a significant increase in blood glucose levels between 0 and 120 minutes. Compared with DC, PST and metformin-treated groups improved considerably oral glucose tolerance (Figure 6A). The rats in these groups had reduced blood glucose levels at all time points, namely 0, 30, 60, 90, and 120 minutes after oral glucose loading. The treatment with PST at 200 mg/kg and 400 mg/kg considerably ($P < 0.001$) lowered blood glucose excursion by 52% and 63%, as revealed by $AUC_{0-120 \text{ min}}$ (Figure 6B), in comparison to DC rats. Metformin administration also significantly ($P < 0.001$) reduced blood glucose excursion ($AUC_{0-120 \text{ min}}$) by 69%.

Table 4. Effect of a phlorotannin-rich fraction of *Sargassum tenerrimum* on lipid, renal, and hepatic biochemical parameters in high-fat diet and streptozotocin-induced diabetic rats (n=10)

Group/Parameter	Non-diabetic control	Diabetic control	Metformin	PST – 200 mg/kg	PST – 400 mg/kg
Total cholesterol (mg/dL)	92.1 ± 8.45	193.3 ± 7.65***	118.9 ± 6.06###	137.7 ± 7.01###	125.3 ± 6.06###
Triglycerides (mg/dL)	84.92 ± 12.64	214.82 ± 7.92***	92.75 ± 7.46###	136.04 ± 6.78###	120.3 ± 6.59###
LDLc (mg/dL)	53.73 ± 2.86	94.42 ± 2.36***	56.81 ± 1.95###	72.5 ± 2.82###	52.53 ± 2.4###
HDLc (mg/dL)	40.11 ± 3.72	21.1 ± 2.79***	41.28 ± 3.21###	30.21 ± 3.97###	38.23 ± 3.82###
Urea (mg/dL)	42.17 ± 4.31	94.55 ± 3.64***	44.07 ± 3.27###	60.44 ± 4.26###	47.73 ± 3.09###
Serum creatinine (mg/dL)	0.55 ± 0.03	1.09 ± 0.03***	0.64 ± 0.02###	0.75 ± 0.03###	0.68 ± 0.03###
SGOT (U/L)	146.61 ± 7.97	498.81 ± 9.95***	152.63 ± 8.64###	215.89 ± 7.93###	189.94 ± 10.43###
SGPT (U/L)	61.96 ± 10.45	261.77 ± 8.92***	66.74 ± 8.15###	88.96 ± 10.71###	72.74 ± 8.4###
ALP (U/L)	159.39 ± 8.88	597.69 ± 16.8***	185.53 ± 10.21###	259.16 ± 9.12###	206.15 ± 5.26###

PST: Phlorotannin-rich fraction of *Sargassum tenerrimum*. ALP: Alkaline phosphatase; SGPT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase. *** $P < 0.001$ vs. NDC group; ### $P < 0.001$ vs. DC group.

Effect of PST on gastrocnemius muscle oxidative stress markers

STZ and HFD significantly enhanced oxidative stress in gastrocnemius muscle by increasing MDA levels and reducing GSH and SOD levels, and CAT activity in diabetic rats. PST administration significantly lowered MDA levels and increased SOD, GSH, and CAT concentrations ($P < 0.001$), the same as metformin (Figure 7) in the treated rats. These findings showed that PST might exert its anti-diabetic effect by strengthening the body's natural antioxidant defences and protecting muscle tissue from oxidative stress-related damage. PST at 400 mg/kg showed intense antioxidant action in reducing the oxidative stress generated during the diabetes condition.

Effect of PST on TNF- α , a serum pro-inflammatory marker

There is growing evidence that prolonged activation of pro-inflammatory pathways in insulin-target cells may play a role in developing insulin resistance, obesity, and

related metabolic diseases, plus T2D (28). The levels of TNF- α in the serum of DC rats were observed to increase significantly. Both PST and metformin treatments significantly ($P < 0.001$) alleviated the TNF- α levels in diabetic rats, and the effect was more pronounced with PST at 400 mg/kg (Figure 8).

Histopathological analysis of pancreas and liver

Histopathological inspection of a diabetic rat's pancreas showed degeneration of pancreatic islets with reduced cell size and number and loss of pancreatic architecture. Inversely, the diabetic rats' pancreas, treated with PST and metformin, protected the islets from degenerating, improved cell proliferation, and repaired the pancreatic architecture (Figure 9). The liver histology of the diabetic control group rats showed moderate congestion and accumulation of lipids. Treatment with PST at 400 mg/kg and metformin for 21 days resulted in mild congestion and reduced accumulation of lipids in the liver (Figure 10).

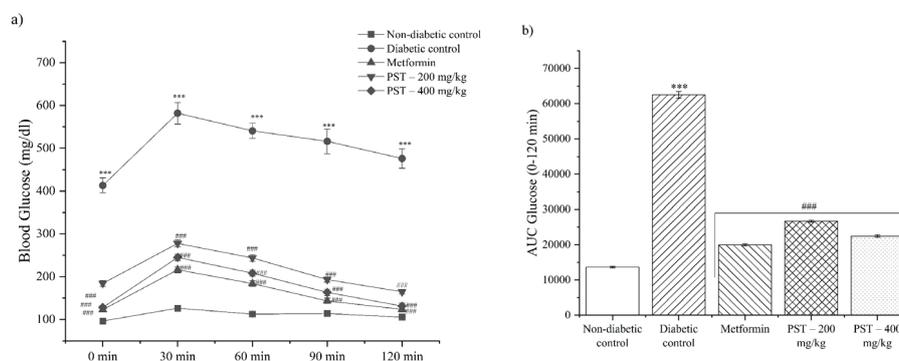


Figure 6. Effect of a phlorotannin-rich fraction of *Sargassum tenerrimum* on glucose tolerance in high-fat diet and streptozotocin-induced diabetic rats (n = 10). (a) OGTT blood glucose levels (b) OGTT area under the curve for glucose (AUC Glucose_{0-120 min}). OGTT: Oral Glucose Tolerance Test; AUC: Area under the curve. *** $P < 0.001$ vs. non-diabetic control group; ### $P < 0.001$ vs. diabetic control group.

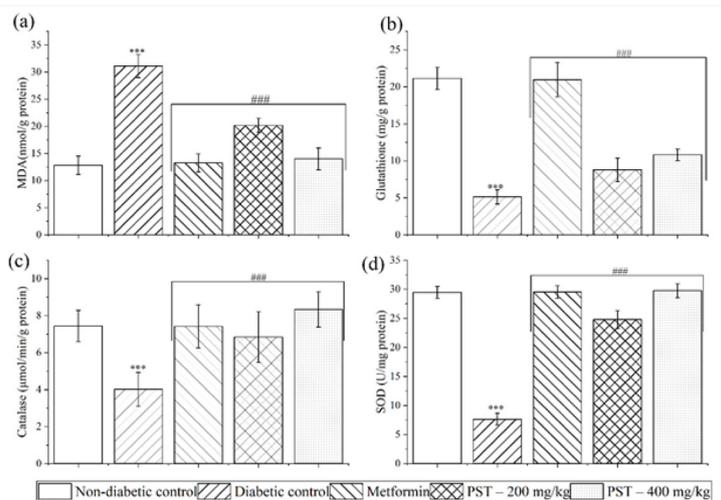


Figure 7. Effect of a phlorotannin-rich fraction of *Sargassum tenerrimum* on gastrocnemius muscle oxidative stress markers (n=10). a) Malondialdehyde (MDA) levels b) Glutathione levels c) Catalase levels and d) Superoxide dismutase (SOD) levels. PST: Phlorotannin-rich fraction of *S. tenerrimum*. *** $P < 0.001$ vs. non-diabetic control group; ### $P < 0.001$ vs. diabetic control group.

Discussion

This study is the first to demonstrate that giving HFD and STZ-induced diabetic rats a PST reduces blood glucose levels and oxidative stress and improves insulin sensitivity. Additionally, PST therapy improves renal, hepatic, and altered lipid levels in diabetic rats along with body weights. It also reduces the levels of the pro-inflammatory cytokine, TNF- α . The histopathological findings indicating the restoration of pancreatic and hepatic architecture further supported the results (Figure 11).

In the present study, the phlorotannin content of ST was higher in ethyl acetate fraction than in crude extract and other fractions, making it ideal for further evaluation. Based on the published literature, the phlorotannin

compounds from ethyl acetate extract of brown algae ST were identified using UPLC-MS/MS (Figure 1 and Table 3). Eckol was the main active ingredient, followed by Fuhalol, Trifuhalol, dibenzodioxins-1,3,6,8, tetraol, and Phlorethohydroxycarmalol. The obtained data were in agreement with a previous study in which 42 phlorotannin compounds of different molecular weights were separated and characterized from *Sargassum fusiforme* (Harvey) Setchell by UPLC-MS/MS technique (10). Likewise, *Fucus vesiculosus* ethyl acetate fraction was subjected to UPLC-MS/MS analysis, which resulted in discovery of the existence of fucophlorethols, fucols, fuhalols, and numerous other phlorotannin derivatives (13).

The effects of phlorotannin-rich fraction of ST on T2D were evaluated using an HFD (3 weeks) and low-dose STZ (35 mg/kg) induced diabetic Wistar rat model, which closely reflected the metabolic traits characteristics of human T2D, although no animal model is completely accurate. HFD causes insulin resistance, and STZ at low doses partially destroys β -cells, resulting in β -cell dysfunction (29). Furthermore, HFD-fed rats were already mildly hyperglycemic due to insulin resistance, making them more vulnerable to the diabetogenic effect of STZ (21). In our study, diabetic rats treated with PST for three weeks showed a decline in serum glucose with an improvement in serum insulin levels, and the effect was dose-dependent and higher at 400 mg/kg of PST. These results are substantiated by histopathological findings in the pancreas of diabetic rats treated with PST, which indicate a positive impact on the proliferation of β -cells and restoration of pancreatic cell architecture. Thus, PST by halting the HFD and STZ-induced degeneration of β -cells in diabetic rats and improving their number might have contributed to its insulinogenic action. The results are in line with those of Gheda et al, who found that giving

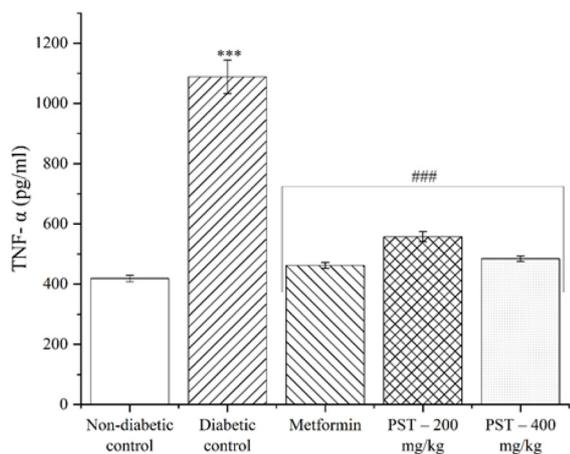


Figure 8. Effect of a phlorotannin-rich fraction of *Sargassum tenerrimum* on serum levels of TNF-alpha high-fat diet and streptozotocin-induced diabetic rats (n=6). PST: Phlorotannin-rich fraction of *S. tenerrimum*; TNF-alpha: Tumor necrosis factor-alpha. *** $P < 0.001$ vs. non-diabetic control group; ### $P < 0.001$ vs. diabetic control group.

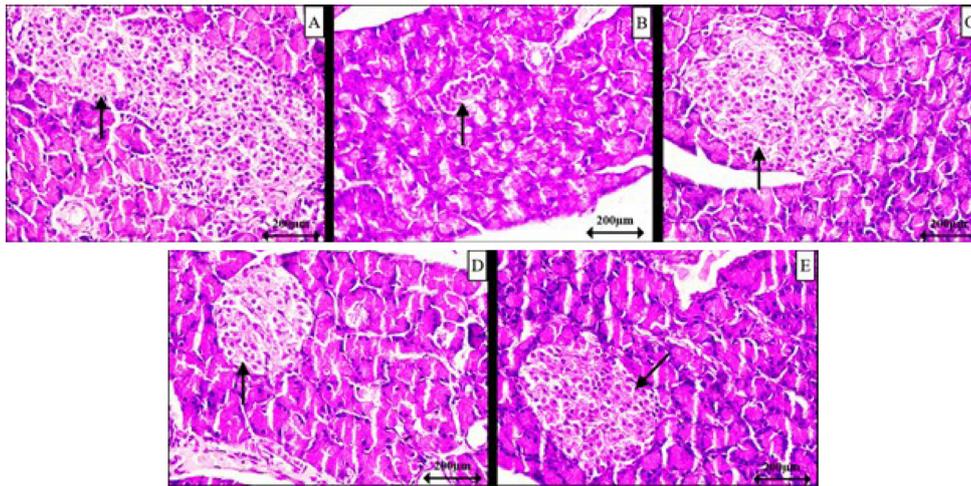


Figure 9. Histopathology of pancreatic tissue samples of diabetic rats treated with metformin and phlorotannin-rich fraction of *Sargassum tenerrimum* (PST) (100x). (A) Non-diabetic control group – arrow shows normal intact islets of the pancreas embedded in acinar cells; (B) Diabetic control group – arrow specifies atrophied, contracted pancreatic islets; (C) Metformin treated group – arrow denotes restored pancreatic islets with normal morphology; (D) PST 200 treated group – arrow shows partially repaired pancreatic islets; (E) PST 400 treated group – arrow shows recovered pancreatic islets with typical structure.

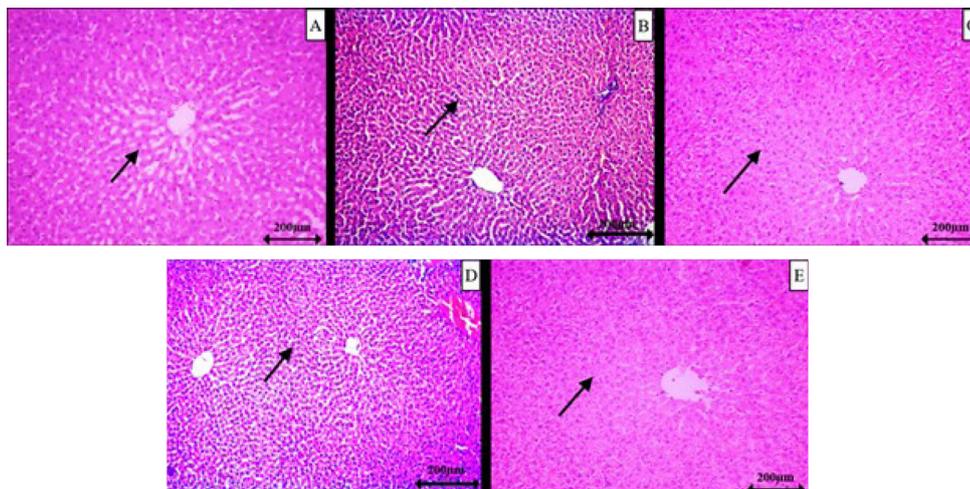


Figure 10. Histopathology of liver samples of diabetic rats treated with metformin and phlorotannin-rich fraction of *Sargassum tenerrimum* (PST) (100x). (A) Non-diabetic control group – arrow shows typical arrangement of hepatocytes surrounding a central vein; (B) Diabetic control – arrow indicates hepatic cellular congestion with lipid accumulations; (C) Metformin treated group – arrow indicates recovered hepatic architecture; (D) PST 200 treated group – arrow indicates mild congestion with few lipid accumulations; (E) PST 400 treated group – arrow indicates recovered hepatic architecture with no lipid accumulations.

diabetic rats, phlorotannins from the brown seaweed *Cystoseira compressa* orally for 28 days caused an increase in insulin levels, a drop in blood glucose levels, and the repair of pancreatic beta-cell damage (14). Additionally, Gotama et al found that diabetic rats receiving oral *Sargassum hystrix* extracts (SHE) for 15 days resulted in a prominent reduction in blood glucose levels and improvement in β -cell number and cell repair (30). Insulin resistance was also attenuated by PST in diabetic rats, as evidenced by the decrease in HOMA-IR values. Further, PST showed comparable efficacy to the standard anti-diabetic medication, metformin, in enhancing glucose tolerance in diabetic rats.

HFD has been a source of insulin resistance via various mechanisms, the most important of which is the glucose-fatty acid cycle (31). In summary, elevated triglyceride levels due to excessive fat consumption may be a cause of augmented fatty acid availability and oxidation. Increased fatty acid oxidation dampens the insulin-mediated decrease in liver glucose production and lowers glucose absorption or utilization in muscular tissues (32). Additionally, increased fat deposition as intramyocellular lipids in muscle tissue has been linked to decreased insulin sensitivity. As a result, dyslipidemia has emerged as an important biomarker for tracking the course of diabetes illness. Diabetic rats showed high serum triglyceride,

cholesterol, and LDL-C levels, with lower HDL-C levels in our study. PST treatment improved serum lipid profiles, with the effect being higher at 400 mg/kg. Hepatic histopathological alterations in DC rats, such as lipid buildup, are consistent with serum lipid modifications. In diabetic rats, PST treatment (200 mg/kg & 400 mg/kg) significantly diminished lipid buildup. Metformin treatment yielded similar results, and the effects of PST at 400 mg/kg was comparable to metformin.

A significant objective of biomedical research has been to stop or delay the evolution of renal disease because diabetic nephropathy is a chronic consequence of diabetes and one of the leading causes of death in people with diabetes mellitus (33). As STZ promotes pathological alterations to the kidney, elevated urea and creatinine levels are recognized as crucial indicators of renal dysfunction by hyperglycemia (34). The reduced levels of serum creatinine and urea in PST and metformin-treated diabetic rats could be understood by minimizing the negative impact on kidney function associated with STZ-induced diabetes.

Skeletal muscles accounts for more than 60% of consumed glucose absorption via insulin-mediated signaling pathways. As a result, skeletal muscle resistance to insulin signals plays a key role in the etiology of T2DM (35). Growing evidence suggests that the augmented oxidative stress brought on by ectopic lipid accumulation and hyperglycemia promotes muscle cell membrane lipid peroxidation, increases ROS production, impairs insulin signalling, reduces GLUT 4 transport to the plasma membrane, and ultimately results in insulin resistance (36). SOD, GSH, and CAT are vital antioxidant markers in our system that guard against oxidative damage. MDA is a by-product of the oxidation of polyunsaturated fatty acids in cells. Elevated MDA indicates increased lipid peroxidation, which leads to high amounts of oxidative stress and may contribute to the onset of diabetes (37). A striking decrease in antioxidant levels and an increase in MDA levels in diabetic rats in this study point to the production of oxidative stress brought on by hyperglycemia and elevated serum-free fatty acid levels. As a result, our findings indicate that PST can effectively prevent oxidative stress in the gastrocnemius muscles of diabetic rats, which may have resulted in the improved insulin sensitivity found in our study. Gheda et al found that *Cystoseira compressa* phlorotannin extracts significantly improved endogenous antioxidant defences in diabetic rats by increasing the activities of CAT and GSH and decreasing lipid peroxidation. Pathophysiology of oxidative stress and diabetes mellitus being closely related, phlorotannins found in marine brown algae can treat both (38).

Insulin resistance is characterized by low-grade systemic inflammation, correlated with increased plasma, adipose tissue, and skeletal muscle levels of TNF- α , a pro-inflammatory cytokine (39). Increasing insulin receptor

substrate-1's (IRS-1) serine phosphorylation, which turns IRS-1 into an inhibitor of insulin receptor tyrosine kinase activity, is thought to be how TNF- α promotes insulin resistance (40). IRS-1 is required for modulating insulin signal transduction along metabolic pathways such as GLUT4 translocation and skeletal muscle glucose uptake (41). In the study, treatment with PST and metformin resulted in attenuation of the increased serum TNF- α levels in diabetic rats suggesting its role in decreasing low-grade inflammation linked to diabetes. This indicates that PST, by diminishing TNF- α levels, may have modulated insulin signal transduction concerning metabolic response pathways, resulting in increased insulin sensitivity.

At the end of the study, DC rats showed a significant increase in body weight compared to the NDC rats despite administering the low dose of STZ. Both Gheibi et al and Srinivasan et al revealed a similar finding, confirming that diabetic rats induced with HFD and low-dose STZ have increased weight relative to their controls at the end of the experiment (4, 21). In contrast, PST and metformin significantly lowered body weight compared to the DC rats. The study results exhibited that improving serum lipid profile and insulin sensitivity by PST in diabetic rats might have reduced blood glucose levels. As a result, the experimental rats' food intake may have been regulated, leading to a decrease in the body weight of PST-treated rats in contrast to the DC rats.

The anti-diabetic activity of the PST might be due to the compounds identified in the fraction. Eckol, a chemical precursor illustrating the dibenzo-1,4-dioxin class of phlorotannins identified in the PST, includes phloroglucinol components connected in various ways (42). Eckol has inhibited α -glucosidase (43), α -amylase, rat lens aldose reductase, and advanced glycation end products formation *in vitro*. (44). Additionally, it improved glucose tolerance and lowered insulin levels, glycoalbumin, fructosamine, and fasting glucose in diabetic KK-A y mice (45). Eckol has also been reported to attenuate reactive oxygen species (ROS) (46), cellular lipid peroxidation, and expression levels of inflammatory cytokines, i.e., TNF- α , IL-1,6, and 8 (47). Additionally, Eckol has been shown to have anti-inflammatory (48), hepatoprotective (49), anti-obesity (50), and anti-hyperlipidemic (51) effects. Fuhadol compounds comprise ether-linked phloroglucinol units with an extra hydroxyl group, making that unit vicinal trihydroxylated. Fuhadol class of compounds were found to inhibit α -glucosidase, α -amylase and pancreatic lipase activities (13) and improve serum glucose and insulin levels in diabetic rats (14). Trifuhadol A extracted from *Agarum cribrosum* has inhibited cyclooxygenase-2, TNF- α , IL-6, and IL-1 β levels in lipopolysaccharide-stimulated RAW264.7 cells (52). Similarly, it reduced the signs of allergic inflammation in mice by dual inhibiting TAK1 and MK2 through the actions of IgE and IL-33 (53). Based on the previous reports of the biological activities of phlorotannin compounds, ST contains compounds

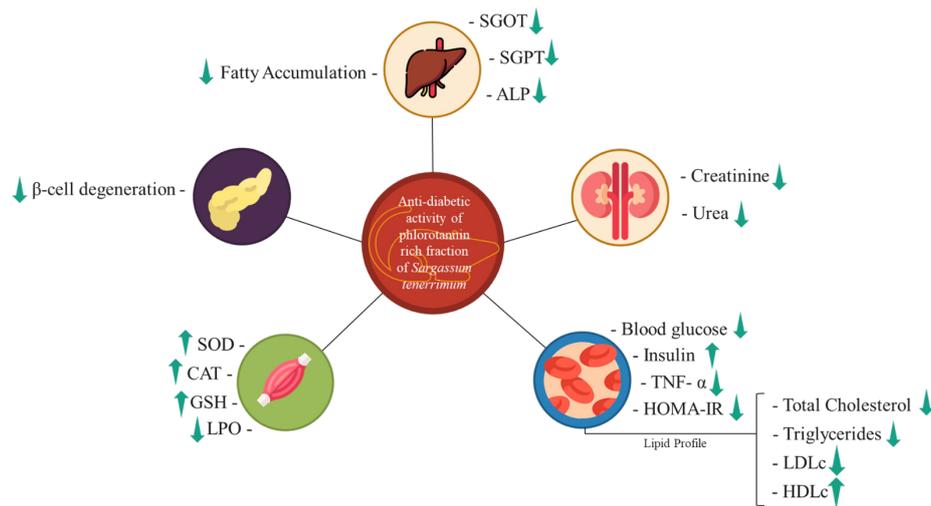


Figure 11. Effects of a phlorotannin-rich fraction of *Sargassum tenerrimum* on various tissues of high-fat diet and streptozotocin-induced diabetic Wistar rats.

accountable for the anti-diabetic activity, which were further confirmed by the preclinical *in vivo* studies.

Conclusion

The present study demonstrated that the PST had potent anti-diabetic potential by reducing blood glucose levels, improving insulin levels, and repairing damaged pancreatic β -cells. Furthermore, PST, which has antioxidant scavenging activity, could play a role in the algae's potential mechanism by aiding in the effective binding of insulin to its receptors, which in turn increase glucose uptake. Our study's findings suggest that ST phlorotannins consumption can help prevent T2D and diabetes-related complications. Further experiments on isolating phlorotannins from ST and investigating their effects on human cell line models are needed to elucidate the mechanism of action and move forward with clinical trials involving such natural drugs to treat T2D.

Authors contributions

NGV: Literature synthesis, methodology, data curation, writing original draft & editing. CV: Conceptualization, reviewing draft, supervision. All authors have read and agreed to the published version of the manuscript.

Conflict of interests

The authors have no relevant financial or non-financial interests to disclose.

Ethical considerations

The study protocol was approved by the institutional animal ethics committee of DAC Regional research institute for Homeopathy, Kolkata (Reg. No.: 2055/GO/RBi/S/19/CPCSEA), with no. DACRRIH/CPCSEA/IAEC/2022/02.

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