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Protective role of marine macroalgae extracts against STZ induced diabetic rats

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ABSTRACT

Objective: To study the anti-diabetic activity of marine macroalgae extracts ($n = 31$), purification and characterization of sulphated galactopyran (SGP) from *Gracilaria opuntia* (FM4) in diabetic rats.
Methods: The animals were separated into groups and STZ (55 mg/kg body weight) was used to induce diabetics. Glucose, HbA1c, insulin, C-peptide levels and *in vivo* antioxidant levels were estimated and histopathological studies were done in STZ-induced diabetic and marine macroalgae treated rats.
Results: Based on glucose and HbA1c levels and *in vivo* antioxidant levels, among the 31 marine macroalgae extracts, FM4 has showed high anti-diabetic activity. Hence, FM4 was purified and characterized by ^{1}H-NMR spectra and FT-IR as sulphated galactopyran. During the survival analysis, SGP at dose of 100 mg/kg showed significant ($P < 0.05$) survival rate and elevations in C-peptide and insulin levels. The histopathological modulations of SGP were observed in diabetic rat tissues such as liver, kidney and brain. Hence obtained results reveal that SGP treated diabetic rats has significant changes in C-peptide and insulin levels which regulates the blood glucose levels and recovered the histopathological changes.
Conclusions: Marine macroalgae have significant anti-diabetic activity. Hence, they could be used as nutraceutical supplement or natural green remedy against diabetes mellitus.

1. Introduction

Diabetes mellitus (DM) is a group of chronic metabolic disorders leading to hyperglycemia. Globally, estimated occurrence of diabetes and projection for the year 2040, as given by International Diabetes Federation (IDF) is 642 million[1,2]. DM is a major risk factor during cardiovascular disorders such as cerebral stroke, ischemic disease and peripheral artery disease that increased mortality during diabetics[3]. DM was characterized by persistent hyperglycemia with changes in carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both[4]. The long term effects of chronic DM include progressive development on the specific complications like retinopathy, nephropathy, neuropathy and cardiovascular diseases[5].

According to Indian Council of Medical Research (ICMR), it has been recently identified that DM is one of the refractory disease in which allopathic system of medicine is not satisfactory, therefore investigations on herbal medicines are preferred for treating DM[6]. Herbal treatments for diabetes have been used in patients with insulin dependent and non-insulin dependent diabetes. Scientific validation of several Indian plant species (nearly 45 000) has proved to be effective in reducing the sugar levels, with different mode of action and phytoconstituents[7]. Seaweeds are group of non-flowering marine plants commonly known as macroalgae having increased demand in cosmetic, pharmaceutical and food additives[8,9]. They are photosynthesizing organisms in marine environment and produce the basic biomass in the intertidal zone and have a rich source of bioactive compounds, such as carotenoids, proteins, oligosaccharides, essential fatty acids, antioxidants, vitamins and minerals useful in medical and pharmaceutical industries[10-12].

Anti-diabetic, anti-inflammatory and anti-hypertensive effects have been reported from salt-resistant marine halophytes and

marine algae. This is due to the presence of oligosaccharides, glycolipids, phenolic compounds and small molecular weight bioactive compounds and their effect was proved in experimental animals[13,14]. Marine algae produce a wide range of new secondary metabolites with various biological activities which may have potential applicability in the field of pharmaceutical industry to provide a stable platform for medicine industries[15]. Several bioactive compounds from red algae such as *Rhodomela confervoides*, *Symphyocladia latiuscula*, *Polysiphonia urceolata* and brown marine algae have been experimentally tested for their biomedical efficacy[16-18].

In the sight of above information, the current study endeavors to (a) evaluate the anti-diabetic properties of the aqueous, ethanolic/methanolic extracts, polysaccharide and oligosaccharide conjugates (*n* = 31) derived from different red and brown seaweeds; (b) analyze the antioxidant enzymes and histopathological modulations during streptozotocin (STZ) induced diabetes; (c) investigate characterization of sulphated galactopyran from *Gracilaria opuntia* (*G. opuntia*) and its efficacy against diabetes.

2. Materials and methods

2.1. Chemicals

STZ, butylated hydroxyl toluene (BHT), thiobarbituric acid (TBA), tris base, ethylene diamine tetra acetic acid (EDTA), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), dinitrophenyl hydrazine (DNPH), glutathione (GSH), trichloro acetic acid (TCA), NADPH, triton X-100, reduced glutathione (GR), oxidised glutathione (GSSG), nitro blue tetrazolium chloride (NBT), epinephrine, tetra butyl hydroperoxide, 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Himedia (Bangalore, India) and Sigma Aldrich (St. Louis, Missouri, USA).

2.2. Collection of marine macroalgae material

Brown marine macroalgae used in this study were *Turbinaria conoides* (*T. conoides*), *Sargassum wightii* (*S. wightii*), *Sargassum myriocystum* (*S. myriocystum*), *Padina gymnospora* (*P. gymnospora*), *Padina tetrastomatica* (*P. tetrastomatica*), and *Turbinaria ornata* (*T. ornata*) (Table 1). The red marine macroalgae were *G. opuntia*, *Kappaphycus alvarezii* (*K. alvarezii*), *Laurencia papillosa* (*L. papillosa*), *Jania rubens* (*J. rubens*), *Hypnea musciformis*, and *Hypnea valentiae*. The identities of the marine macroalgae considered in the present study were ascertained with the sample specimens maintained in the Marine Biodiversity Museum of Central Marine Fisheries Research Institute. The marine macroalgae were collected freshly from the Gulf of Mannar in Mandapam region located between 8°48' N, 78°9' E and 9°14' N, 79°14' E on the south east coast of India during the months spanning between August and April. Samples collected (2 kg) were washed in running water and shade dried before being pulverized to a minimum particle size.

Table 1
Solvent (methanol, ethanol) and aqueous extracts of red and brown seaweeds.

Extracts	Sample code	Genus name	Sample code	Genus name
		Red seaweeds		Brown seaweeds
Aqueous				
	FS4	*G. opuntia*	PS1	*T. conoides*
	FS1	*K. alvarezii*	A1	*S. wightii*
	EM2	*L. papillosa*	A2	*S. myriocystum*
	EM3	*J. rubens*	G1	*P. gymnospora*
	FM1	*K. alvarezii* (Polysaccharide conjugate*)	EM9	*P. tetrastomatica*
	FM4	*G. opuntia* (Oligosaccharide conjugate*)	S3	*T. ornata*
Methanolic				
	F1	*G. opuntia*	SF1	*T. conoides*
	K1	*K. alvarezii*	PH1	*S. wightii*
	MV1	*L. papillosa*	S2	*S. myriocystum*
	MP1	*J. rubens*	F1	*P. gymnospora*
	FS2	*Hypnea musciformis*	S1	*P. tetrastomatica*
	EM1	*Hypnea valentiae*	S4	*T. ornata*
Ethanolic				
	FM2	*G. opuntia*	S7	*S. wightii*
	FM3	*K. alvarezii*	AE3	*T. conoides*
	FS5	*L. papillosa*	SF	*S. myriocystum*
			S1	*T. ornata*

*Polysaccharide and oligosaccharide conjugates of seaweeds were derived from the ethanol precipitated residue of the supernatant of the aqueous extracts. For detailed description the methodology section to be referred.

2.3. Preparation of marine macroalgae extracts

The powdered marine macroalgae samples (1 000 g) were extracted with *n*-hexane (600 mL × 2), at room temperature for 24 h, and the pigments were separated. The residue was filtered through Whatman No. 1 filter paper and extracted three times with methanol (MeOH) and ethanol (EtOH) (50–60 °C, 3 h), respectively, filtered through Whatman No. 1 filter paper, and the obtained filtrate was concentrated at 50 °C in rotary vacuum evaporator (Heidolf, Germany) to get the dark brown viscous mass of MeOH (112 g) and EtOH fractions (96 g), respectively. The aqueous extracts of the seaweed were prepared by extracting the dried marine macroalgae powder (500 g) with 80–90 °C hot water for 3–4 h. The contents were thereafter cooled (4 °C) and centrifuged at 8 500 r/min for 15 min (Sorvell Biofuge Stratos, Thermo Scientific, USA) to remove the solid residues that were freeze-dried to get the crude aqueous extract (27 g). By using rotational vacuum concentrator (Martin Christ RVC 2-33 IR, Martin Christ, Germany) the latter of *K. alvarezii* (FM1) was condensed upto 1/4th of the original volume, cooled, and incubated at 4 °C for overnight by adding three volumes of ethanol for the precipitation of polysaccharide conjugates. The aqueous extract of *G. opuntia* was concentrated before precipitated with alcohol (500 mL). The precipitate was lyophilized to get a dried oligosaccharide fraction of *G. opuntia* (FM4). This was then powdered and packed in vacuum packed bags and stored in refrigerator until further use.

2.4. Purification and characterization of oligosaccharide fraction of G. opuntia

The precipitated oligosaccharide conjugate of *G. opuntia* (FM4)

was purified by anion exchange column chromatography using DEAE (diethylamineoethyl)-cellulose anion exchange resin. A glass column (25 cm × 4 cm) with a mesh sieve was mounted vertically on a stand and rinsed with water. DEAE-cellulose (3 g) was made into slurry with minimum amount of water and loaded into the column. The polysaccharide fraction (1 g) was dissolved in 2–3 mL of water to make a suspension and loaded into the previously packed column. The column was initially eluted with water to get the first fraction. This was followed by step-wise elusion with 0.1, 0.3, 0.5 and 1 mol/L sodium chloride (NaCl) gradient until the absence of positive reaction of the phenol-sulphuric acid assay in the test tubes containing eluted fractions. The eluted fractions were lyophilized to get the purified oligosaccharide fractions. The oligosaccharide enriched fraction was referred as sulphated galactopyran and the motif was characterized from *G. opuntia* by using fourier-transform infrared (FT-IR) (Perkin-Elmer 16 PC spectrometer, Boston, USA) and nuclear magnetic resonance (NMR) spectroscopy[19,20].

2.5. Maintenance of animals

The study was carried out on male albino Wister rats (*Rattus norvigicus*) weighing (190 ± 10) g selected as experimental animals, which were housed in cages separately. The animals were purchased at Sri Venkateswara Enterprises Pvt. Ltd. (Bangalore, Karnataka, India) and maintained at room temperature of (23 ± 2) ℃, humidity of 45%–55% with 12 h light-dark cycle and allowed to eat and drink. The rats were fed with standard rodent diet during the experimental procedure (Sri Venkateswara Enterprises Pvt. Ltd., Bangalore, Karnataka, India). All experimental procedures involving animals were conducted in accordance to CPCSEA guidelines and approved by Institutional Animal Ethical Committee (IAEC) of PES Medical College, Kuppam, India (Vide No. PESIMSR/Pharma/IAEC/20/2014-2015/date 08.12.14).

2.6. Preparation of STZ

55 mg of STZ was dissolved in 100 mmol/L cold sodium citrate buffer (pH 4.5)[21].

2.7. Induction of hyperglycemia and treatment

After acclimatization of the rats to the lab conditions the treatment was started. Diabetes was induced by a single intraperitonial administration of STZ (55 mg/kg body weight). Control rats received the buffer as vehicle alone. After inducing diabetes 10% glucose water was given in the night to protect the rats from sudden hypoglycemic period that occurs due to pancreatic islets cells lysis by STZ[22]. After 48 h of development of diabetes the rat's glucose levels were checked in fasting by using Accu-check meter (Roche Group, Mannheim, Germany) developed from the basic method of glucose oxidase[23] and HbA1c method[24]. Glucose levels are more than 200 mg/dL and HbA1c levels more than 6.3% were considered as diabetic rats and used for the experiment[21,24].

2.8. Experimental design

2.8.1. Treatment with crude marine macroalgae extracts in STZ induced rats

The treatment was initiated after inducing diabetes with STZ by selecting different marine macroalgae extracts (*n* = 31). The animals were separated into 5 groups with 5 rats in each group. The experimental design is as follows:

Group-1: healthy control, Group-2: diabetic control (STZ), Group-3: diabetic + glibenclamide, Group-4: diabetic + marine macroalgae extracts at 175 mg/kg body weight (*n* = 31 extracts), Group-5: diabetic + marine macroalgae extracts at 125 mg/kg body weight (*n* = 31 extracts).

2.8.2. Treatment with sulphated galactopyran (SGP) of G. opuntia

The treatment of sulphated galactopyran of *G. opuntia* (FM4) extract was initiated after inducing diabetes with STZ in the rats. The animals were separated into 9 groups with 6 rats in group. The experimental design is as follows:

Group-1: Control, Group-2: FM4 alone treated, Group-3: STZ, Group-4: STZ + glibenclamide, Group-5: STZ + 60 mg/kg body weight, Group-6: STZ + 80 mg/kg body weight, Group-7: STZ + 100 mg/kg body weight, Group-8: STZ + 125 mg/kg body weight and Group-9: STZ + 150 mg/kg body weight.

2.9. Determination of ED_{50} and survival analysis of G. opuntia

Rats were divided into groups and the SGP of *G. opuntia* was administered orally by following a standard method[25]. Survival rate was observed continuously for 60 days to detect the mortality rate and behavioral changes. After induction of diabetes, a group was treated with saline and maintained as control. Based on the preliminary toxicity tests, the doses of 60, 80, 100, 125 and 150 mg/kg body weight of *G. opuntia* (FM4) extract were chosen for further experiments. By using the graphpad prism (6.07) the Kaplan-Maier survival analysis was estimated. ED_{50} was calculated by recording the mortality rate during the treatment by Miller and Tainter method[26].

2.10. Sample collection for analysis

The blood samples were collected weekly once from the retro-orbital vein and used for the estimation of blood glucose levels by Accu-check method (Roche Group, Mannheim, Germany) developed from the basic method of glucose oxidase method[23]. The glycosylated hemoglobin was estimated by HbA1c method[24].

2.11. Isolation of tissues

After treatment, rats were sacrificed by cervical decapitation and liver, kidney and brain tissues were collected and rinsed of any adhering blood. The tissues fragments were homogenated in phosphate buffers saline for further studies. The unused tissues were stored at –40 ℃ refrigerator for further analysis.

2.12. Biochemical analysis

The superoxide dismutase[27], catalase[28], glutathione peroxidase[29], glutathione *S*-tansferase[30], reduced glutathione[31], lipid peroxidation[32] activity levels were resoluted in liver, kidney and brain tissues. Insulin and C-peptide were estimated by using the INVITRO 250 automated clinical chemistry analyser (Ortho clinical diagnostics, Bangalore, India) from the blood sample of diabetic and treated rats.

2.13. Histopathology

For histological examination, tissues like liver, kidney and brain were excised by euthanizing the rats after treatment. The tissues were fixed in 10% formaldehyde and dehydrated in 50%–100% ethanol, cleared in xylene and paraffin embedding. The sections (10 µm) were stained with haematoxylin and eosin (H & E) dye and examined under microscope.
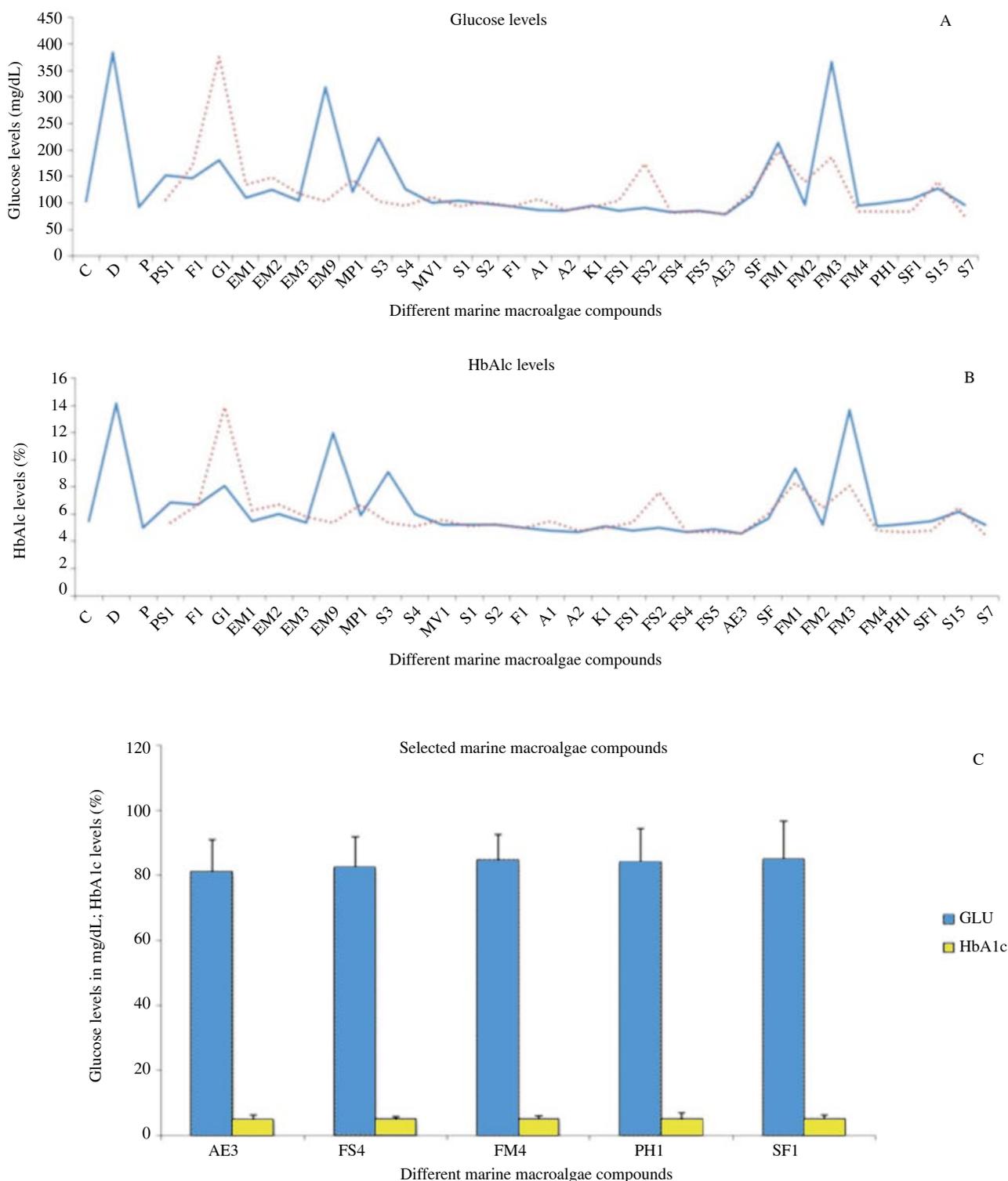
Figure 1. Estimation of (A) blood glucose levels, (B) HbA1c levels in different experimental groups treated with extracts of different marine macroalgae, and (C) selected 5 potential bioactive extracts.
Male wister albino rats were injected with 55 mg/kg body weight of STZ to induce diabetes. The induction was confirmed after 48–72 h by measuring the blood glucose and HbA1c levels. The average glucose and HbA1c levels were ploted in the graphs. The marine macroalgae extracts were given to different experimental groups based on the solubility. C: Healthy control, D: Diabetic control, P: Positive control treated with glibenclamide (20 mg/kg body weight). The remaining groups were treated with different marine macroalgae extracts (*n* = 31) with two different concentrations such as 175 mg/kg body weight (solid line) and 125 mg/kg body weight (dotted line). Among these two concentrations, 125 mg/kg body weight showed more efffect on reducing glucose and HbA1c levels in STZ induced rats. (C) Five potential bioactive extracts were selected based on the glucose and HbA1c levels during STZ-induced diabetes.

2.14. Statistical analysis

The obtained data were analyzed by using the statistical package of social sciences (SPSS, 16.0 version). Comparison between the control and experimental animals results were done by One-way ANOVA followed by Tukey's multiple comparison test. $P < 0.01$ was considered as significant difference. All the values were expressed as mean ± SD ($n = 5$).

3. Results

In the present investigation a total of thirty-one different marine macroalgae extracts were selected for screening of the anti-diabetic properties in STZ induced diabetic rats (Table 1). In earlier reports, some of the red and brown marine algae have showed significant recovery at 50, 100, 125, 150 and 200 mg/kg body weight in STZ induced diabetic rats. Hence, 125 and 175 mg/kg concentrations of marine macroalgae were selected to treat the STZ induced diabetic rats in our studies (Table 1). The present study demonstrates that all the marine macroalgae extracts showed recovery in altered blood glucose and HbA1c levels in STZ induced diabetic rats (Figure 1A

and 1B). Among the 31 marine macroalgae, ethanolic extract of *T. conoides* (AE3, 84.4 ± 8.2 mg/dL; 4.6% ± 0.2%), aqueous extract of *G. opuntia* (FS4, 82.2 ± 9.7 mg/dL; 4.7% ± 0.3%), oligosaccharide extract of *G. opuntia* (FM4, 84.4 ± 8.2 mg/dL; 4.8% ± 0.1%), methanolic extracts of *S. wightii* (PH1, 83.8 ± 10.7 mg/dL; 4.7% ± 0.3%) and *T. conoides* (SF1, 84.8 ± 11.9 mg/dL; 4.80% ± 0.21%) showed significant ($P < 0.01$) reduction in glucose and HbA1c levels in 125 mg/kg body weight treated groups compared to diabetic group (383.7 ± 42.3 mg/dL; 14.2% ± 1.3%).

Among 31 marine macroalgae extracts AE3, FS4, FM4, PH1 and SF1 were selected. The five compounds were selected mainly based on the blood glucose and HbA1c levels (Figure 1C). The perturbations of the *in vivo* antioxidant enzyme levels were studied by using the selected five potential extracts during STZ induced diabetic rats.

The modulations of *in vivo* antioxidant enzyme levels of STZ induced diabetic and marine macroalgae extracts (AE3, FS4, FM4, PH1 and SF1) treated rat tissues such as liver, kidney and brain were evaluated (Figure 2). The antioxidant enzyme levels such as SOD (3.01 ± 0.19, 4.65 ± 1.03, 0.98 ± 0.28 IU/mg of protein), CAT (63.08 ± 6.13, 59.55 ± 5.72, 8.58 ± 1.24 μmol of H_2O_2 metabolised/mg
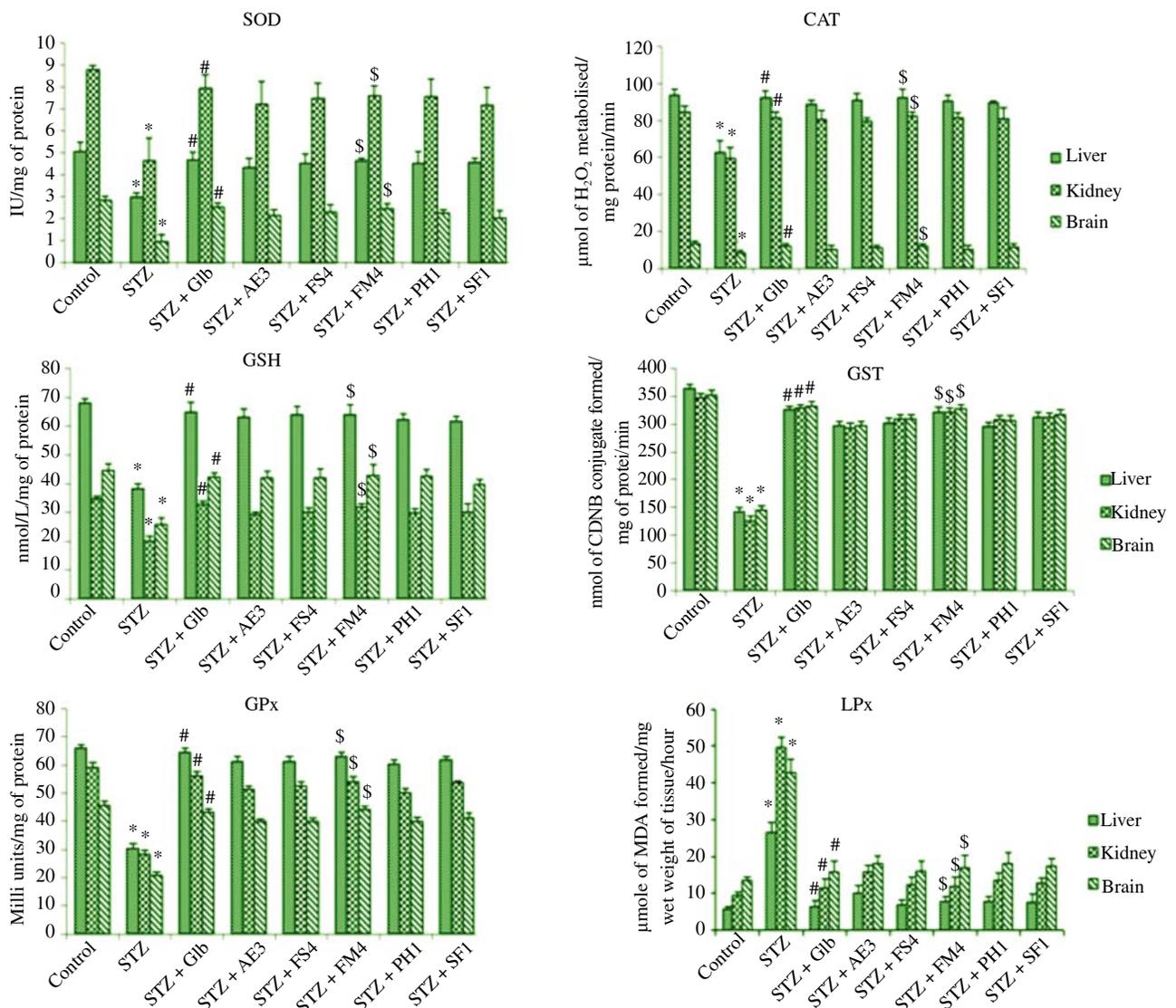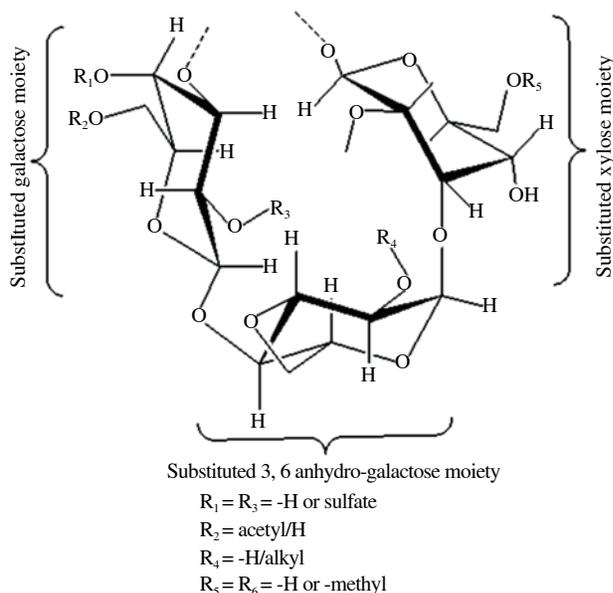

Figure 2. Analysis of marine macroalgae compound on antioxidant enzyme activities in liver, kidney and brain tissues of STZ induced diabetic rats. After induction of diabetes by STZ, the SOD, CAT, GPx, GST, GSH levels were decreased and MDA levels were increased compared to normal rats. In the treatment with the five potential macroalgae extracts, the enzymes activities were recovered in STZ-induced diabetes compared to their controls. Data were expressed in mean ± SD ($n = 6$). *: Comparison between healthy control and diabetic control (STZ), $P < 0.01$. #: Comparison between STZ + Glb and FM4 $P < 0.01$. $: Comparision between STZ and FM4 $P < 0.01$. There is no significance between STZ + Glb and FM4 treated groups.

protein/min), GPx (30.42 ± 0.68, 28.42 ± 0.39, 20.86 ± 1.05 Milli units/mg of protein), GST (142.48 ± 6.56, 126.42 ± 8.46, 146.23 ± 6.76 nmol of CDNB conjugate formed/mg of protein/min), GSH (38.41 ± 1.69, 26.02 ± 2.13, 20.20 ± 1.72 nmol/L/mg of protein) were significantly ($P < 0.01$) decreased, at the same time as the levels of malondialdehyde (MDA) in LPx (26.58 ± 2.77, 49.63 ± 2.86, 42.9 ± 3.62 μmole of MDA formed/mg wet weight of tissue/h) activity in STZ induced diabetic liver, kidney and brain tissues were increased respectively. The AE3, FS4, FM4, PH1 and SF1 (125 mg/kg body weight/day) recovers the altered enzyme activities significantly in diabetic rat tissues. Among these five treatments, oligosaccharide extract of *G. opuntia* (FM4) showed greater ability in reducing the altered levels of antioxidant enzymes activities such as SOD (46.65 ± 0.11, 7.59 ± 0.46, 2.48 ± 0.21 IU/mg of protein), CAT (92.66 ± 4.23, 82.68 ± 2.08, 11.96 ± 1.32 μmol of H_2O_2 metabolised/mg protein/min), GPx (63.26 ± 0.20, 54.28 ± 0.68, 44.26 ± 0.20 Milli units/mg of protein), GST (321.68 ± 8.98, 322.46 ± 6.76 nmol of CDNB conjugate formed/mg of protein/min, 328.46 ± 6.76), GSH (64.14 ± 3.27, 43.07 ± 3.53, 31.96 ± 0.98 nmol/L/mg of protein) and LPx (7.02 ± 1.43, 12.12 ± 2.26, 16.12 ± 3.20 μmole of MDA formed/mg wet weight of tissue/h) in liver, kidney and brain tissues respectively (Figure 2).

Further studies were carried out on oligosaccharide conjugate derived from *G. opuntia* (FM4) because of its high anti-diabetic activity, based on *in vivo* antioxidant enzyme activities, glucose, HbA1c levels and solubility of the extracts. Hence FM4 extract is purified and characterized.

The oligosaccharide fraction of FM4 from the red marine macroalgae *G. opuntia* (FM4) was isolated by column chromatographic method and followed by step-wise elusion with sodium chloride gradient until the dearth of a positive reaction of the phenol-sulphuric acid assay. The eluted fractions were lyophilized to get the purified oligosaccharide fractions. The carbohydrate enriched fraction re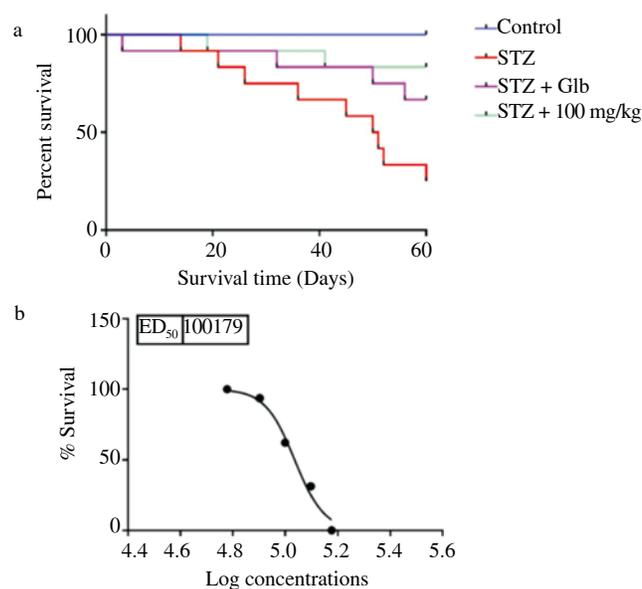ferred as sulphated polygalactan. The structure of the sulphated polygalactan was elucidated by the extensive analysis of ¹H-NMR spectra. The polysaccharide fraction has resolved signals of anomeric protons (δ 4.4 – 5.5), methylene and methane hydrogens (δ 3.6 – 4.9) of the sulphated polygalactan moiety in ¹H-NMR spectra. Characteristic signal at δ 3.4 has revealed the region of additional number of alkoxy (-OCH₃) replacements in the sulfated polygalactans. In addition to the characteristic peaks for sulphated polygalactan units, xylose and anhydrogalactose units were in minor constituents in the fraction, which were often found in red seaweed polysaccharides. The presence of positively and negatively charged ions of sulphate groups in the sulphated galactan moiety has sustained the structure. The FT-IR spectra demonstrated a strong absorption band at 1 210–1 260 cm⁻¹ corresponds to S=O groups (sulphate ester groups). Characteristic bands for –OH groups at 3 200–3 400 cm⁻¹ has substantiated the structure of the sulphated polygalactan (Figure 3).

The survival analysis results demonstrated that the survival percentage in control (100%), STZ (25%), STZ + Glb (66.6%) and STZ + 100 mg/kg (83.3%) was observed (Figure 4a). STZ + FM4 100 mg/kg has shown more survival rate compared to other treated groups. The SGP of *G. opuntia* when orally administrated in the possible doses of 60, 80, 100, 125 and 150 mg/kg in STZ induced diabetic rats, the mortality rate was obtained. Among five concentrations, 100 mg/kg body weight has shown significant survival rate (83.3%) compared to STZ (25%) group (Figure 4a).

The ED₅₀ of sulphated galactopyran (SGP) of *G. opuntia* was studied in STZ induced diabetic rats. The rats treated with five different concentrations such as 60, 80, 100, 125 and 150 mg/kg body weight. The present study showed that all the concentrations have significant recovery compared to STZ group. Based on the survival analysis, among the five concentrations 100 mg/kg body weight has shown significant ($P < 0.05$) recovery compared to the other treated groups (Figure 4a). ED₅₀ was measured for SGP as 100 mg/kg body weight based on glucose and HbA1c levels respectively,

Figure 3. Structural representation of galactopyranan motifs of the sulfated galactan from *G. opuntia*.

The sulphated galactopyran motif of *G. opuntia* was designated as →3)-4-O-sulfonato-(6-O-acetyl)-β-D-galactopyranosyl-(1→4)-3,6-anhydro-(2-Osulfonato)-α-D-galactopyranosyl-(1→3)-4-O-sulfonato-(6-O-acetyl)-β-D-xylosyl-(1→3)-4-Osulfonato-(6-O-acetyl)-β-D-galactopyranosyl-(1→4)-3,6-anhydro-(2-O-sulfonato)-α-D galactopyranan.


Figure 4. Survival analysis of STZ-induced diabetic rats after treatment with SGP of *G. opuntia* (FM4) extract.

(a) Kaplan–Meier estimates of survival between healthy, STZ and SGP treated groups. Survival percentage of groups calculated was as control (100%), STZ (25%), STZ + Glb (66.6%) and STZ + 100 mg (83.3%). Thus, 100 mg/kg treated group has potentially reduced the STZ-induced diabetes compared to STZ group ($n = 12$). (b) Determination of effective dose (ED₅₀) for SGP: STZ-induced diabetic rats were treated with different concentrations of SGP (60, 80, 100, 125, 150 mg/kg body weight). ED₅₀ was determined by survival rate of the animals, glucose and HbA1c levels. ED₅₀ of SGP was determined as 100 mg/kg body weight ($n = 6$).

in STZ induced diabetic rats (Figure 4b). After determining ED_{50} for SGP as 100 mg/kg body weight, the perturbations of the biochemical changes were studied by using the selected concentration *i.e.*, 100 mg/kg body weight during STZ induced diabetic rats.

The insulin and C-peptide levels were significantly ($P < 0.01$) decreased in STZ induced diabetic rats (15.5 ± 1.96 μU/mL; 2.14 ± 0.56 ng/mL) compared to control rats (26.84 ± 2.46; 6.68 ± 0.26). Upon treating with SGP (100 mg/kg body weight) the insulin and C-peptide levels were altered (22.46 ± 2.04; 4.72 ± 0.48) (Figure 5). Based on the insulin and C-peptide levels, the results duly revealed that the SGP of *G. opuntia* showed greater anti-diabetic properties. Hence SGP treated animal tissues were selected for further histopathalogical studies.

Histopathalogical changes in liver, kidney and brain tissues of diabetic and treated (100 mg/kg body weight) rats are shown in Figure 6. The disordered structure of liver in diabetic rats was observed because of necrosis of hepatocells, extensive vacuolization with disappearance of nuclei and microcellular fatty changes (Figure 6A–6C). In diabetic rat kidney, uneven thickening of the basement membrane, endolysis, local fusion of the foot process of the podocytes and glomerular capillary endothelium swelling were observed (Figure 6D–6F). In brain moderate glial shrubberies, necrosis of multifocal neurons and deterioration of cells were observed in diabetic rats. However these changes were reestablished with SGP derived from *G. opuntia* compound treatment (Figure 6G–6I).
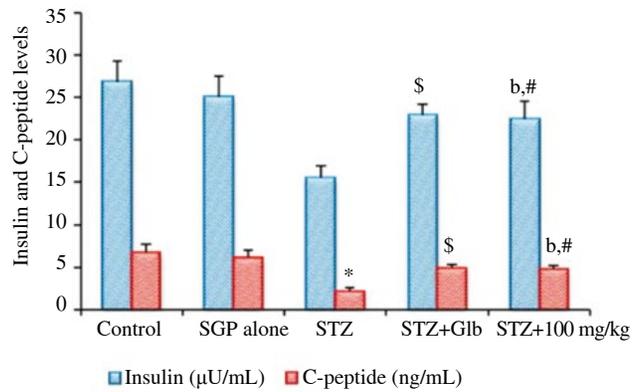
Figure 5. Effect of SGP of *G. opuntia* (FM4) on insulin and C-peptide levels of normal and STZ induced diabetic rats.

Insulin and C-peptide levels were decreased in STZ induced diabetes. After treating with SGP at 100 mg/kg body weight, it has shown the significant recovery compared to STZ groups. There was no significant change in SGP alone treated group compared to control group. Data were expressed as mean ± SD of 6 individual observations. *: Comparison between control and STZ group ($P < 0.01$). $: Comparison between STZ and STZ + Glb ($P < 0.01$). b: Comparision between STZ + Glb and SGP + 100 mg/kg($P < 0.01$). #: Comparision between STZ and SGP (100 mg/kg).
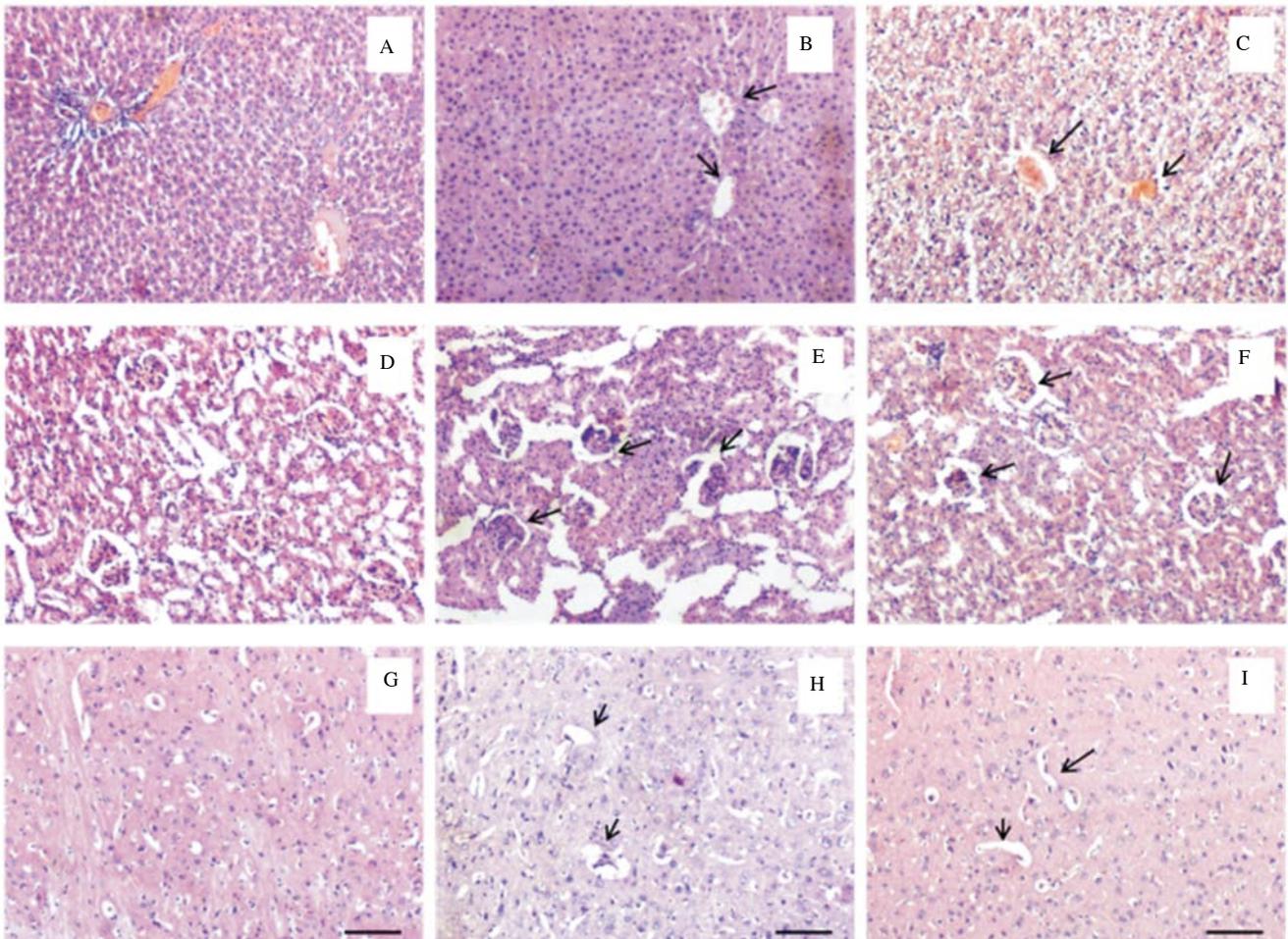
Figure 6. Photomicrographs of liver, kidney and brain.
A: Control; B: Diabetic control; C: Diabetic + SGP (100 mg/kg body weight) treated in liver tissue, showing degradation of hepatocytes and necrosis of cells in diabetic control and recovered by treating with SGP. D: Control; E: Diabetic control; F: Diabetic + SGP (100 mg/kg body weight) treated in kidney, showing mesangial accumulation and necrosis in diabetic control and this was regenerated after treating with SGP. G: Control; H: Diabetic control; I: Diabetic + SGP (100 mg/kg body weight) treated in brain, showing multifocul neural necrosis and moderate glialshrubberies and degeneration of cells in diabetic control and this was recovered with the SGP compound treatment. Scale bar = 10 μm; Lens = 10×.

4. Discussion

To study the diabetogenic activities, STZ induced diabetic rats serves as an useful experimental model[33]. In the present study the effect of marine macroalgae extracts in STZ induced diabetic rats were revealed and oligosaccharide conjugate of *G. opuntia* (FM4) was purified and characterized.

STZ induced hyperglycemia is mainly due to the depletion of insulin producing β-cells of pancreas which leads to reduction in insulin secretion[34]. Our results demonstrated that the increased blood glucose and HbA1c levels were significantly decreased upon treatment (125 mg/kg body weight) with 31 marine macroalgae extracts (Figure 1A and 1B). This might be due to regeneration of pancreatic β-cells and increases of the insulin secretion that directly reflects on conversion of stored glucose to glycogen[35]. This was previously reported in some of the marine algae such as *Sargassum ringgoldianum*, *S. wightii* and *Ulva fasciata*[36,37]. The free amino group of hemoglobin (Hb) reacted with glucose is a non-enzymatic reaction that produces HbA1c (glycated haemoglobin)[38]. The decrease in Hb and increase in HbA1c is directly proportional to the blood glucose levels[39]. Decrease in HbA1c levels might be due to improved glycemic control produced by marine macroalgae extracts (Figure 1B). Mohapatra *et al.*[37] and El-Desouki *et al.*[40] had been reported that HbA1c levels were decreased upon treating with *Ulva fasciata* and *Spirulina*. Of the tested 31 marine macroalgae extracts, the ethanolic extract of *T. conoides* (AE3), methanolic extract of *S. wightii* and *T. conoides* (PH1 and SF1), aqueous extract of *G. opuntia* (FS4) and oligosaccharide conjugate of *G. opuntia* (FM4) have showed more activity in reducing the STZ induced diabetes in rats (Figure 1C)[41].

Antioxidant enzymes play a prominent role in protecting the cell/tissue from STZ induced diabetic damage. SOD plays an important role in all physiological conditions that convert superoxide anions to H_2O_2 and O_2 and then converted into H_2O_2 by CAT and GPx enzymes which protect the cells against detoxification of oxidative free radicals[42]. GPx diminishes H_2O_2 and reacts against superoxide anion to obtain the prolonged half life[43]. CAT plays an important role in elimination of H_2O_2 and inactivation of superoxide radicals and glycation of enzymes. The reduced CAT activity leads to the reduction of GPx levels in the tissues[44]. This might be due to increased consumption of GPx and GSH, and increased utilization of free radical scavenging activity. GSH synthesizes important macromolecules and implication in protection against oxygen compounds[45]. The malondialdehyde (MDA) is an indicator of lipid peroxidation (LPx). The earlier studies reported that in diabetes hypoinsulinemia increases the activity of enzymes that initiated the oxidation of fatty acids, results in lipid peroxidation impairment of membrane fluidity which leads to cell injury[46]. In the present study, our results demonstrated that the selected five potential marine macroalgae extracts (AE3, FS4, FM4, PH1 and SF1) modulated the antioxidant enzymes and lipid peroxidation levels in liver, kidney and brain tissues of STZ induced diabetic rats (Figure 2). This was previously reported in *Ulva pertus*, *Ulva lactusa*, *Spirulina* and

sulphated polysaccharide of *Porphyra haitanesis*[47-49]. Unnikrishnan *et al.*[50] has reported that some of the green marine algae such as *Chaetomorpha aerea*, *Enteromorpha intestinalis*, *Cladophora rupestris* also recovered the antioxidant activities in diabetic rats.

The present study illustrates that among five potential marine macroalgae extracts, the oligosaccharide conjugate of red macroalgae, particularly oligosaccharide conjugate of *G. opuntia* (FM4) exhibited greater anti-diabetic properties. The potential anti-diabetic activity of FM4 indicates the fact that they are the reservoirs for anti-diabetic agents and are used as food as well as pharmaceutical products in place of synthetic derivatives. Therefore, FM4 was selected to characterize and to evaluate further studies in STZ induced diabetic rats. The structure of FM4 was characterized by ^{1}H-NMR and FT-IR analysis and demonstrated as sulphated galactopyran (SGP).

The survival analysis and ED_{50} was measured for SGP in diabetic rats. Among the five different concentrations 100 mg/kg has shown significant recovery of altered C-peptide and insulin levels; and the survival rate was more compared to STZ induced diabetic rats (Figure 4). 100 mg/kg body weight is determined as ED_{50} for SGP and this concentration has been chosen for the further analysis.

C-peptide and insulin are the important products in proinsulin enzymatic cleavage that secreted into the circulation in equimolar concentrations[51]. C-peptide and insulin levels were decreased significantly in diabetes rats. This might be due to the destruction of pancreatic β-cells and thereby induces hyperglycemia[52]. In the present study the significant increase in the levels of insulin and C-peptide was observed in SGP administrated diabetic rats (Figure 5). This might be due to increased insulin secretion from pancreatic β-cells. This was previously reported in STZ induced diabetic rats treated with synaptic acid[53].

The histopathalogical changes of liver, kidney and brain were observed in purified oligosaccharide fraction of FM4 in diabetic rats. The organs such as liver and kidney play a prominent role in detoxification and excretion of many metabolites[54]. Hence these tissues may be damaged intracellularly during STZ induced diabetes. Brain is an important organ of nervous system and the central nervous system was damaged during the STZ induced diabetes[55]. The necrosis of hepatocells, changes in microcellular fats, extensive vacuolization with disappearance of nuclei was observed in diabetic rat liver[56]. This was salvaged after treatment with SGP (Figure 6A–6C). It was previously hypothesized that hepatoprotective activity of *Sargassum polycystum* has recovered the damaged liver tissues[57]. In diabetic kidney, due to increased glucose levels H_2O_2 was produced in murine mesangial cells which causes swelling in glomerular capillary endothelium that leads to endolysis[58]. The tissues were regenerated after treating with the SGP (Figure 6D–6F). It was previously reported that some of the marine algae such as *Kappaphycus alvarizii* and *Sargassum polycystum* and some brown seaweeds have also shown the recovery of kidney tissue damages caused due to diabetes mellitus[59,60]. The cerebral tissue of diabetic rats showed necrosis in multifocul neurons and moderate glialshrubberies and cell degeneration[14]. This was recovered after

treating with SGP derived from *G. opuntia* (Figure 6G–6I). It was previously reported that some of the marine algae such as *Ulva rigida* have also shown the recovery of damaged brain tissue caused by diabetes mellitus[60].

In conclusion, marine algae were demonstrated to be the valuable natural source of bioactive compounds with potential activities against type-2 diabetes. This study showed that the ethanolic extract of *T. conoides* (AE3), methanolic extracts of *S. wightii* and *T. conoides* (PH1 and SF1), aqueous extract of *G. opuntia* (FS4) and oligosaccharide conjugate of *G. opuntia* (FM4) have significantly reduced the glucose, HbA1c and antioxidant enzymes activity levels. Among the five extracts, the FM4 has showed higher anti-diabetic activity. The FM4 was purified by anion exchange column chromatography method. The SGP derived from FM4 showed a significant anti-diabetic activity. The ED_{50} of SGP was found to be 100 mg/kg body weight. The present study reveals that SGP has potent anti-diabetic activity in STZ induced diabetic rats. Further comprehensive chemical and pharmacological investigations are needed to elucidate the exact mechanism of the hyperglycemic effect of SGP of *G. opuntia*.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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