Muscle extract of *Arothron immaculatus* regulates the blood glucose level and the antioxidant system in high-fat diet and streptozotocin induced diabetic rats

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ABSTRACT

In the present study pufferfish, *Arothron immaculatus* muscle methanol extract (AIME) was used to evaluate the antidiabetic activity against the high-fat diet (HFD) in streptozotocin (STZ) induced diabetic rat models. Initially, the *In vitro* antioxidant activity of the different muscle extract was evaluated which showed that AIME has higher efficiency to scavenge the free radicals. The animal study results revealed that the AIME could decrease the blood glucose level after 14 days of oral treatment and recover the animal from the severe progression of the disease. The LC-ESI/MS analysis of AIME extract revealed the presence of compounds such as docosahexaenoic acid, adrenic acid, docosanol, codeine and metoprolol. Among these compounds, docosahexaenoic acid, adrenic acid and docosanol are reported for its antidiabetic studies. Hence, the muscle is recommended to consume by humans as natural food in order to overcome the development of diabetes. This is the first study on the muscle extract of marine pufferfish which is used as antidiabetic agent to treat the diabetes-induced in the animal model.

1. Introduction

Diabetes mellitus (DM) and its obvious secondary complications such as kidney, nervous system and microvascular disorders and further threatened health conditions of nephropathy, neuropathy, retinopathy, diabetic foot, ketoacidosis, cardiovascular diseases and hypertension are a serious concern of today [1]. Diabetes is classified as type-1 resulting due to complete necrosis of beta cells in response to triggered autoimmunity and type-2 caused by genetics and lifestyle factors [2]. Globally 90–95% of people were affected with type-2 diabetes. Obesity-related to high calorie diet and sedentary way of life causes biochemical abnormalities and high glucose concentration in the blood which reduces β-insulin sensitivity and leads to insufficient secretion of insulin by β-cells of the pancreas [3,4].

A global survey stated that the expected incidence of diabetes and projection for the year 2030 is 350 million [5]. In spite of difficulties in maintaining proper glycemic control with available pharmaceutical approaches [6], the serious side effects include hypoglycemic coma, hepatorenal dysfunction, etc [7]. Therefore, replacing the pharmaceutical products with supplementary medicines and through the diet was of great interest. Nowadays, increasing diabetic and its related complication with their limited anti-dotes has led to a search for novel compounds, particularly from marine sources have attracted the scientific group of people and produce a number of novel scaffolds often with unusual skeletons [8].

Marine plants and animal species have been screened for potential antihyperglycemic and antidiabetic activities. Derivatives from marine fishes have a promising antioxidant and antidiabetic properties [9]. The collagen peptides from marine wild fish decrease free fatty acids and regulate nuclear receptor metabolism in type-2 diabetic patients [10]. Among, the family Tetraodontidae, commonly called as puffers, mostly found in shallow, warm, tropical and temperate water habituated fish, is the largest family in the order Tetraodontiformes comprises 184 species belonging to 27 genera [11]. Puffers have a long and rich

cooking history in East Asian countries including China, Korea, and Japan that have been locally harvested for food and medicine for centuries. In East Asia and elsewhere, modern technologies have enabled the demand of puffers supply in global aquaculture trade [12]. Hence, the present study was aimed to explore the antidiabetic potential of *Arothron immaculatus* muscle methanolic extract in the high-fat diet (HFD) and streptozotocin (STZ) induced diabetic rats.

2. Materials and methods

2.1. Drugs and chemicals

2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), α amylase and α glucosidase enzyme, starch, dinitrosalicilicacid (DNS), p-nitrophenyl-β-D-glucopyranoside (p-NPG) and acarbose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Streptozotocin (STZ) was obtained from Sisco Research Laboratory, India, Metformin from Merck, Germany. All the chemical solution was prepared using double distilled water. Animal feed was procured from Sai Enterprises, Bangalore, India.

2.2. Animal collection and preservation

Pufferfish specimens of *Arothron immaculatus* Fig. S1 (Order: Tetradontiformes; Family: Tetradontidae) were purchased from the fisherman in fresh condition, Mandapam fish landing centre, Gulf of Mannar Biosphere Reserve, India (Latitude 9°16′14″N; Longitude 79°7′10″E) during the period of January to March 2017. The total of 10 specimens was brought to the laboratory, washed and stored at −20° C until further analysis. The standard length (SL) and body weight (BW) of specimens range from 7.5 to 28 cm and 65.9 to 302 g respectively. The specimens were identified following the standard publications [13].

2.3. Sample extraction

From the stored fish sample, the muscle was removed and dried by exposing the sample in a hot air oven at 60° C to remove the moisture content. The dried sample is ground to powder by using mortar and pestle. Extraction of bioactive compounds from the fish muscle powder is done using various solvents like ethanol (AIEE), methanol (AIME), 50% ethanol methanol (AIEM), acetone (AIAE), hexane (AIHE), petroleum ether (AIPE) and chloroform (AICE) using soxhlet apparatus. The muscle sample of 20 gm and 200 mL of respective solvents were used for the extraction process for 12 h. The extracted sample was condensed to dryness using rotary evaporator at 50 °C. The extract was further dried, stored and used for the study.

2.4. In vitro antioxidant activity

The ability of the different fish muscle extract to quench the free radicals was checked by performing different *In vitro* antioxidant activities like ABTS, DPPH, ferric reducing power activity.

2.4.1. 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS. +) radical scavenging assay

The amount of ABTS free radicals scavenged by the *A. immaculatus* muscle extract was performed following Re et al. [14] Various concentration of *A. immaculatus* muscle extract (20–100 μg/mL) was added and allowed to react with the ABTS solution (1 mL) and incubated for 10 min. The absorbance of the resultant reaction mixture was measured in UV spectrophotometer at 734 nm against the blank solution. The scavenging ability was calculated by

Scavenging ability (%)

$$= (A734 \ \text{control} - A734 \ \text{sample}/A734 \ \text{control}) \times 100$$

Where, A734 control – absorbance of control at 734 nm, A734 sample– absorbance of the sample at 734 nm.

2.4.2. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

To the 1 mL of 20 mM DPPH, the various concentration of *A. immaculatus* muscle extract (20–100 μg/mL) was added and the reaction was preceded by incubating at 37 °C for 30 min. The absorbance of the resultant mixture was measured using UV spectrophotometer at 517 nm against the blank solution. The scavenging activity was calculated by

Scavenging ability (%)

$$= (A517 \ \text{control} - A517 \ \text{sample}/A517 \ \text{control}) \times 100$$

Where, A517 control – Absorbance of control at 517 nm, A517 sample – absorbance of the sample at 517 nm [15].

2.4.3. Ferric reducing power activity

Various concentrations of *A. immaculatus* muscle extract were allowed to react with potassium ferricyanide and phosphate buffer (pH–6.6) and were reacted at 50 °C for 20 min. To the above mixture, 10% TCA was added, centrifuged at 340g for 10 min. The supernatant was collected and it was mixed with distilled water and ferric chloride. The resultant reaction mixture was then measured at 700 nm using spectrophotometer [16].

2.5. LC-ESI/MS analysis

In order to identify the bioactive compounds present in the *A. immaculatus*, LC-MS analysis was performed using Ultra performance liquid chromatography apparatus Waters UPLC-TQD-ESI–MS. PDA detector was used for the study is UPLC LG 500 nm detector, Waters, USA, and C18 column was used to separate the compound (ACCUCORE C18 100 × 3 mm, 2.6 μm column, Waters, USA). Water with 0.1% formic acid was used as solvent A and methanol was used as solvent B. Gradient program was obtained from the modified procedure of Ramirez et al. [17]. The program used was 93% B (0 min), 85% B (7 min), 75% B (12 min), 60% B (21 min), 41% B (28 min), 51% B (34 min), 2% B (45 min) and 93% B (50 min). The flow rate was set at 1.0 mL/ min and the compound was detected at 100 – 1200 nm with a monitoring wavelength of 220 nm. The MS analysis was achieved in an ESI method (ACQUITY TQD mass spectrometer, Waters, USA), the detected compounds were identified based on mass and previous literature reports.

2.6. In vitro antidiabetic activity

2.6.1. Alpha-amylase inhibitory activity

To the 50 μl of phosphate buffer (20 mM phosphate buffer pH = 6.8), various concentrations of AIME extract (50–250 μg/mL), 10 μl of α–amylase enzyme (2 U/ml of enzyme dissolved in 20 mM PBS buffer) were added and allowed to react at 25° C for 30 min. To the above reaction mixture, 20 μl of soluble starch (1% starch dissolved in 20 mM of phosphate buffer at pH 6.8) was added and incubated at room temperature for 30 min. To the reaction mixture, 100 μl of Dinitrosalicilic acid (DNS) was added and incubated at 95 °C for 10 min. The change in color was measured at 540 nm in UV spectrophotometer. The above reaction with the various concentration of Acarbose (50–250 μg/mL) was used to obtain the standard solution value. The experiments were carried out in triplicate. Percentage of inhibition was calculated by

% of inhibition = (A540 control − A540 sample/A540 control) × 100

Where, A540 control – absorbance of control (without sample) at

540 nm, A540 sample –absorbance of the sample [18].

2.6.2. Alpha-glucosidase inhibitory activity

Alpha-glucosidase enzyme inhibitory activity was done following the modified protocol of Hemalatha et al. [19]. In brief, to the different concentration of AIME extract (50–250 μg/mL), 50 μl of phosphate buffer (20 mM at pH 6.8) was added. To the above mixture 10 μl of alpha-glucosidase (1 U/ml of enzyme dissolved in 20 mM phosphate buffer at pH 6.8) was added and the resultant mixture was incubated at 37 °C for 15 min. 5 mM 4-nitrophenyl α-ᴅ-glucopyranoside (P-NPG) was used as a substrate at a concentration of 20 μl was added and incubated at 37 °C for 20 min. 0.1 M sodium carbonate (Na_2CO_3) stopping solution, was added at a concentration of 50 μl. The absorbance of the colored solution was measured at 405 nm using UV spectrophotometer. The above experiment is repeated with the various concentration of Acarbose (50–250 μg/mL) to obtain the standard solution value. Control reaction was prepared using the above mixture without AIME extract. The experiments were carried out in triplicate. Percentage of inhibition was calculated by

% of inhibition = (A405 control − A405 sample/A405 control) × 100

Where, A405control – Absorbance of control (without sample) at 405 nm, A405 sample – absorbance value of the sample.

2.7. In vivo antidiabetic activity – Animal experiments

2.7.1. Animals and diets

To check the potential of AIME against diabetes, the antidiabetic study was performed in male albino Wistar rats (150–180 g of body weight (BW)). The animals were purchased from Indian Institute of Science (IISc), Bangalore-560 012 (Reg. No-48/99/CPCSEA) and maintained in the animal husbandry following laboratory procedures at 25° C, relative humidity maintained at 60–70% and 12 h of dark light cycles. Animal numbers and experimental procedures were permitted by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). All the animal experiments were carried out in accordance with relevant guidelines and regulations which are approved by Bharathidasan University Institutional Animal Ethical Committee (IAEC No: BDU/IAEC/2017/NE/28/Dt.21.03.2017). Prior to the start of the animal experiments, the animals were adapted for one week by supplying feed and water.

2.7.2. Induction of diabetes

The animals were induced with diabetes following the procedure of Gayathri et al. [20] In brief, after the period of initial adaptation, 12 animals were randomly selected and were distributed into two groups with six animals in each group and were termed as a Non diabetic control group and Non diabetic vehicle control (DMSO) group. These two groups of animals were allowed to nourish normal diet (feed) throughout the study. High-fat diet (HFD) was prepared by following Jeszka-Skowron et al. [21] procedure (Table S1). The other animals were allowed to nourish on HFD (45% energy from fat) for a period of two weeks and were administered with a single intraperitoneal injection of streptozotocin (STZ) at 35 mg/kg concentration to induce diabetes. Three days after induction, fasting blood glucose was analyzed in the tail vein blood using Sugerchek Glucometer, Wockhardt manufacture, India. The animals with the blood glucose range 300–350 mg/dL were chosen and were subjected to an oral glucose tolerance test (OGTT). The animals were fasted for 16 h and administered with glucose (3 g/kg) orally to check the oral tolerance capacity of the animals. Blood glucose concentration was reviewed at 0, 30, 60, 90 and 120 min time interval using Sugerchek Glucometer. The animals having blood glucose level ≥350 mg/dL were selected randomly and grouped as the diabetic control group (Disease control), standard drug-treated group (Metformin), A. immaculatus treated groups (AIME250 and AIME500).

2.8. Treatment

The non-diabetic control group animals were left untreated and the non-diabetic vehicle control (DMSO) group animals received 10% DMSO orally on a daily basis. The diabetic control group (disease control) animals were left untreated and standard drug-treated group animals were received metformin (single dose at 300 mg/kg BW) dissolved in double distilled water were administered orally for a period of 21 days. The last two groups of animals were administered with A. immaculatus muscle extract dissolved in 10% DMSO daily at 250 and 500 mg/kg BW concentrations respectively for a period of 21 days. During the experimental period, the feed and water consumption by the animals were recorded daily and the change in the body weight and the blood glucose concentrations were recorded on 0th, 7th, 14th and 21st day of treatment. After 21 days of treatment, the animals were fasted and sedated. The blood was collected by cardiac puncture method where the animals were anesthetized using pentobarbital (45 mg/kg) as per CPCSEA regulations and the animal was sacrificed. Liver, kidney, and pancreas were collected from the animal, washed in saline and stored at −80 °C for further analysis.

2.8.1. Plasma insulin estimation

The blood samples were collected by heart puncture method and were stored in the anticoagulated coated tube (heparin-coated tube). Plasma was separated from blood by centrifuging at 340 g, for 15 min at 4 °C. The plasma insulin was estimated by using ELISA kit method (Linco Research, USA)

2.8.2. Analysis of lipid profile, hepatic and renal function test

The collected blood was centrifuged at 340g for 20 min to obtain the serum from blood. The separated serum was stored and used for the quantification of total cholesterol (TC), triglycerides (TGL), serum glutamic oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase (ALP), urea (U) and creatinine (C) following the instructions in kit manufactured by Nice Chemicals [P] Ltd., Cochin, Kerala, India.

2.8.3. Post mitochondrial supernatant preparation

The stored tissue samples of all the group animals were homogenized using the homogenizing buffer with the composition of ice-cold phosphate buffer (0.1 M, pH − 7.4) Dithiothreitol (DTT), 1.61 mM of Ethylenediaminetetra acetic acid (EDTA), 1 M sodium chloride, Phenylmethanesulfonyl fluoride (PMSF) and Triton X 100. The sample was centrifuged at 9390g for 30 min. The supernatant was collected and stored at −20 °C and used for further studies.

2.8.4. Estimation of oxidative stress markers levels

The tissue (liver, kidney and pancreas) homogenates were used to analyze the oxidative stress markers which include superoxide dismutase (SOD) was performed following modified method of Kakkar et al. [22], reduced glutathione (GSH) was experimented using modified method of Moron et al. [23], glutathione peroxidase (GPx) was done using modified method of Rotruck et al. [24] and Lipid peroxidation (MDA) was done following the modified method of Ohkawa et al. [25].

2.9. Histopathology analysis

The liver, pancreas, and kidney tissue samples were embedded into 5 μm thick paraffin sections using 20% paraformaldehyde and with 10% neutral buffered saline. Haematoxylin and eosin (H&E) stain was used to observe the morphological changes of the tissue samples under the light microscope.

2.10. Statistical analysis

In all the experiments, the data were obtained in triplicate value and statistically analyzed using One Way ANOVA method with mean significance ($p < 0.05$) value calculated using Duncan's Post hoc test in SPSS software.

3. Results

3.1. In vitro antioxidant activity

In this study, it was attempted to explore the *In vitro* antioxidant activity of the pufferfish *A. immaculatus*. The muscle samples were extracted through the ethanol (AIEE), methanol (AIME), 50% ethanol methanol (AIEM), acetone (AIAE), hexane (AIHE), petroleum ether (AIPE) and chloroform (AICE) solvents. Out of the various solvents, *A. immaculatus* methanol extract (AIME) showed strong inhibition of the ABTS free radicals. Fig. S2a shows the ABTS free radical scavenging activity of various extracts of *A. immaculatus*. AIME exhibited the IC50 of about 165.07 µg/mL. The standard BHT showed the IC50 of about 39.45 µg/mL. The IC50 value of other samples were mentioned in Table S2.

In DPPH radical scavenging activity, higher scavenging activity was observed in the AIME whereas the *A. immaculatus* petroleum ether extract and hexane extract (AIPE and AIHE) exhibited poor scavenging activity (Fig. S2b). The AIME exhibited scavenging activity in a dose-dependent manner. The IC50 value of AIME was found to be 152.36 µg/mL (Table S2). The ability of the molecules to donate electrons is validated in the reducing power assay. The results obtained in this assay proves that *A. immaculatus* has the ability to donate the electron to ferric ion and convert them into ferrous ion. *A. immaculatus* exhibited reducing power activity in a dose-dependent manner. AIME showed potent reducing activity compared to other extracts (Fig. S2c). AIME exhibited strong *In vitro* antioxidant activity compared to the other extract. Hence, we proceeded with AIME for further experiments.

3.2. LC-ESI/MS analysis

In order to identify the bioactive compounds which possess antioxidant activity, LC-ESI/MS analysis was performed. The LC-ESI/MS analysis showed the presence of various compounds in the AIME extract. The AIME were analyzed in the positive ionization mode where the protonated ions in the sample were spotted. Tentative identification of the compounds present in AIME extracts were docosahexaenoic acid, adrenic acid, docosanol (Behenyl alcohol), Codeine ((5α,6α)-7,8-didehydro-4,5-epoxy-3-methoxy-17-methylmorphinan-6-ol) and metoprolol (1-[4-(2-methoxyethyl)phenoxy]-3-(propan-2-ylamino)propan-2-ol) (Table S3). The base peak mass chromatogram and the positive ionization chromatogram of *A. immaculatus* muscle extract compounds were given in the Fig. S3.

3.3. In vitro antidiabetic activity

3.3.1. Alpha-amylase inhibitory activity

The digestion process involves the digestive enzymes – alpha-amylase and alpha-glucosidase for the breakdown of the foods. If this process is slowed down, the immediate build-up of glucose in the circulating blood can be reduced. Hence, the ability of AIME to inhibit these digestive enzymes was checked. It was observed that AIME inhibited the alpha amylase enzyme in a dose-dependent manner. Fig. S4a shows the inhibition of alpha-amylase by AIME. It exhibited 50% of inhibition at 200 µg/mL concentration. The IC50 value of standard acarbose was found to be 98.36 µg/mL.

3.3.2. Alpha-glucosidase inhibitory activity

Alpha-glucosidase inhibitor reduces the breakdown of carbohydrate in the small intestine. AIME exhibited inhibition of the alpha-glucosidase enzyme. It was observed that AIME inhibited 50% of the enzyme at a concentration of 230.96 µg/mL. Acarbose was used as a standard. It showed 50% inhibition at a concentration of 151.77 µg/mL. Both the muscle extract and the standard exhibited inhibition of the enzyme in a dose-dependent manner (Fig. S4b).

3.4. In vivo antidiabetic activity

3.4.1. Effect of AIME on body weight, feed intake, and water intake

In vitro antidiabetic activity confirmed that the muscle extract has the ability to control the diabetes progression. This was further confirmed in rats induced with diabetes using HFD and STZ. The non-diabetic control group and nondiabetic vehicle DMSO group animals showed an increase in body weight (Fig. 1a). Due to the induction of the disease, all the experimental group animals showed a reduction in body weight. Treatment with AIME significantly slowed down the progression of the disease and managed the animal to regain the body weight to normal. AIME significantly elevated body weight after 10 days of oral treatment with AIME (p < 0.05). In spite of the decrease in body weight, the induced experimental group animals showed an increased intake of feed and water. AIME 500 treatment for 21 days significantly reduced the level of feed and water intake by the animals and brought back to normal and also brought back the body weight to the normal level which was found to be absent in the diabetic control group animals (Fig. 1b & c). The standard metformin-treated group animals showed an increase in body weight on the 7th day of the treatment.

3.4.2. Effect of AIME in blood glucose

The blood glucose level of AIME 250 and AIME 500 group animals was found to be 328.66 ± 21.5 mg/dL and 376.33 ± 26.5 mg/dL respectively on the 0th day. Treatment of AIME 500 had successfully reduced the blood glucose level on the 14th day to 170.33 ± 7.09 mg/dL. On the 21st day of the study, treatment with AIME 500 successfully reduced the blood glucose level to 137.69 ± 12.069 mg/dL whereas the treatment with AIME 250 has reduced the blood glucose level to 210.85 ± 2.88 mg/dL. In the diabetic control group animals, the blood glucose level was observed to be high throughout the study. Compared to the diabetic control group, AIME 500 treated group displayed a significant difference in the blood glucose level (p < 0.05). Fig. 1d shows the change in the blood glucose level of the experimental group of animals. The standard metformin-treated group animals also exhibited the reduction in the blood glucose level on the 7th day of the study and the animals were brought back to normal. The efficacy of the AIME extract to reduce the blood glucose level has increased at higher concentration (500 mg/kg) than the lower concentration (250 mg/kg). The change in blood glucose in the standard drug and extract treated group animals showed that the administration of metformin and AIME recovered the animals from diabetes.

3.4.3. Effect of AIME on plasma insulin

In all the induced group animals – diabetic control, metformin, AIME250 and AIME500, there was no significant change in the level of plasma insulin observed at the end of 21 days of study. These results reveal that HFD and STZ have developed diabetes without alterations in plasma insulin level.

3.4.4. Effect of AIME on hepatic and renal function test

Liver enzymes such as SGOT, SGPT, and ALP are important to study the liver functioning ability while the urea and creatinine level in the serum helps us to understand the kidney functioning. Increased level of these enzymes in the serum shows the impairment in the function of the liver and kidney. Table 1 shows the level of SGOT, SGPT, ALP, urea and creatinine levels in our study. It was documented that at the end of 21 days of treatment, the level of SGOT, SGPT, ALP enzymes was increased in diabetic control. Similarly, the level of urea and creatinine
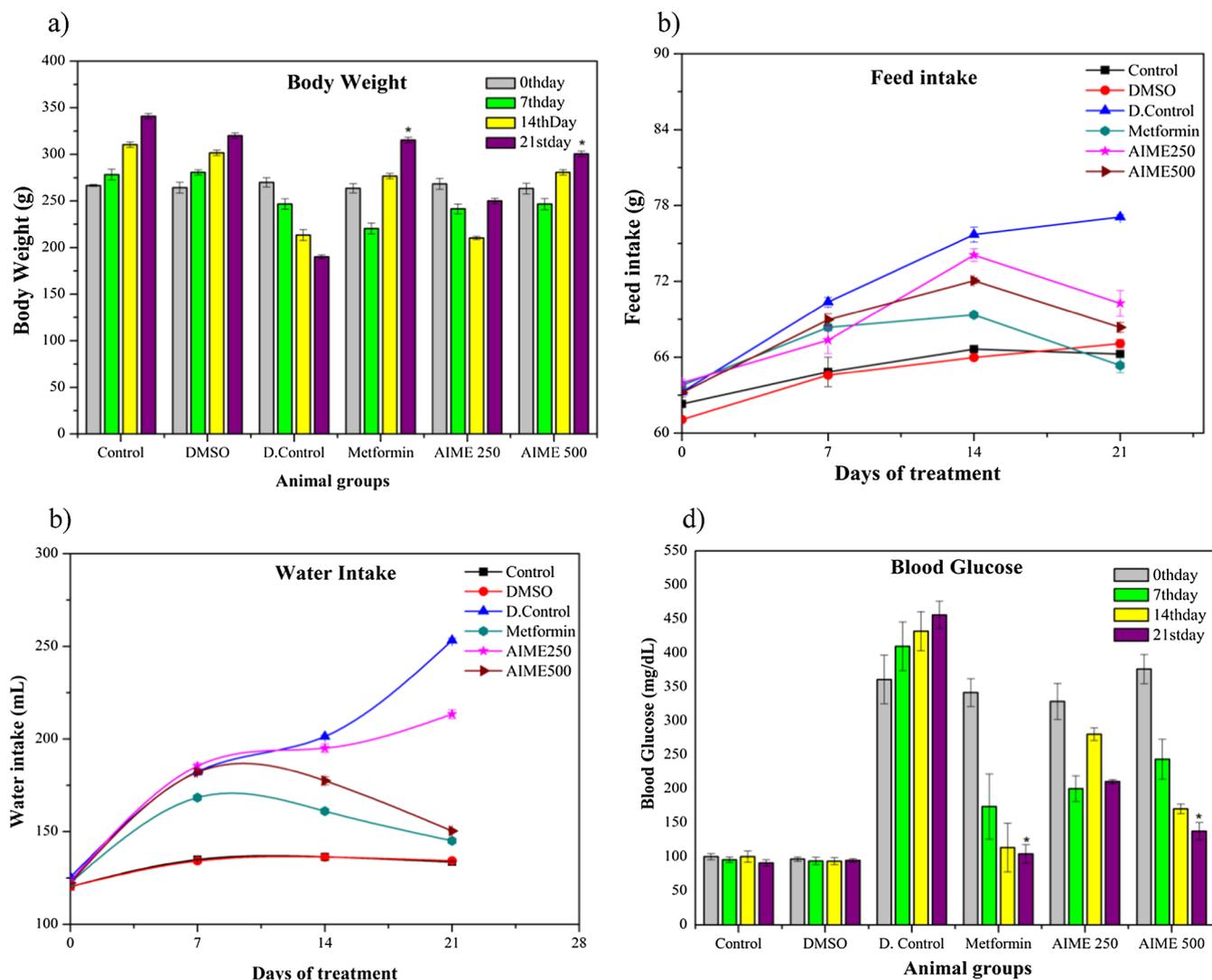
Fig. 1. (a) Change in the body weight of experimental animal groups (b) Feed intake capacity of experimental animal groups (c) Water intake capacity of experimental animal groups (d) Change in blood glucose level of experimental animal groups Each value is expressed as "mean $\pm$ SD" (N = 6).* indicates the significance difference between the treatment group (AIME500 and metformin) with the disease control group ($p < 0.05$).

was found to be increased in the diabetic control group animals. This depicts that the development of diabetes in the animal group has caused noteworthy damage to the liver and kidney. Treatment with AIME250 and AIME500 has recovered the animals from liver and kidney damage successfully. Significant reduction in the serum SGOT, SGPT, ALP content, the urea and creatinine content ($p < 0.05$) in the AIME500 treated animal groups was observed. This confirms that AIME500 has the potential to overcome the liver and kidney damage caused due to

the development of diabetes. The standard metformin-treated group animals also showed an increase in the liver and kidney function test and recovered the animals from kidney and liver damage at the end of the study.

3.4.5. Effect of AIME on triglycerides and cholesterol

The level of the triglycerides and cholesterol was found to be higher in the diabetic control group. At the end of 21 days of the study, it was

Table 1
Estimation of fasting insulin and biochemical parameters in experimental animals on 21st day.

Number of groups	Insulin (mIU/L)	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	Total cholesterol, mg/dL	Triglycerides, mg/dL	Urea, mg/dL	Creatinine, mg/dL
Control	0. 25 ± 1.03	84.66 ± 1.52[e]	25.78 ± 3.33[c]	127.07 ± 1.19[c,d]	119.36 ± 2.6[d,]	115.78 ± 2.28[d]	20.22 ± 0.60[c]	0.22 ± 0.12[d]
DMSO	0. 23 ± 0.51	86.33 ± 3.51[e]	25.49 ± 1.20[c]	125.06 ± 1.91[d]	120.43 ± 1.69[c,d]	117 ± 3.00[c]	20.50 ± 1.50[c]	0.24 ± 0.02[d]
D. Control	0.22 ± 0.99	146.66 ± 1.35[a]	63.90 ± 3.65[a]	288.6 ± 0.89[a]	210.63 ± 2.6[a]	239 ± 1.35[a]	42 ± 1.19[a]	1.21 ± 0.10[a]
Standard Metformin	0.23 ± 2.02	96.33 ± 3.05[d]	23.11 ± 1.83[d]	129.79 ± 2.60[c]	121.66 ± 3.2[c]	118.66 ± 1.52[c]	20.69 ± 0.92[c]	0.28 ± 0.16[c]
AIME 250	0. 22 ± 0.01	109.66 ± 2.08[b]	33.71 ± 3.36[b]	175.36 ± 4.54[b]	157.3 ± 2.08[b]	131.33 ± 4.50[b]	34.62 ± 0.65[b]	0.89 ± 0.07[b]
AIME 500	0. 23 ± 0.02	99.66 ± 4.50[c]	26.13 ± 3.46[c]	128.53 ± 3.23[c]	121.2 ± 1.31[c]	116.24 ± 4.50[c,d]	20.47 ± 0.51[c]	0.23 ± 0.05[d]

Each value is expressed as "mean $\pm$ SD" (N = 6).
Mean values with different superscripts are significantly different from each other as revealed by Duncan post hoc test ($p < 0.05$).
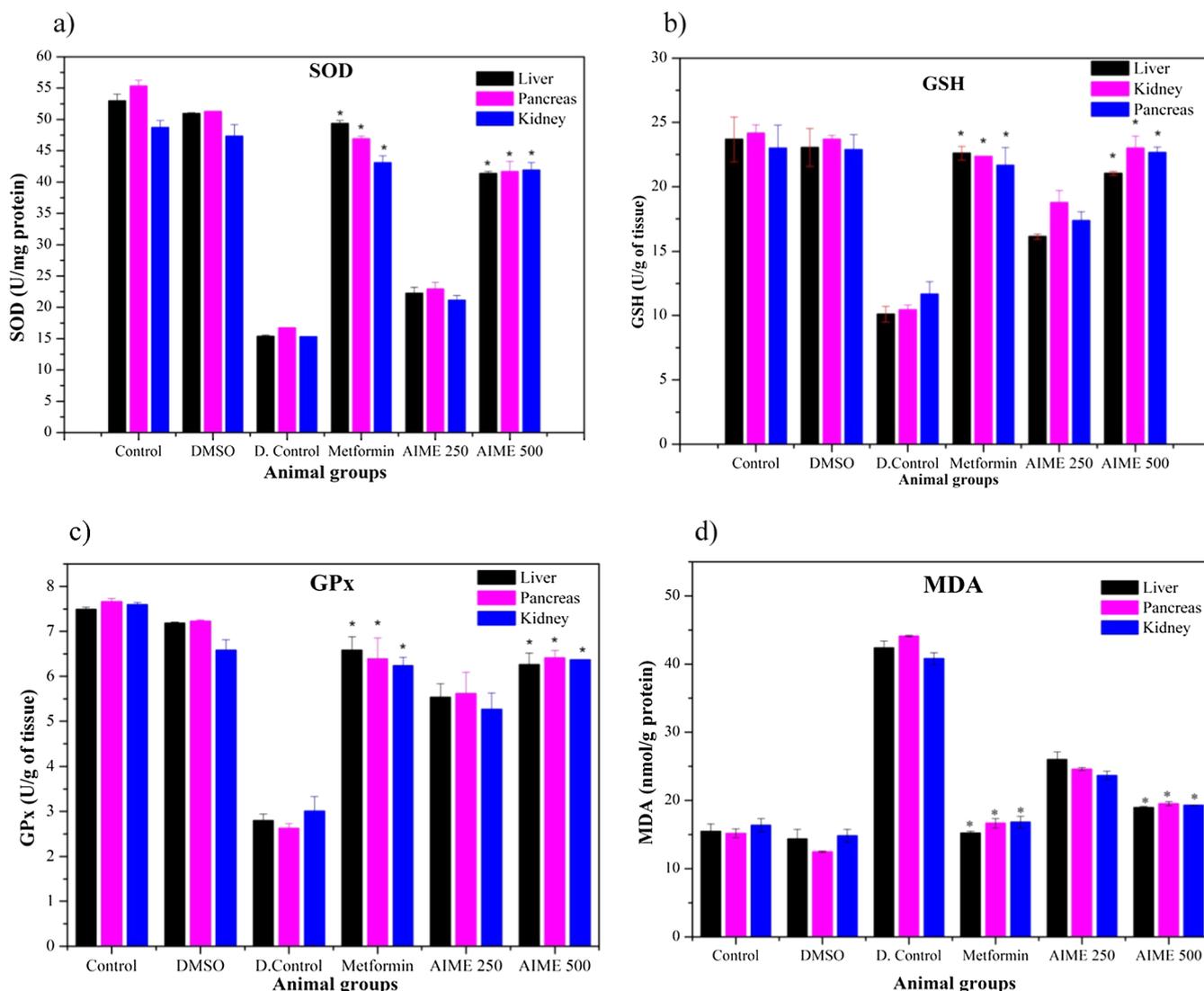
Fig. 2. (a) Estimation of SOD level in experimental rats. (b) Estimation of GSH in experimental rats. (c) Estimation of GPx in experimental rats (d) Estimation of MDA in experimental rats each value is expressed as the "mean ± SD" (N = 6). * indicates the significance difference between value of the treatment group (AIME500 and metformin) with the disease control group ($p < 0.05$).

recorded that treatment with AIME250 and AIME500 showed an improvement in the triglyceride and cholesterol level. AIME500 treated animal group significantly reverted back the triglyceride level in the animal serum compared to the diabetic control group animals ($p < 0.05$). The serum cholesterol level was significantly influenced by the treatment with AIME 500 and standard drug metformin. The level of cholesterol was observed to be reduced on treatment with AIME500 and metformin at the end of the study (Table 1).

3.4.6. Effect of AIME on oxidative stress markers of liver, kidney, and pancreas

The *in vivo* antioxidant potential of AIME in the liver, kidney and pancreatic tissues were given in Fig. 2. It was observed that at the end of 21 days of the observation, the diabetic control group animal showed a decrease in the concentration of SOD, GSH and GPx (Fig. 2a, b & c) whereas the MDA level was observed to be increased (Fig. 2d). This clearly indicated that oxidative stress has developed in the animal on HFD + STZ induction, and has contributed to the progression of the disease. Oral treatment with AIME250, AIME500, and metformin for 21 days has controlled the level of oxidative stress developed successfully and has created a protective state for the animals to overcome diabetes.

3.5. Histopathology analysis

The morphology of the liver was observed in Fig. 3. The diabetic control group showed the swelling of the hepatic cells with the accretion of the lipids. The AIME extract treated animals (AIME250 and AIME500) and metformin-treated group animals indicated the regular sized and normal hepatic cells development similar to that of the normal nondiabetic control and nondiabetic DMSO treated group animals.

Fig. 4 shows the histopathological observation of pancreas under the light microscope. The image was observed with the damage in the islet of Langerhans in the pancreas in the diabetic control group. The β cell size was shrunken and the cell structure was destructed with their numbers reduced. In the nondiabetic control and the nondiabetic DMSO treated group animals, the healthy and proliferating cells were noticed. AIME250, AIME500, and metformin-treated group animals were observed significant recovery with the regenerating healthy cells from the disease.

The morphology of the kidney cells was shown in Fig. 5. The disease control group animals showed collapsed nephron cells and glomeruli. Development of endocytic vacuole was observed in the disease control cells. Whereas the control and DMSO group animals showed normal
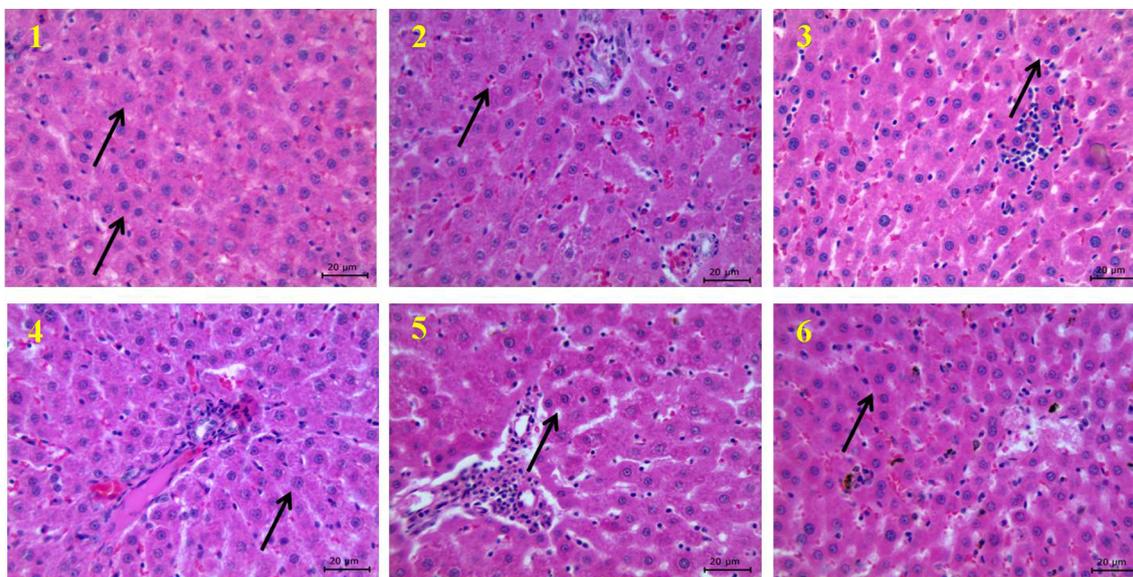
Fig. 3. Histology of the liver tissue sample of diabetes induced animal groups. 1 – Control group, 2 – Vehicle DMSO group, 3 – D.control group, 4 – Metformin treated group, 5 – AIME 250 treated group and 6 – AIME 500 treated group.

well developed nephron cells and glomeruli. The animal group treated with AIME250, AIME 500 and metformin respectively showed the development of nephron cells and glomeruli. Vacuoles development was not seen in the treated group of animals.

4. Discussion

The aim of our study is to check the antidiabetic potential of the muscle extract of *A. immaculatus* in the HFD and STZ-induced diabetic rat models. Our results demonstrated that *A. immaculatus* muscle extract has greatly reduced the blood glucose level on 14 days of treatment and recovered the animal from diabetes and reverted back the animals to normal. Type 2 Diabetes mellitus – a chronic disorder which requires the constant medical check and proper self-management by a patient for the control of disease and to avoid complications developed through the disease/diabetics. Many drugs are commercially available to overcome this disorder. Now the researchers focus on the marine organisms

using their bioactive compounds to treat against the development of the various diseases. Keeping in mind the medicinal value of marine compounds, fish-derived products such as the peptide obtained from the fish muscle, fish skin gelatin and collagen, bones, internal organs, oil, exoskeleton shells have gained a lot of interest to explore the bioactive compounds which can be of medicinal values [26].

Free radicals are the byproducts developed during the metabolic process which leads to damage the DNA and the proteins by causing severe oxidative stress. These free radicals were the basic reason for the development of the various medical complications such as cancer, diabetes, neurodegenerative disorders and so on [27]. Even though many synthetic drugs are available to control the oxidative damage, control through diet also plays an equal role [28]. Ali et al. [29] have reported the antioxidant potential of *Katsuwonus pelamis* heart muscle using 70% ethanol extract. He has further reported that *K. pelamis* heart muscle could scavenge the free radicals efficiently. Similarly, in our study, it was observed that among various extracts tested, AIME could
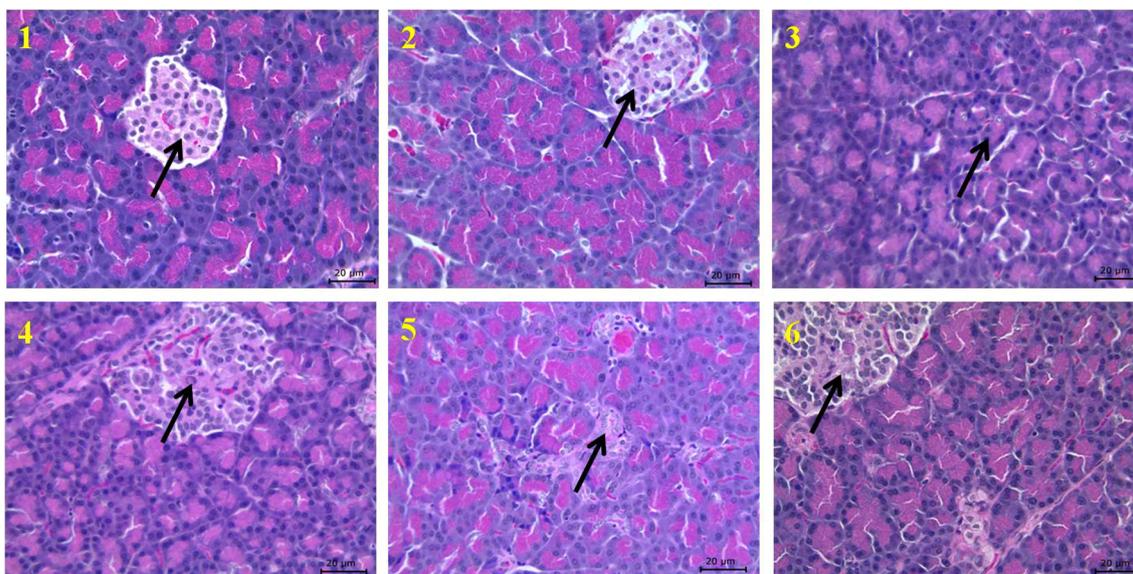

Fig. 4. Histology of the pancreas tissue sample of diabetes induced animal groups. 1 – Control group, 2 – Vehicle DMSO group, 3 – D.control group, 4 – Metformin treated group, 5 – AIME 250 treated group and 6 – AIME 500 treated group.
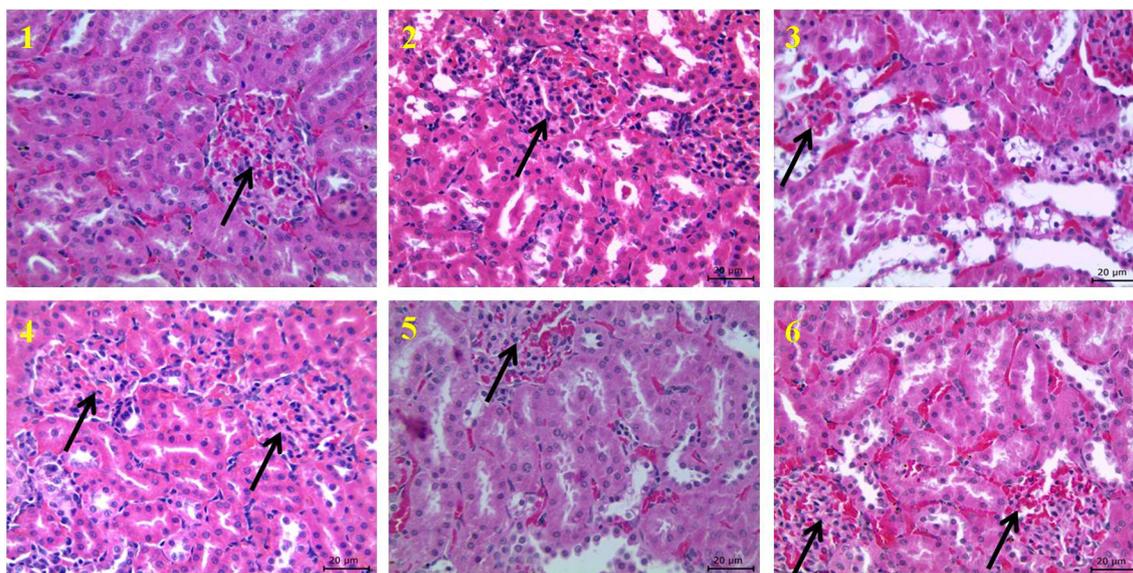
Fig. 5. Histology of the kidney tissue sample of diabetes induced animal groups. 1 – Control group, 2 – Vehicle DMSO group, 3 – D.control group, 4 – Metformin treated group, 5 – AIME 250 treated group and 6 – AIME 500 treated group.

scavenge the ABTS and DPPH free radical and possess the reducing power activity in a dose-dependent manner. The identification of the antioxidant property in puffers may be due to the presence of the bioactive compounds. Our result is well supported by Centenaro et al. [30], who have reported that fish hydrolysate exhibits free radical scavenging property.

The LC-ESI/MS analysis recorded the presence of compounds like docosahexaenoic acid, adrenic acid, docosanol (Behenyl alcohol), co-deine ((5α,6α)-7,8-didehydro-4,5-epoxy-3-methoxy-17-methylmor-phinan-6-ol) and metoprolol (1-[4-(2-methoxyethyl)phenoxy]-3-(propan-2-ylamino)propan-2-ol). Some of these compounds are reported for displaying antidiabetic property. Docosahexaenoic acid (DHA) is a polyunsaturated fatty acid. DHA is essential for maintaining the structure and function of the brain. It is reported for possessing many medicinal properties such as proper fetal development, improved cardiovascular functioning, anti-inflammatory property, improved platelet levels and also recovers from Alzheimer's disease [31]. Fishes show an increased content of DHA. DHA has a role in the control of blood glucose and lipid levels thus showing antidiabetic activity. Li et al. [32] have demonstrated that the antidiabetic activity of doc-osahexaenoic acid in STZ-induced diabetic mice. Adrenic acid is also known as docosatetraenoic acid is one of the omega 6 fatty acids which are biologically active in nature. It is also remembered the presence of adrenic acid in jumbo squid liver has been reported by Rubio-Rodríguez et al. [33]. The essential role of adrenic acid in the control of type 2 diabetes is already reported [34]. Jhong et al. [35] has demonstrated the *In vitro* antidiabetic activity in the docosanol and has reported the ability of docosanol to inhibit the digestive enzymes. They have also demonstrated the binding affinity of docosanol through *in silico* studies. Janaki et al. [36] have reported that the antidiabetic activity of *Fusinus nicobaricus* might be due to the presence of one of the bioactive compound docosanol.

α-amylase is a digestive enzyme which has a major role in the conversion of complex starch into smaller oligosaccharides. Inhibitor of α-amylase enzyme helps to delay the hydrolysis of the starch which in turn leads to the reduction of glucose absorption and ultimately leads to lesser postprandial blood glucose level [37]. AIME expressed a better activity in the inhibition of the α-amylase enzyme. Similarly, AIME could also inhibit another important digestive enzyme, α-glucosidase effectively. The enzyme α-glucosidase present in the borders of the small intestine were involved in the reduction of disaccharides into

simple sugar so that it can be easily absorbed into the small intestine. Subsequently, inhibition of these enzymes may aid in controlling the circulating blood glucose [19]. Similar results of inhibition of α-glu-cosidase enzyme activity were observed in the *K. pelamis* heart muscle treatment [29].

Based on the inhibition of the digestive enzymes by AIME, it was attempted to explore the antidiabetic activity of AIME in HFD + STZ induced diabetic rats. Notably, literary reports are available which states that animal administrated with a low dose of streptozotocin leads to the development of diabetes. However, this model does not progress the insulin resistance typically seen in T2DM. Hence, a combination of HFD with STZ is involved in this study for the development of the disease where HFD leads to the development of insulin resistance and low dose STZ induction will lead to the destruction of pancreatic β cells which mimics the type 2 diabetes pathogenesis [38]. HFD for a period of 14 days followed by a single intraperitoneal injection of STZ at 35 mg/kg concentration forms the best model for the development of type 2 diabetes. Previously, Wang et al. [39] have reported that HFD induction for 4 weeks and STZ injection at 30 mg/kg forms the best model for the development of diabetes. Increasing the period of HFD for the animals may lead to the death of the animals. Hence in our study, the modified procedure of [39] has been adopted.

The general symptom of diabetes is the loss in the body weight with the polyphagia and polydipsia symptoms. The reason for the weight loss in diabetes patients may be due to the excessive structural protein de-gradation due to the progression of the disease in spite of excessive feed and water intake [40]. In our study AIME500 treatment has recovered the animals from weight loss by decreasing protein degradation. No-tably, decrease in the feed and water intake by the animals was observed. Whereas the disease control group animal showed that the body weight of the animal kept on decreasing throughout the study.

Hyperglycemia is the dominant symptom of diabetes. In our study, the blood glucose level of the diabetic group found to be predominantly increased throughout the study. AIME500 treatment could significantly control the blood glucose level maintained the animal to normal. It is noteworthy that throughout the study, there is no alteration in the level of plasma insulin. In all the induced group animals, HFD and STZ have developed diabetes which was noticed by increasing blood glucose level, but the level of plasma insulin remains unchanged. The reason behind the maintenance of the plasma insulin was that the nearly 15% of the β-cell mass was present in the animals which can produce the

insulin and maintained the level to normal but insulin resistance condition was developed which has caused an increase in the blood glucose [41].

Previous literature by Huang and Wu [42] also support our results. He has reported that the peptide extracted from the shark liver is also able to control the blood glucose level. Cui-Feng et al. [41] have reported that oligopeptide from marine Solomon skin has reduced the blood glucose in the HFD + STZ-induced diabetic rats, but there was no significant change in the level of insulin throughout the study. Diabetes may also lead to the development of the lipid abnormalities in the body. The lipid abnormalities may include hypertriglyceridemia and increased free fatty acids levels [43]. In our study, the level of triglycerides increased in diabetic condition. Treatment with AIME500 shows reduced triglyceride and cholesterol content. A similar result was reported by Keapai et al. [44] who have reported that the consumption of fish oil reduced the triglyceride and total cholesterol content in diabetic rats.

Increase in the serum intracellular enzyme such as SGOT, SGPT, ALP, Urea and creatinine indicates that the streptozotocin has caused damage to the liver and kidney tissue. Liver forms vital organ for detoxification and excretion of the toxic materials. STZ cause damage to the liver which leads to the release of these enzymes from the liver cytosol and hence the enzyme concentration founds to be increased in the serum [45]. In diabetic condition, these enzyme concentrations found to be higher in the serum. Treatment with AIME500 has caused the animal to recover from the damage caused during the induction of diabetes. Similarly, one of the complications of diabetes is kidney damage/ failure. Failure of the kidney may lead to an increase in the level of urea and creatinine in the serum. In the present study, the animals induced with diabetes have caused the kidney damage and hence in the diabetic control group, the level of urea and creatinine is found to be higher. Treatment with AIME500 has recovered the animal from kidney damage hence the urea and creatinine content was found to be decreased in the treated group animals.

The prolonged hyperglycaemic condition has lead to increased oxidative stress condition in the diabetic group. Our body produces antioxidants to take control of the oxidative stress created by the free radicals. Superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH) are some of the antioxidant enzymes produced in our body [46]. In this study, changes in the oxidative stress markers due to the onset of diabetes were recorded. SOD is one of the antioxidant enzymes which play an important role in safeguarding the cells against the oxidative stress. The superoxide radical which forms because of oxidative stress gets reduced to hydrogen peroxide. The hydrogen peroxide form gets reduced to water molecules [47]. The GSH helps in hindering the free radical to oxidize through lipid peroxidation and GPxreduces the peroxidative stress. Likewise, fatty acids on breakdown release MDA. Increased MDA concentration shows a higher level of lipid peroxidation occurring in our body which causes increased oxidative stress [48]. In our results, oxidative stress created in the animal due to the development of disease has been reduced on treatment with AIME500 and the animals were saved from the mortality. Huang et al. [49] have reported the shark fish livers role in reducing the oxidative stress during the diabetes progression.

5. Conclusion

In conclusion, the emergence of the population with diabetes has been increasing day by day. Hence, the search for a suitable drug with the antidiabetic potential is in great demand. Since diet plays a key role in controlling and the management of the disease by the consumption of the food rich in medicinal value has been increasing. The fish *A. immaculatus* showed the presence of many bioactive compounds which has antidiabetic property. This fish can be consumed by people for the control of diabetes when it is carefully handled (TTX) and properly cooked. However, the detailed study is required in order to explore the mechanism in which the bioactive compounds of *A. immaculate* influence to control diabetes.

Declaration of Competing Interest

We declare that we have no conflict of interest

Appendix A. Supplementary material

Supplementary data to this article can be found online at https:// doi.org/10.1016/j.bioorg.2019.103072.

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