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Article

Seaweed *Chara baltica*: Isolation, Characterization and *In vivo* Antidiabetic Study

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For the first time, five known metabolites - **1** and **5** are reported from the ethyl acetate extract from seaweed *Chara baltica* **(EAE)**. Both the metabolites and **EAE** were screened against free-radicals, α-glucosidase enzyme and glycaemia in albino rats. **4, 5** and **EAE** depicted significant antiradical and α-glucosidase inhibitory profile. Particularly, compound **5** showed equivalent inhibition of superoxide free radical as that of the standard drug with IC_{50} value of 32.0 µg/mL. In addition, the **EAE** (200 mg/kg b.w) revealed significant reduction in plasma glucose, body weight, total cholesterol, total glycerides and LDL levels in Streptozotocin-induced diabetic rats. The HDL levels were markedly augmented in **EAE** treated diabetic rats, when compared with control group. **EAE** abolished the increased lipid peroxidation content in both liver and kidneys. The histopathological examination of pancreas of **EAE** protected the Langerhans islets with the number of islet cells were found statistically significant, when compared to diabetic control pancreas. This is the first *in vitro* and *in vivo* antidiabetic report on *C. baltica*.

Keywords: Antioxidant activity. α-Glucosidase inhibitory. Langerhans islets. Glycaemia.

INTRODUCTION

Diabetes mellitus (DM) is an endocrine metabolic disorder triggered from an impact between gene and habitat (Tuomi *et al*., 2014). The epidemiology studies of DM demonstrated from free radicals (oxidative stress) evolution and advancement of DM and its complications like atherosclerosis, hypercholesterolemia, hypertension, ischemic attacks, myocardial infraction, nephropathy and retinopathy (Ghoul *et al*., 2012; Tatipamula *et al*., 2017). During hyperglycaemia, rapid augment in the free radicals takes place by various mechanisms which include glucose auto-oxidation, activation of polyol pathway and non-enzymatic protein glycation. This increase in free radicals causes lipid peroxidation and protein oxidation which eventually leads to cell injury and necrosis (Ghoul *et al*., 2012).

DM is chiefly subdivided into Type 1 (insulin dependent) which is an autoimmune damage of pancreatic insulin producing cells and Type 2 (non-insulin dependent/ insulin resistance in cells) diabetes. In known cases of DM patients, only 10% of patients are believed to suffer from Type 1 diabetes, rest of all would be distressing with Type 2 diabetes. Type 1 DM can be managed insulin, whereas Type 2 DM by oral medication of natural/synthetic drugs (Valko *et al*., 2007). Natural sources play a prominent role in the origin of new biological agents that include antioxidants and anti-diabetes, which examined to be effectual as well as safe alternative method in the therapy for DM, instead of synthetic molecules. As a result, the searching and identifying new bioactive species and their derivatives for treating DM has become a point of interest. According to recent research studies, several aquatic organisms like seaweeds, seagrasses, sponges and corals had been screened for their antidiabetic potentiality (Lauritano, Ianora, 2016).

In folklore, seaweeds (macro algae) are used as a food source in Asian counties. Based on the pigmentation, seaweeds are classified as red, brown and green algae. From the past few decades, seaweeds have been highly

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acknowledged to possess prominent biological activities, which include antineoplastic, antimicrobial and antiviral activities (Patterson, Larsen, Moore, 1994) due to their specific functional compounds (which are not available in land plants) (Pietra, 1997; Cabrita, Vale, Rauter, 2010). Particularly, seaweed of *Chara* genus (family: Characeae – charophyte green algae) possess to have antioxidant enzyme and allelopathic activities and lipid peroxidation capabilities along with high levels of photosynthetic pigment content (Berger, Schagerl, 2003). Earlier the experiment which we had performed has established the phytochemical analysis along with antioxidant activities of seaweed *Chara baltica* (Naidu *et al*., 2018). Till date, there are no proper chemical and pharmacological evaluation has been carried out on *C. baltica*. In continuation of our research on chemical and pharmacological evaluation of seaweed and also based on the aforementioned properties of *Chara* genus, as well as, *C. baltica*, we examined by recollecting the *C. baltica* specimens (in July 2018) for its chemical and pharmacological properties which are reported on this research paper.

MATERIAL AND METHODS

Collection of Seaweed

The specimens of seaweed *Chara baltica* was collected near Korangi coast, Kakinada, Andhra Pradesh, India at a depth of 8-10 ft (16°80'N and 82°08'E with 3 m elevation) in 3 July, 2018. The seaweed was authenticated by Dr. G. Mohan Narasimha Rao, Professor of Botany Department, Andhra University, Visakhapatnam, Andhra Pradesh, India. A voucher specimen was deposited in the Marine Organisms Museum in the University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India (No. AU-SW-2018-947).

Chemicals

All the chemicals used in the present study were of analytical grade. Streptozotocin (STZ) was from the Himedia Laboratories Pvt. Ltd. (Mumbai, India); glibenclamide from the Avantis Pharma Ltd. (Mumbai, India); and rat feed from the Hindustan Lever Ltd. (Mumbai, India).

Extraction and Isolation

The seaweed was cleansed from extraneous substance and stored in ethanol-water (1:1) at the site of collection. The animal material (about 250 g) was extracted at least thrice with ethanol-water and concentration at a reduced pressure and the combined extracts were dispersed in water and re-extracted with ethyl acetate. The ethyl acetate fractions were dried on anhydrous sodium sulphate and evaporated under a reduced pressure to obtain ethyl acetate extract (**EAE**); as a reddish brown gummy residue (9.2 g) (Chitturi *et al*., 2016; Rao *et al*., 2015). The **EAE** (2 g) was subjected to column chromatography (hexane-ethyl acetate) resulted in three fractions. Fraction I (236 mg) a yellowish liquid obtained from 10% ethyl acetate in hexane, subjected to column chromatography yielded **1** (90 mg) as a pale yellowish liquid and **2** (100 mg) as a colourless liquid. Fraction II (207 mg) obtained from 20% ethyl acetate in hexane as green coloured residue was subjected to column chromatography to obtain **3** (60 mg) as a greenish solid. Fraction III (350 mg) a dark greenish solid obtained from 40% ethyl acetate in hexane as green coloured residue was subjected to column chromatography to obtain **4** (150 mg) as a pale yellowish solid and **5** (130 mg) as a greenish solid.

Antioxidant activity

ABTS radical scavenging assay

The metabolites **(1-5)** and **EAE** was subjected to 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radical scavenging assay (Sastry, Vedula, Tatipamula, 2018; Tatipamula, Vedula, Sastry, 2019). Firstly, the stock solution was prepared by adding 7 mM of ABTS+· to 2.45 mM potassium persulfate in water at 25˚C and standardized for 16 h. Then to each 1 mL of ABTS+· solution added different concentrations of extract and standard (ascorbic acid), incubated for 6 min and the absorbance was measured at 750

nm spectrophotometrically (Spectra MAX plus 384, Molecular Devices Corporation, Sunnyvale, CA, USA) and the experiment was triplicated and the data was expressed as percentage inhibition.

Superoxide radical scavenging assay

In the radical method (Tatipamula, Vedula, Sastry, 2019; Haritha, Patnaik, Tatipamula, 2019), the superoxide radicals generated from non-enzymatic phenazine methosulfate/nicotinamide adenine dinucleotide (PMS/NADH) reduces nitro blue tetrazolium (NBT) to a purple formazan. To 1 mL of reaction mixture contained 20 mM phosphate buffer (pH 7.4), 73 µM NADH, 50 µM NBT, 15 µM PMS added various concentrations of extract/isolates/ascorbic acid and incubated for 10 min at room temperature and the absorbance was noted at 562 nm spectrophotometrically (Spectra MAX plus 384, Molecular Devices Corporation, Sunnyvale, CA, USA) against blank and the experiment was triplicated and the data was data was expressed as percentage inhibition.

Percentage inhibition (%) = (Ac – As)/Ac × 100

where Ac is the absorbance of the control.

As is the absorbance of sample.

A graph is plotted between concentrations of the extract and their percentage inhibition to determine the IC_{50} values of particular extract.

α-Glucosidase inhibitory assay

The assay of α-glucosidase inhibitory activity was estimated by using modified procedures of Tatipamula group (Tatipamula *et al*., 2019) in triplet (n=3). 2.0 µL of α-glycosidase from rat intestine acetone powder solution (a stock solution of 1.0 mg/mL in 10 mM phosphate buffer, pH 6.8, diluted 40-fold with same buffer) was mixed with 20 µL of the samples at different concentrations and added 100 µL of 50 mM phosphate buffer (pH 6.8) in 96 well microplate and incubated for 5 min at 37˚C. After incubation, 50 µL of substrate (5 mM of p-nitrophenyl-α-D-glucopyranoside prepared in 50 mM of phosphate buffer, pH 6.8) were added and the entire reaction mixture was again incubated for 20 min at 37˚C. Thereafter the reaction was terminated by adding 50 µL of Na_2CO_3 (1 M) and made up the final volume to 150 µL. The amount of *p*-nitro phenol released from substrate was noted at 405 nm spectrophotometrically (Spectra MAX plus 384, Molecular Devices Corporation, Sunnyvale, CA, USA). DMSO and acarbose were used as control and standard, respectively. The percentage of enzyme inhibition was calculated by using below formula

Percentage of inhibition (%) = (C-S)/C × 100

where C is the absorbance of the control.
S is the absorbance of sample.
IC_{50} values of the samples were determined by plotting percentage inhibition against concentrations.

Animals

Healthy albino Wistar rats weighing between 180-200 g of either sex were maintained on standard rat feed were utilized for this study. All the experiment protocols were according to the Organisation for Economic Co-operation and Development (OECD) guidelines and regulations of Institutional Ethical Committee (Regd No. 516/01/A/CPCSEA).

Acute toxicity studies

The male albino rats are grouped into one containing five animals, were allowed free access to water and diet under room temperature for one week before the experiment. The **EAE** extract 2000 mg/kg body weight (b.w) were administered orally and the rats were observed at regular intervals of time (1, 2, 6, 12 and 24 h) for aggressiveness, morbidity, oral secretions, respiratory movements, sensitivity and their mortality (Talluri *et al*., 2018; Tatipamula, Vedula, 2017). The mortality number triggered by the **EAE** within this time duration was observed, form which log dose response plots were calibrated and median lethal dose (LD_{50}) of the sample was determined (Babu, Prabuseenivasan, Ignacimuthu, 2007).

Hypoglycaemic activity (Oral Glucose Tolerance Test)

The plasma blood glucose levels of overnight fasted normal animals were noted using tail vein puncture and glucometer method (Tatipamula *et al*., 2017). These animals were grouped (n=6) orderly and treated with **EAE** (100 and 200 mg/kg b.w) and glibenclamide (standard, 1 mg/kg b.w) which were deliquesced in 0.5% carboxy methyl cellulose (CMC) in distilled water. Only 0.5% CMC in distilled water was dosed to a group which serves as a control. By tail vein puncture method, the blood glucose levels were determined at 0, 30 and 60 min. After obtaining the reading at 60 mins, all the animals were administered orally 20% glucose solution in distilled water (2 g/kg) using polyethylene gastric tube, then the blood glucose levels were noted at 90, 120, 240 and 360 min using above method. The percentage reduction of plasma blood glucose was calculated using the below formula (Ghezzi *et al*., 2012).

$$\% \text{ reduction in glycaemia} = (G_0 - Gx)/G_0 \times 100$$

Where G_0 is initial glycaemia (0 h); Gx is glycaemia at 30, 60, 90, 120, 240 and 360 min.

Anti-Hyperglycaemic activity

Induction of diabetes

Diabetes was inducted to the animals (overnight fasted) by injected intraperitoneally the freshly prepared solution of Streptozotocin (STZ, 55 mg/kg b.w) in 0.1 M cold citrate buffer (pH 4.5). In order to overcome drug induced hypoglycaemia the animals were allowed free access to drink 5% glucose solution overnight. The control group animals received only citrate buffer. The blood glucose values of induced animals were monitored for three successive days after STZ injection. The animals with above 250 mg/dL blood glucose values on third day were considered as diabetic and used for the treatment from fourth day onwards (Tatipamula *et al*., 2017; Tatipamula *et al*., 2019).

Antidiabetic activity

The diabetic animals were divided into five groups (each n=6) as indicated below, overnight fasted and treated with **EAE** (100 and 200 mg/kg b.w) and glibenclamide (standard, 1 mg/kg b.w) which were deliquesced in 0.5% CMC in distilled water for once a day for a period of three consecutive weeks. During this time, the animals were observed for their blood glucose levels and body weight at regular intervals of time i.e., 1, 7, 14 and 21 day. *Group I.* Control, with 0.5% CMC in distilled water; *Group II.* Diabetic control, with 0.5% CMC in distilled water; *Group III.* Standard, with glibenclamide (1 mg/kg b.w/day); *Group IV.* **EAE** (100 mg/kg b.w/day); *Group V.* **EAE** (200 mg/kg b.w/day). The fasting blood glucose levels were scrutinized at particular intervals days i.e., 1, 7, 14 and 21 and the effect of test samples on body weight of animals were also monitored on these days. On 21 day, blood samples were collected from overnight fasted animals by puncturing of retro-orbital plexus and these blood samples used for estimation of plasma total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol using Randox diagnostic kits (Tatipamula *et al*., 2019). The absorbance was measured and calculated using fully smart semi-automated analyzer and finally animals were anaesthetized (sodium pentobarbital, intraperitoneally, 60 mg/kg b.w) and sacrificed and then kidneys, pancreas and liver tissues were detached for measurement of lipid peroxidation (TBARS) and histopathological studies.

Lipid Peroxidation (TBARS) in Tissues

The isolated liver and kidneys were immediately excised and washed with 0.9% sodium chloride (NaCl), wet tissue homogenized in 4.5 mL of 0.25 M sucrose using homogenizer. The cytosolic fraction was collected by a two-step centrifugation, first at 1000 × g for 10 min and then at 2000 × g for 30 min at 4°C. A volume of homogenate (0.2 mL) was transferred to a vial and was mixed with 0.2 mL of 8.1% (w/v) sodium dodecyl sulphate solution, 1.5 mL of a 20% acetic acid solution (pH 3.5) and 1.5 mL of 0.8% (w/v) solution of thiobarbutiric acid

and the final volume of solution was adjusted to 4.0 mL with distilled water and then vials were heated in boiling water bath for 60 min. After cooling, equal volumes of tissue blank or test sample and 10% trichloroacetic acid were transferred into a centrifuge tube and centrifuged at 1000 × g for 10 min and the absorbance of the supernatant fraction was measured at 532 nm (Ohkawa, Ohishi, Yagi, 1978; Tatipamula *et al*., 2017).

Histopathological Studies

Histopathological analysis of pancreas was performed by cutting 2 mm sections of the tissues by using a microtome and fixing in 10% formalin solution and staining with haemotxylene and eosin.

Statistical Analysis

The experimental values were given in mean±SEM and statistical differences in extracts, standard and control groups were compared by two-way ANOVA followed by Dunnett's test. The p value <0.05 were considered statistically significant.

RESULTS

Chemistry

The isolated **1** was identified as (E)-4-hydroxynon-2-enal (Figure 1) which is pale yellowish liquid; R_f: 0.4 (hexane:ethyl acetate, 9:1); Purity: above 95% pure; Mol. Formula: $C_9H_{16}O_2$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 0.99-1.02 (*t*, 3H, *J*= 3.2 Hz, 9-CH_3), 1.25 (*s*, 1H, 4-OH), 1.28-1.43 (*m*, 6H, 6,7,8-CH_2), 1.48-1.53 (*m*, 2H, 5-CH_2), 4.18-4.22 (*m*, 1H, 4-CH), 6.41-6.45 (*dd*, 1H, *J*= 4 Hz, 2=CH), 6.59-6.63 (*dd*, 1H, *J*= 4 Hz, 3=CH), 9.69-9.71 (*d*, 1H, *J*= 8 Hz, 1-CHO). ^{13}C NMR ($CDCl_3$, 400 MHz) δ_C: 14.00 (C-9), 22.92 (C-8), 25.15 (C-6), 31.49 (C-5), 36.31 (C-7), 73.31 (C-4), 129.17 (C-2), 154.96 (C-3), 191.99 (C-1). ESI-MS (positive mode) *m/z*: 157.13 ($M+H^+$), calcd. *m/z* for $C_9H_{16}O_2$: 156.12.

The isolated **2** was obtained as colourless liquid and identified as (*Z*)-3,3-dimethyl-5-methylene-4-(3-methylpenta-2,4-dien-1-yl)cyclohex-1-ene (Figure 1) with R_f: 0.6 (hexane: chloroform, 4:1); Purity: above 95% pure; Mol. Formula: $C_{15}H_{22}$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 1.00 (*s*, 6H, 13,14-CH_3), 1.78 (*s*, 3H, 15-CH_3), 1.79-1.81 (*m*, 1H, 8b-CH_2), 2.04-2.08 (*m*, 2H, 4-CH, 8a-CH_2), 2.56-2.60 (*dd*, 1H, *J*= 4.8 Hz, 6b-CH_2), 2.67-2.71 (*dd*, 1H, *J*= 4.8 Hz, 6a-CH_2), 4.75 (*s*, 2H, 7=CH_2), 5.02-5.04 (*d*, 1H, *J*= 8 Hz, 12b=CH_2), 5.14 (*s*, 1H, 12a=CH), 5.14 (*m*, 1H, 9=CH), 5.44-5.46 (*m*, 1H, 5=CH), 5.88-5.90 (*d*, 1H, *J*= 8 Hz, 4=CH), 6.13-6.18 (*dd*, 1H, *J*= 8 Hz, 14=CH). ^{13}C NMR ($CDCl_3$, 400 MHz) δ_C: 17.82 (C-15), 24.92 (C-13/14), 26.74 (C-8), 38.48 (C-3), 39.72 (C-6), 50.04 (C-2), 110.64 (C-7), 112.77 (C-12), 122.23 (C-5), 133.23 (C-9), 135.27 (C-10), 135.57 (C-11), 137.55 (C-4), 144.71 (C-1). ESI-MS (positive mode) *m/z*: 203.13 ($M+H^+$), calcd. *m/z* for $C_{15}H_{22}$: 202.17.

The isolated **3** was identified as (*E*)-8-(5-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl)-6-methyloct-5-en-2-one (Figure 1) which is greenish solid; R_f: 0.4 (hexane:ethyl acetate, 3:1); Purity: above 95% pure; m.p.: 163-164˚C; Mol. Formula: $C_{18}H_{30}O_2$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 1.15 (*s*, 6H, 7,8-CH_3), 1.20 (*s*, 1H, 1-OH), 1.87 (*s*, 3H, 18-CH_3), 1.90 (*s*, 3H, 17-CH_3), 2.08-2.09 (*m*, 1H, 6b-CH_2), 2.10-2.12 (*m*, 5H, 5b,9,10-CH_2), 2.18 (*s*, 3H, 16-CH_3), 2.20-2.23 (*m*, 1H, 5a-CH_2), 2.30-2.33 (*m*, 2H, 13-CH_2), 2.51-2.54 (*t*, 2H, *J*= 4, 8 Hz, 14-CH_2), 3.57-3.60 (*t*, 1H, *J*= 4, 8 Hz, 1-CH), 5.29-5.31 (*m*, 1H, 12=CH). ^{13}C NMR ($CDCl_3$, 400 MHz) δ_C: 16.53 (C-18), 20.12 (C-17), 23.42 (C-7/C-8), 24.53 (C-9), 24.63 (C-13), 27.69 (C-6), 28.56 (C-16), 29.10 (C-5), 38.76 (C-10), 40.66 (C-2), 41.76 (C-14), 74.45 (C-1), 124.74 (C-12), 129.25 (C-4), 135.71 (C-11), 137.81 (C-3), 206.74 (C-15). CHNS analysis: anal. C 77.60, H 10.45(%), calcd C 77.65, H 10.86(%). ESI-MS (positive mode) *m/z*: 279.16 ($M+H^+$), calcd. *m/z* for $C_{18}H_{30}O_2$: 278.22.

The isolated **4** was obtained as pale yellowish solid and identified as 1-(4-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl)butane-1,3-dione (Figure 1) with R_f: 0.6 (hexane: chloroform, 1:1); Purity: above 95% pure; m.p.: 176-177˚C; Mol. Formula: $C_{13}H_{20}O_3$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 1.05 (*s*, 6H, 11,12-CH_3), 1.16 (*s*, 1H, 4-OH), 1.57-1.61 (*m*, 1H, 3a-CH_2), 1.88-1.94 (*m*, 2H, 5a,3b-CH_2), 1.98 (*s*, 3H, 13-CH_3), 2.15 (*s*, 3H, 10-CH_3), 2.17-2.21 (*m*, 1H, 5b-CH_2), 3.96-4.02 (*m*, 1H, 4-CH), 4.06 (*s*, 2H, 8-CH_2). ^{13}C NMR ($CDCl_3$, 400 MHz) δ_C: 21.56 (C-13), 28.63 (C-11/12), 28.73

(C-10), 35.19 (C-2), 38.87 (C-5), 44.80 (C-3), 56.99 (C-8), 65.78 (C-4), 133.37 (C-1), 142.67 (C-6), 192.67 (C-7), 194.89 (C-9). CHNS analysis: anal. C 70.75, H 8.44(%), calcd. C 70.56, H 8.55(%). ESI-MS (positive mode) *m/z*: 225.11 ($M+H^+$), calcd. *m/z* for $C_{13}H_{20}O_3$: 224.30.

The isolated **5** was identified as (3*E*,5*E*)-1-(4-hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]heptan-1-yl)-3-methylocta-3,5-diene-2,7-dione (Figure 1) which is greenish solid; R_f: 0.4 (hexane:chloroform, 1:1); Purity: above 95% pure; m.p.: 280-281˚C; Mol. Formula: $C_{18}H_{26}O_4$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 1.15 (*s*, 6H, 17,18-CH_3), 1.46-148 (*m*, 1H, 5b-CH_2), 1.47 (*s*, 3H, 15-CH_3), 1.65-1.67 (*d*, 1H, *J*= 8 Hz, 3b-CH_2), 1.70-1.71 (*d*, 1H, *J*= 8 Hz, 5a-CH_2), 1.89 (*m*, 1H, 3a-CH_2), 2.11 (*s*, 3H, 18-CH_3), 2.39 (*s*, 3H, 14-CH_3), 2.52 (*s*, 1H, 1-OH), 2.97-3.00 (*d*, 1H, *J*= 12 Hz, 7b-CH_2), 3.22-3.25 (*d*, 1H, *J*= 12 Hz, 7a-CH_2), 3.63-3.69 (*m*, 1H, 4-CH), 6.46-6.49 (*d*, 1H, *J*= 12 Hz, 12=CH),. 24 (*s*, 1H, 9=CH), 7.64-7.67 (*d*, 1H, *J*= 12 Hz, 11=CH). ^{13}C NMR ($CDCl_3$, 400 MHz) δ_C: 12.43 (C-18), 20.17 (C-15), 27.05 (C-14), 27.64 (C-16/C-17), 34.99 (C-6), 36.07 (C-7), 45.18 (C-3), 46.73 (C-5), 64.40 (C-2), 66.15 (C-4), 67.73 (C-1), 128.28 (C-12), 136.24 (C-9), 140.07 (C-10), 140.87 (C-11), 198.89 (C-13), 200.87 (C-8). CHNS analysis: anal. C 70.20, H 10.65(%), calcd C 69.89, H 10.32(%). ESI-MS (positive mode) *m/z*: 307.12 ($M+H^+$), calcd. *m/z* for $C_{18}H_{26}O_4$: 306.18.

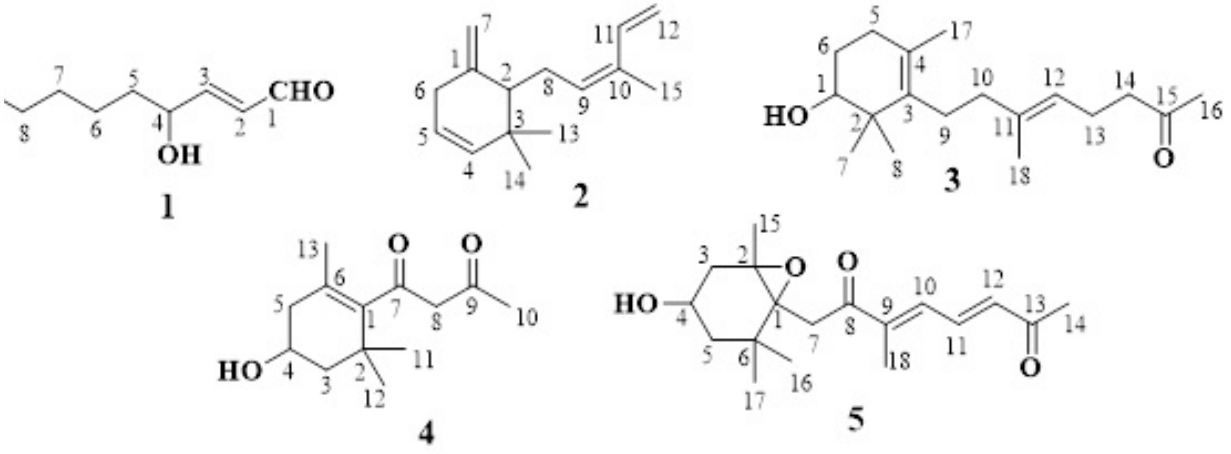

FIGURE 1 – Identified secondary metabolites from seaweed *Chara baltica*.

Antioxidant activity

Generally, natural antioxidants have great forbearance to humans and are without of considerable side effects (Tatipamula, Vedula, Sastry, 2019). The free radical-quenching assays of ethyl acetate extract of *C. baltica* **(EAE)** against ABTS and superoxide radicals were illustrated in (Figure 2). The inferior IC_{50} values indicates superior inhibition of free radicals. From the antioxidant results it is established that the **EAE** depicted to have a promising antiradical scavenging capacities.

The principal of ABTS radical assay in which decoy of the radical cation $ABTS^{\cdot+}$ takes place (Ambigaipalan, Shahidi, 2017). The IC_{50} values of **4, 5** and **EAE** were 85.0, 66.0 and 143.0 µg/mL, respectively, whereas standard value was 30.1 µg/mL (Figure 2).

In the same way, the superoxide radical generally arises from metabolic process/ROS, which interact with other substrates in presence of enzyme/metal catalysed processes to generated hydroxyl radical, H_2O_2 and $^1O_2$. These radicals induce oxidative damage in DNA, lipids and proteins (Poprac *et al*., 2017). The concentration of **4, 5** and **EAE** needed for 50% inhibition of superoxide radical were found to be 63.0, 32.0 and 112.0 µg/mL, respectively, while standard (ascorbic acid) was 32.0 µg/mL (Figure 2). From all the anti-radical assays, it can be concluded that the antioxidant capability of **EAE** was due to the presence of the metabolites **4** and **5**.

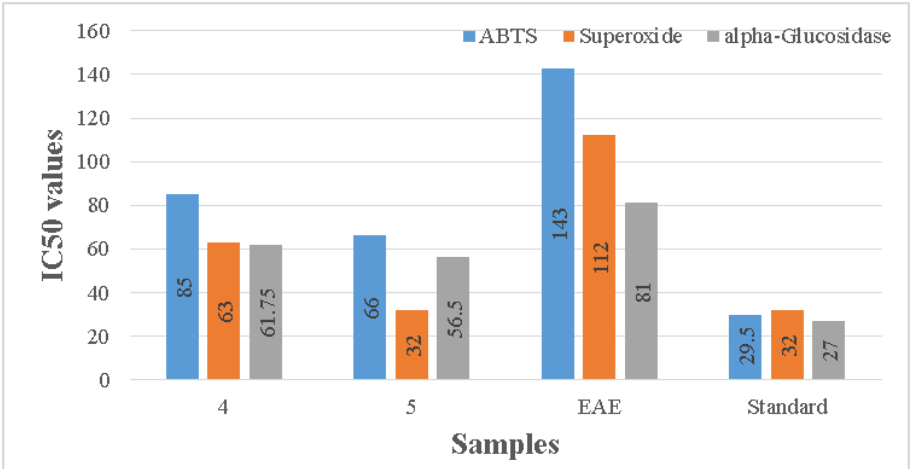

FIGURE 2 – IC_{50} values of metabolites and **EAE** against ABTS, superoxide free radicals and α-glucosidase enzyme.

α-Glucosidase inhibitory assay

The *in vitro* antidiabetic activity was assessed by α-glucosidase inhibitory assay (Tatipamula *et al*., 2019) using *p*-nitrophenyl-α-D-glucopyranoside and acarbose as substrate and standard, respectively, and the results as IC_{50} values were presented in (Figure 2). From the assay, it was estimated that the **EAE** exhibited prominent inhibition of α-glucosidase enzyme with IC_{50} values of 81.0 µg/mL, whereas standard (acarbose) with 27.0 µg/mL (Figure 2). Additionally, the 50% concentration needed for compound **4** and **5** to inhibit α-glucosidase enzyme were determined to be 61.75 and 56.5 µg/mL (Figure 2), respectively. From the outcomes of this assay, it has been confirmed that the key agents were responsible for the *in vitro* antidiabetic activity of **EAE** from *C. baltica* were **4** and **5**.

Mortality

The acute toxicity studies of **EAE** from *C. baltica* exhibited no mortality or symptoms of toxicity up to 2000 mg/kg b.w. Hence the LD_{50} value of **EAE** found to be above 2000 mg/kg b.w and the dosage was fixed as $1/10^{th}$ and $1/20^{th}$ part i.e. 100 and 200 mg/kg b.w.

Biochemical analysis

Based on the toxicity studies, the **EAE** (100 and 200 mg/kg b.w) subjected to hypoglycaemic and anti-hyperglycaemic activity by using oral glucose tolerance (OGT) test and STZ-induced diabetes in rats.

In OGT test, the control group animals (dosed with 0.5% CMC) showed linear level of blood glucose levels in normal rats for the initial 60 min. Subsequently, the normal control rats showed sharp rise in plasma sugar levels due to the regulation of 20% glucose solution using polyethylene gastric tube for 240 min, thereafter following that, the fall in blood glucose levels is observed and is established due to the physiological metabolism of animals (Table I). In normal rats, the **EAE** showed significant reduction of plasma blood glucose levels (Table I). Normal animals treated with **EAE** (200 mg/kg b.w) reduced plasma sugar levels as that of the standard group of animals treated with glibenclamide, 1 mg/kg b.w.

In glucose loaded rats, the **EAE** showed augment reduction of the blood glucose levels for 240 min with respect to standard. Thereafter the animals are relieved from hypoglycaemic activity due to biological degradation and excretion of the samples from the animal body (Table I). Among all the animal groups, the **EAE** (200 mg/kg b.w) group animals showed 18.55% reduction of plasma glucose levels in glucose loaded rats at the accurate 240 min, whereas, the standard group of animals revealed potent reduction (48.12%) and causes severe hypoglycaemic conditions in glucose loaded rats (Table I).

In STZ-induced diabetes model, it was observed that the normal group of animals showed a slight variation

of blood sugar levels in respective intervals of time i.e., 1, 7, 14 and 21 day (Table II). In addition, the diabetic controlled animals radically intensified their plasma glucose levels from 334.17±5.20 to 433.34±6.36 mg/dL with 29.68% raise at 21 day (Table II). Additionally, animals treated with **EAE** at 100 and 200 mg/kg b.w dosage for three consecutive weeks resulted in gradual reduction of plasma sugar levels from 346.34 ±7.92 to 229.5±7.20 mg/dL and 330.17±6.94 to 167.34±3.12 mg/dL with 33.74 and 49.34% reduction, respectively, at 21 day (Table II). However, standard (glibenclamide, 1 mg/kg b.w) group of animals exhibited 62.11% reduction in their blood glucose levels at 21 day (Table II). Analysing anti-hyperglycaemic activity, it can be interesting to note that **EAE** at 200 mg/kg b.w displayed exceptional antidiabetic potentiality with respect to standard and control.

Body weight

Throughout the antidiabetic experimental protocol, all the animal groups were noted for their body weight at 1, 7, 14 and 21 day (Table III). Monitoring these observations, it can be noticed that there was a chronological increase in their body weight in the normal control animals (10.08% at 21 day), whereas the diabetic controlled showed a drastic decline in their body weight of about 26.34% reduction at 21 day (Table III). The diabetic animals supervised with **EAE** (200 mg/kg b.w) and standard (glibenclamide, 1 mg/kg b.w) showed a minimal reduction in their body weight with 6.52 and 5.30% reduction at 21 day, whereas the rats treated with **EAE** at 100 mg/kg b.w exhibited 12.96% reduction in their body weight (Table III).

Plasma analysis and TBARS content in tissues

The variations in biochemical parameters: total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL), thiobarbituric acid reactive substances (TBARS) in liver and kidneys of diabetes in normal, treated (**EAE**, 100 and 200 mg/kg b.w) and standard treated (glibenclamide, 1 mg/kg b.w) rats were estimated and presented in Table IV. The analysis of blood samples of treated rats (**EAE**, 100 and 200 mg/kg b.w) at various degrees displayed large variation in their levels of TC, TG, LDL and HDL (Table IV). When compared to the 100 mg/kg b.w dosage, the **EAE** at 200 mg/kg b.w exhibited better recovery from TC, TG, LDL and HDL levels with respect to the normally controlled and diabetically controlled animals, whereas the standardly treated animals became normal in all calibrated parameters (Table IV).

In addition, the higher doses of **EAE** (200 mg/kg b.w) revealed 32.51±2.96 and 10.02±0.32% reduction of lipid peroxidation in liver and kidney tissues of rats, respectively, than the lower doses of **EAE** (100 mg/kg b.w). On the other hand, the standard treated animals exhibited 40.10±0.97 and 9.15±0.12% depletion in TBARS content in liver and kidneys (Table IV).

Histopathological studies

The histopathological studies of pancreas in normal rats, a clear and well arrangement of endocrine islets and exocrine acini were observed in normal rats (Figure 3A) whereas huge necrosis of islets of Langerhans were observed in diabetic (diseased) rats (Figure 3B), due to the oxidative stress conditions produced by the STZ. The STZ-induced diabetic rats, when treated with **EAE** (200 mg/kg b.w) and standard (glibenclamide, 1 mg/kg b.w) for three consecutive weeks, resulted in the regeneration of islets of Langerhans in the slides of pancreas (Figure 3C & 3D). From the observation of biological parameters and histopathological study of pancreas it can be suggested that the *C. baltica* possess to have a great aptitude to act against free-radicals and diabetes.

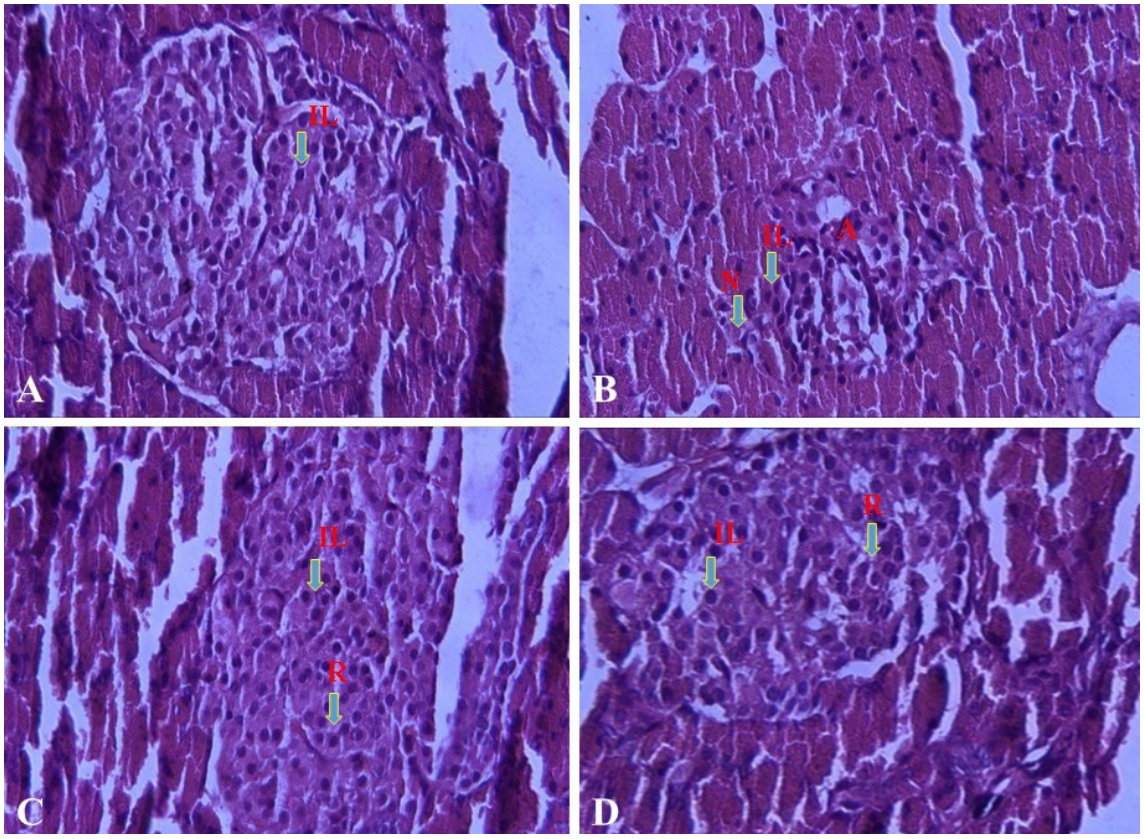

FIGURE 3 – Histopathological study of pancreatic tissues in rats **(A)** Normal Control pancreas; **(B)** Diseased pancreas; **(C)** Glibenclamide treated pancreas; **(D)** EAE treated pancreas at 200 mg/Kg b.w.

(A: Acini; IL: islets of Langerhans; N: necrosis, R: regeneration)

DISCUSSION

DM is a chronic metabolic disorder well-defined by hyperglycaemia. It is considered by alteration in the metabolic rate of carbohydrate, lipid and proteins (Babu, Prabuseenivasan, Ignacimuthu, 2007; Tatipamula *et al*., 2019). Additionally, oxidative stress conditions assumed to be augmented in the system where the level of free radical production is elevated. According to the recent studies, the oxidative stress-induced free radicals have been associated in the pathology of Type 2 DM (Luo *et al*., 2004; Valko *et al*., 2007).

In the current analysis of the study, we have evaluated the protective effect of *Chara baltica* on STZ-induced diabetes and oxidative stress in albino rats. We quantified the free-radical quenching potentiality of ethyl acetate extract of *C. baltica* **(EAE)** and identified the higher scavenging capacity towards ABTS and superoxide free radicals (Figure 2). The **EAE** at 100 and 200 mg/kg b.w exhibited remarkable hypoglycaemic effect in normal, glucose loaded and STZ-induced diabetic rats by reducing the plasma glucose levels (Table I). The outcomes of the anti-hyperglycaemic study showed that the **EAE** is time dependant. In observations, a major weight loss was detected in the diabetic of group animals while rats administered with **EAE** (100 mg/kg b.w) revealed notable increase in the body weight in contrast to diabetic group of animals but, lesser than in the normally controlled rats (Table II & Table III). This type of effect on the body weight was not detected at given higher doses of **EAE**. From this observations, it can be defined that the **EAE** reduces hyperglycaemia with increase in dosage.

Also, it is depicted that the STZ administration has augmented glycaemia and varied in lipid metabolic

parameters such as TC, TG. LDL and HDL levels and TBARS content in liver and kidneys (Table IV). The STZ-induced diabetic rats showed increase in plasma triglycerides and cholesterol (Table IV). More prominently, we have estimated and showed that **EAE** reduced plasma glucose, metabolic parameters and TBARS content variations in STZ-induced diabetic rats (Table IV), indeed, the effectiveness of **EAE** is assumed due to the presence of antioxidant properties of *C. baltica*. Measurement of plasma parameters (TC, LDL and HDL) and TBARS in liver and kidneys clarify the part of the hypo and anti-hyperglycaemic capabilities of the **EAE**.

The histopathological examination of diabetic animal pancreas showed the degeneration of the pancreas and decline in size and islets of Langerhans (in number). In the experimental study, the damage of pancreas in diabetic rats and regeneration of Langerhans islets by **EAE** was noticed. Additionally, the number of islet cells and their diameter significantly augmented in **EAE** treated group compared to the diabetic group of animals (Figure 3). Histopathologically, the **EAE** treated rats exhibited partial regeneration of the Langerhans islets and necrosis of the residual cells. From all these observations, it can be revealed that the potentiality of *C. baltica* can be used as a natural oral agent with both hypo and anti-hyperglycaemic effects.

TABLE I – Plasma blood glucose levels in nomral and oral glucose loaded (OGL) rats due to effect of **EAE** at 100 and 200 mg/kg b.w doses

Time (min)	Plasma glucose levels (mg/dL) (% reduction)			
	Control	**EAE100**	**EAE200**	**Glibenclamide**
0	103.34±5.45	97.84±6.67	94.34±7.84	101.84±6.47
30	98.67±5.78	90.17±2.47* (7.84)	82.17±6.47 (12.90)	88.50±5.58* (13.09)
60+OGL	99.17±4.57	86.17±7.47 (11.93)	68.84±8.81 (27.03)	74.17±5.75* (27.17)
90+OGL	123.84±6.78	102.84±4.79* (-5.11)	90.17±5.31* (4.42)	94.84±6.47 (6.87)
120+OGL	157.34±7.21	100.17±8.14 (-2.39)	85.67±9.45 (9.19)	78.34±4.15* (23.08)
240+OGL	162.34±9.14	94.5±3.11* (3.41)	76.84±6.51* (**18.55**)	52.84±4.37* (48.12)
360+OGL	149.34±8.74	112.17±3.72* (-14.65)	105.34±2.19* (-11.66)	---

#Values are mean±SD (n=6), *p<0.05

TABLE II – Blood glucose levels of STZ-induced diabetic rats administered with **EAE** observed within 21 days of treatment

Sample	Plasma glucose levels (mg/dL) (% reduction)			
	Day 1	**Day 7**	**Day 14**	**Day 21**
Normal control	87.34±2.09	97.0±5.56 (+11.06%)	95.17±4.97 (+8.97%)	94.67±5.74 (+8.39%)
Diabetes control	334.17±5.20	380.5±10.51 (-13.86%)	422.34±12.57 (-26.39%)	433.34±6.36 (-29.68%)
EAE100	346.34±7.92	313.17±5.25* (+9.58%)	274.5±7.32* (+20.74%)	229.5±7.20* (+33.74%)
EAE200	330.17±6.94	279.17±12.09 (+15.45%)	211.84±9.11* (+35.84%)	167.34±3.12* **(+49.32%)**
Glibenclamide (1 mg/kg b.w)	339.17±4.22	255.34±9.09* (+24.72%)	189.0±11.47 (+44.28%)	128.5±8.59* **(+62.11%)**

#Values are mean±SD (n=6), *p<0.05

TABLE III – Effect of **EAE** on body weight in STZ-induced diabetic rats

Sample	Body weight# (g) (% change)			
	Day 1	**Day 7**	**Day 14**	**Day 21**
Normal control	196.4±6.30	200.4±4.69 (+1.83%)	205.4±5.78 (+4.48%)	216.2±6.5 **(+10.08%)**
Diabetes control	194.4±6.5	182.0±6.3 (-6.38%)	169.2±4.5 (-12.96%)	143.2±3.6 (-26.34%)
EAE100	195.3±7.2	183.6±6.5 (-5.99%)	175.5±2.0* (-10.14%)	170.0±3.8* (-12.96%)
EAE200	199.1±0.9	194.6±2.9* (-2.26%)	189.2±1.8* (-4.97%)	186.1±2.0* **(-6.52%)**
Glibenclamide (1 mg/kg b.w)	199.8±0.2	195.3±0.9* (-2.25%)	191.0±1.1* (-4.41%)	189.2±1.8* **(-5.30%)**

#Values are mean±SD (n=6), data were analyzed using one way ANOVA analysis; *p<0.05

TABLE IV – The serum glucose concentrations, total cholesterol (TC), triglycerides (TG), High density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol levels and TBARS contents in diabetic rats due to the effect of ethyl acetate extract of *C. baltica* **(EAE)**

Sample	TC (mg/dL)	TG (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TBARS in µmol/g±SEM (% change)	
					Kidney	**Liver**
Control	64.1±0.20	44.1±0.74	29.0±0.11	65.2±0.92	183.67±1.78	349.17±1.84

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TABLE IV – The serum glucose concentrations, total cholesterol (TC), triglycerides (TG), High density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol levels and TBARS contents in diabetic rats due to the effect of ethyl acetate extract of *C. baltica* **(EAE)**

Sample	TC (mg/dL)	TG (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TBARS in µmol/g±SEM (% change)	
					Kidney	Liver
Diabetic Control	155.9±1.82	173.0±1.46	127.1±0.20	38.9±0.47	355.67±2.65 (+93.80±3.27)	394.5±2.26 (-13.0±0.1)
EAE100	135.2±1.64	81.2±0.74	70.4±0.18	48.7±0.38	290.167±3.52* (-18.02±0.97)	382.67±2.92* (-2.99±0.62)
EAE200	95.4±0.44	60.3±0.38	52.4±0.18	53.3±0.45	239.17±2.63* **(-32.51±2.96)**	358.5±2.61* **(-10.02±0.32)**
Glibenclamide (1 mg/kg b.w)	74.0±0.54	45.2±0.29	36.02±0.38	63.0±0.83	207.67±1.98* (-40.10±0.97)	354.34±2.29* (-9.15±0.12)

*p<0.05; The values are in mean±SD (n=6)

CONCLUSION

To draw to a close, the experimental findings suggest that the administration of ethyl acetate extract of seaweed *C. baltica* (at a safe dose) suppresses free radicals and α-glucosidase enzyme along with STZ-induced diabetes in albino rats. The main metabolites - **4** and **5,** claimed to be responsible for these activities. The outcomes of the histopathological study revealed better effect on regeneration of islets of Langerhans and this, *in vivo* study explained the biological importance of *C. baltica* in the production of novel agents like **4** and **5** which, clarify the treatment of oxidative stress conditions. This study is the first report of α-glucosidase inhibitory, hypoglycaemic, anti-hyperlipidaemia and anti-hyperglycaemic properties. The results of the current study remain useful for further research on diabetes to identify the potential bioactive molecules from different aquatic fauna which includes seaweeds like *C. baltica*.

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CONFLICT OF INTEREST

No conflict of interest between any of the authors.

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