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Effect of *Begonia malabarica* fraction on streptozotocin induced insulitis and β-cell damage

P Pandikumar, S Muthukanagavel, S Mutheeswaran, P Ganesan, S Ignacimuthu*
Xavier Research Foundation, St. Xavier's College, Palayamkottai, Tirunelveli, Tamil Nadu, India

Abstract
The stems of *Begonia malabarica* Lam. (Begoniaceae) is used as a food supplement and remedy for diabetes by the *Malasar* tribes in Tamil Nadu. The work assessed the protective effect of a bioactive fraction from *B. malabarica* on pancreatic β-cells of Multiple Low Dose of Streptozotocin (MLDS) induced diabetic mice. The animals were treated orally either with vehicle or with 50-200 mg/kg of the bioactive fraction (EtSF-BM) from the day of diabetes induction upto 21 days. Biochemical parameters such as glucose, MDA, MPO, insulin as well as pancreatic histopathology were assessed. By the end of the study 80% of the animals in control group were diabetic; while EtSF-BM (200 mg/kg) treatment reduced it to 20%. The treatment with EtSF-BM at 200 mg/kg concentration significantly reduced MDA (65%) and MPO (45.1%) levels. The insulin level of the pancreatic tissue was significantly increased by 2.3 fold at 200 mg/kg concentration. Histological analysis also revealed a decrement in insulitis. The results of our experiment suggest that the antidiabetic effect of *B. malabarica* might be through β-cell protection. Further experiments are required to assess potential application of *B. malabarica* as a food supplement and remedy for diabetes.

Keywords: diabetes mellitus, MLDS, pancreas, traditional food supplement

Introduction
Diabetes mellitus is one of the most common non-communicable diseases globally. Plasticity of β-cells is one of the important criteria in the etiopathogenesis of diabetes [1]. In both Type 1 and Type 2 diabetes, a progressive β-cell failure has been observed. Though many differences occur between the mechanisms leading to nutrient- and cytokine-induced β-cell death in Type 1 and Type 2 diabetes, they share some commonalities among the activation of a common pathway invoving 1L-1 β , NF-κB and Fas [2].
Novel drug targets proposed to preserve the β-cell mass and its function might allow the cell to compensate better for peripheral insulin resistance and even to avoid progression of the disease condition [3]. Therapeutic molecules capable of increasing β-cell mass *in vivo* by systemic use have the advantage of eliminating the need of invasive surgical procedures [4]. In animal models, expansion of islet mass and stimulation of insulin secretion have been shown to occur in response to glucagon like peptide-1 (GLP-1) and its receptor agonist, exendin-4 [5, 6].
Begonia malabarica Lam. (Begoniaceae) is a sub-shrub occurring in occasional clumps above 900 m, in peninsular India and Sri Lanka. The stems are used as a food supplement and remedy for diabetes by the *Malasar* tribe, in Coimbatore District [7]. The hypoglycemic and antihyperglycemic effects of this plant were reported by us previously [8]. This work was aimed to assess the protective effect of a bioactive fraction from *B. malabarica* on pancreatic β-cells of MLDS diabetic mice.

Materials and methods
Chemicals
The solvents (hexane, ethylacetate, methanol and ethanol) were purchased from Hi-Media, Mumbai, India. Streptozotocin (STZ) was purchased from Sigma chemicals, India. All the other chemicals were purchased from Rankem, Mumbai, India and were of analytical grade.

Extraction
Plant material
The stems of *B. malabarica* were collected from Coimbatore District, Tamil Nadu, in January, 2012. The voucher samples (ERI-P4b) were collected at reproductive stage in duplicate, processed and deposited in the herbarium of Entomology Research Institute, Loyola College, Chennai. The botanical identity was confirmed with the help of taxonomist at Loyola College, Chennai.

Preparation of Bioactive Fraction
Anthocyanin rich fraction was prepared according to the method of Wang and coworkers with modifications [9]. Fresh stems (1 kg) of *B. malabarica* were washed, ground well and extracted with methanol (3 l). The extracts were filtered using Whatman number No. 1 filter paper and condensed under reduced pressure. The condensed methanol extract (11.5 g) was dissolved with equal volume of water and washed with hexane (3 x 100 ml) followed by ethylacetate (3 x 100 ml) to remove non-polar compounds. The hexane (0.31 g) and ethylacetate (1.1 g) soluble portions were condensed under reduced pressure and stored separately. The hexane, ethylacetate insoluble portion was lyophilized to remove water and it (8.2 g) was dissolved in absolute ethanol. The sugars present in the extract were removed as an insoluble residue (4.5 g). The ethanol soluble portion contained high amount of anthocyanins (3.2 g); it was decanted and condensed under reduced pressure. This fraction was named as EtSF-BM and used for further studies.

Determination of Total Anthocyanin Content in the Fractions
Total anthocyanin content of the ethanol soluble and insoluble fractions was measured using the pH differential method [10, 11]. The samples (100 µg) were dissolved in

potassium chloride buffer (KCl, 0.025 M, pH 1.0) and sodium acetate ($CH_3CO_2Na.3H_2O$, 0.4 M, pH 4.5) with a pre-determined dilution factor. The absorbance of each sample was read at 520 and 700 nm against a blank (deionized water). The absorbance (A) of the diluted sample was calculated as follows: $A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$. The concentration of the monomeric anthocyanin pigment in the original sample was calculated using the formula:

$$\text{Anthocyanin content (mg/g fraction)} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

in which the molecular weight of cyanidin-3-*O*-glucoside (MW = 449.2), the dilution factor or dilution multiple and the molar absorptivity constant (ε = 29,600) were used. The results were expressed as the mean values for five replicates.

Animals

Three to four weeks old, male mice (Balb/c strain) weighing 22 ± 1 g were used in this study. The animals were maintained in polypropylene cages at 21 ± 1°C with a relative humidity of 50 ± 5% and 12/12 light/dark cycles, fed (except in fasting condition) with commercial pellet diet purchased and watered *ad libitum*. The feed contained 74% carbohydrate, 22% protein and 4% fat. The animals were fasted from 7.00 a.m. to 11.00 a.m., if required. All these protocols were reviewed and approved by the Institutional Animal Ethics Committee (Clearance number: IAEC-ERI-LC-11).

Induction of Diabetes and Treatment

The induction of diabetes was carried out according to standard procedure [12]. The animals were fasted briefly and injected intra peritoneally with 40 mg/kg of STZ (Ice-cold citrate buffer, pH 4.5) for five consecutive days. Two hours after induction, the animals were treated with the fraction or with the vehicle. The EtSF-BM was dissolved in a vehicle containing 0.9% NaCl, 0.2% Polysorbate-80, 0.5% Carboxy Methyl Cellulose, 0.9% benzyl alcohol and 97.2% double distilled water. The vehicle alone served as a negative control. On the basis of body weight, the animals were randomly assorted into five groups containing ten animals in each group. Group I consisted of normal control animals treated with the vehicle; group II consisted of normal control animals treated with EtSF-BM at higher dose (200 mg/kg); group III consisted of MLDS induced diabetic animals treated with the vehicle; and group IV-VI consisted of MLDS induced diabetic animals treated with 50, 100 and 200 mg/kg of EtSF-BM, respectively. The treatment was given orally for 21 days from the first day of induction [13].

End of the Study

Blood samples were taken by tail clipping and blood glucose levels were measured at 1st, 7th, 14th and 21st day using one touch glucometer. At the end of the study, the animals were euthanized between 10.00 a.m. and 11.00 a.m. to minimize the diurnal variations and blood samples were collected in EDTA containing tubes. Pancreatic biopsy specimens were taken and stored either in 10% phosphate buffered formalin for histopathological analysis or snap-freezed in liquid nitrogen and stored at -70° C for biochemical analysis. The pancreatic islet specimens were stained with hematoxylin-eosin for histopathological analysis [13].

Estimation of Pancreatic Insulin Content

The pancreatic biopsy specimen (25 mg) was homogenized in 225 µl of acidified ethanol (75% ethanol, 1.5% 12 mol/l HCl, and 23.5% distilled water), incubated for 72 h at 4° C and centrifuged. The insulin content of the supernatants was estimated using an ELISA kit (Calbiochem, USA) and the results were expressed as ng insulin/mg protein.

Estimation of Malondialdehyde (MDA) Content

The pancreatic biopsy specimen (25 mg) was ground in 225 µl of 1.15% KCl buffer. Two hundred microliters of the homogenate were added to a reaction mixture containing 1.5 ml of 0.8% thiobarbituric acid, 200 µl of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 600 µl of distilled water. The mixture was then incubated at 90° C for 45 min. After cooling to room temperature, the sample was cleared by centrifugation at 10,000 x g for 10 min. The absorbance was measured at 532 nm and the results were expressed as pM MDA/mg protein.

Estimation of Myeloperoxidase (MPO) Activity

The pancreatic biopsy specimen (25 mg) was ground in 0.5 % hexadecyltrimethyl ammonium bromide dissolved in 10 mmol/L 3-N-morpholinopropane sulfonic acid (225 µl) and centrifuged at 15,000 x g for 40 min. The aliquot was mixed with a solution containing 1.6 mmol/l tetramethybenzidine and 1 mmol of hydrogenperoxide. MPO activity was measured spectrophotometrically at 650 nm and the results were expressed as mU of MPO activity per milligram of protein [14]. In all the above mentioned estimations, the protein levels were estimated using Bradford method [15].

Statistics

The values were presented as mean ± SEM for ten animals. The analysis for significance was determined separately for each biochemical parameter. One way analysis of variance (ANOVA) was done, primarily. Individual comparisons between the groups were done by two-tailed Student's *t*- test (SPSS version 11.5). The values were considered significant at $P \leq 0.05$.

Results

Anthocyanin Content of the B. malabarica Fractions

The anthocyanin content of the ethanol soluble fraction (EtSF-BM) was 523.5 ± 12.3 mg cyanidin-*O*-glycoside equivalents per gram fraction; the ethanol insoluble fraction contained only 34.5 ± 5.7 mg of cyaniding-*O*-glycoside equivalents per gram fraction.

Effect of EtSF-BM on Hyperglycemia and Diabetic Incidence of MLDS Diabetic Mice

Initially, the antidiabetic effect of all the extracts and the fractions were evaluated using streptozotocin induced diabetic animals. Among them, the methanol extract and its anthocyanin rich fraction have shown a significant antidiabetic effect (data not presented). Based on this preliminary work, the anthocyanin rich bioactive fraction of *B. malabarica* was taken and its pancreatic β-cell protective effect was evaluated using MLDS diabetic model.

Treatment with EtSF-BM significantly reduced the plasma glucose level (Figure 1a), and significantly delayed the diabetic incidence compared with the diabetic control animals (Figure 1b).

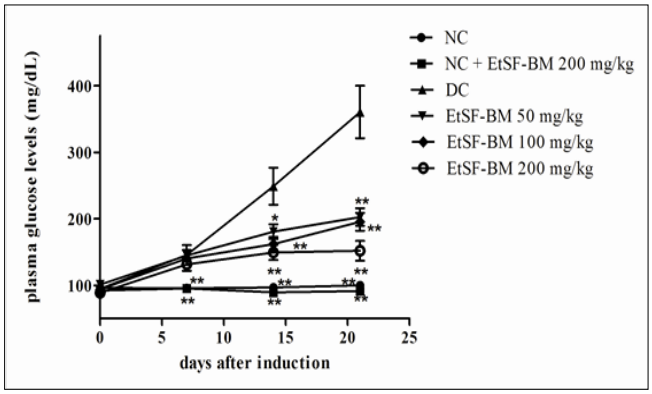


Fig 1a: Effect of the EtSF-BM on blood glucose level of MLDS induced diabetic mice.

All values represent mean ± SEM for ten animals. [NC – normal control, NC + EtSF-BM – normal animals treated with EtSF-BM, DC – Diabetic control, EtSF-BM (50-100 mg/kg) – Diabetic animals treated with varying doses of EtSF-BM]. * Values vary significantly from diabetic control animals ($P \leq 0.05$) and ** values vary very significantly from diabetic control animals ($P \leq 0.005$)**.**

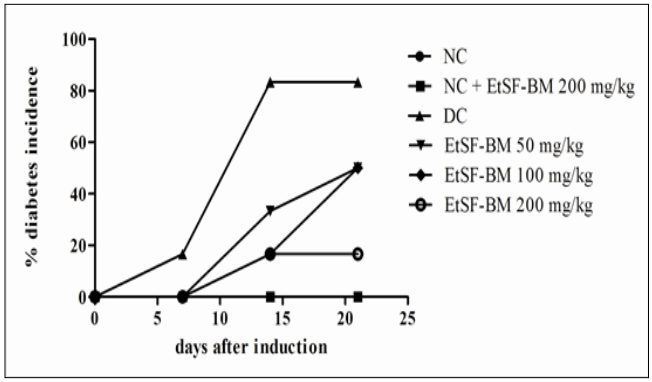
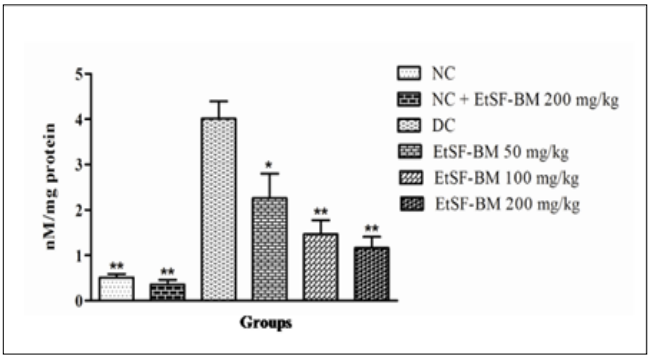


Fig 1b: Effect of the EtSF-BM on percent diabetic incidences of MLDS induced diabetic mice.

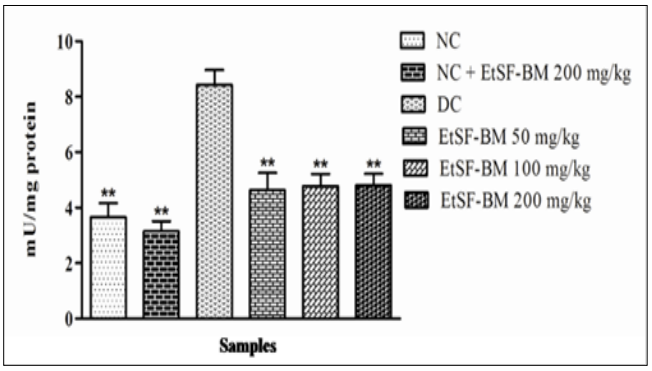
[NC – normal control, NC + EtSF-BM – normal animals treated with EtSF-BM, DC – Diabetic control, EtSF-BM (50-100 mg/kg) – Diabetic animals treated with varying doses of EtSF-BM]. Percent diabetic incidence was expressed as a cumulative percentage of mice with a blood glucose ≥200 mg/dL.

Effect of EtSF-BM on MDA, MPO and Insulin Levels of Pancreatic Specimen of MLDS Diabetic Mice

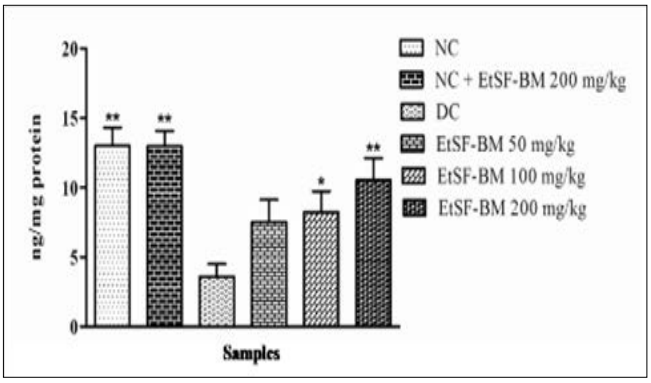
In the present study the levels of MPO and MDA in the pancreatic specimens were measured to determine the extent of inflammation and membrane damage occurred in the pancreatic tissues. EtSF-BM treated animals showed significant decrease in the MDA and MPO levels (Figures 2a & 2b) and increase in insulin levels (Figure 2c) compared with that of animals in diabetic control group.



(a)



(b)



(c)

Fig 2: Effect of the EtSF-BM on a) MDA, b) MPO and c) insulin levels of pancreatic tissue taken from MLDS induced diabetic mice.

All values represent mean ± SEM for ten animals. [NC – normal control, NC + EtSF-BM – normal animals treated with EtSF-BM, DC – Diabetic control, EtSF-BM (50-100 mg/kg) – Diabetic animals treated with varying doses of EtSF-BM]. * Values vary significantly from diabetic control animals ($P \leq 0.05$) and ** values vary very significantly from diabetic control animals ($P \leq 0.005$).

Effect of EtSF-BM on Histopathology of Pancreas of MLDS Diabetic Mice

In order to evaluate the protective effect of EtSF-BM on pancreatic islets, the biopsy specimens were subjected to histological examination. The most consistent findings in the diabetic control animals were infiltration of mononuclear cells, degenerative and necrotic changes and islet shrinkage. These changes were dose-dependently reduced with EtSF-BM treatment (Figure 3).

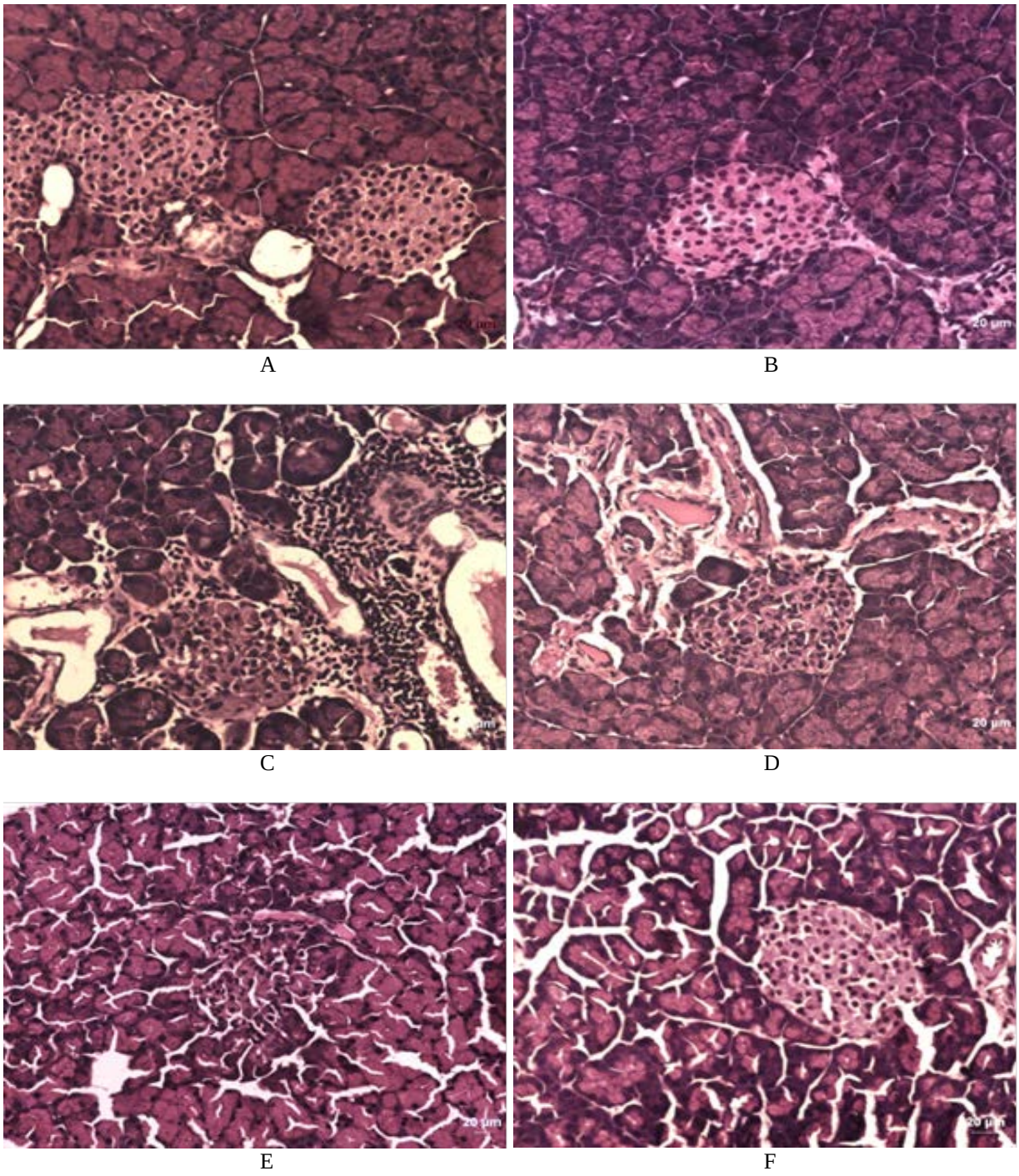


Fig 3: Effect of the EtSF-BM on the histopathology of the pancreas of MLDS induced diabetic mice.

Pancreatic biopsy specimens were stained with hematoxylin-eosin; (A)–Normal control animals (no detectable abnormalities), (B)–Normal control treated with EtSF-BM 200 mg/kg (C)–STZ control (showing islet degeneration and high MNC infiltration), (D) to (F)–treated with EtSF-BM at 50, 100 and 200 mg/kg concentrations (showing a dose dependent reduction in islet cell degeneration and MNC infiltration).

Discussion

Various animal models have been described in the literature to study immune-mediated β-cell death [16, 17]. The MLDS diabetic model was extensively used to study the immune pathways involved in the destruction of islet β-cells [18]. This animal model is characterized by extreme insulin deficiency as a result of a decrease in the number of functional β-cells not only by a direct toxic effect of STZ on β-cells but also by inflammatory reaction against damaged β-cells [19].

Administration of multiple low doses of STZ induces progressive islet cell death and hyperglycemia [20]. Our results showed that the treatment with EtSF-BM could counteract the rise in blood glucose levels. The treatment was given on days 0 to 21, and with this regimen, the blood glucose levels of the treated animals remained lower than the MLDS control animals. The treatment also lowered the diabetic incidence, while 80% of the animals in MLDS group were hyperglycemic.

Previous studies indicated that the auto-reactive cytolytic T-cells, as well as soluble ediators including proinflammatory cytokines such as TNF-α, IL-1β and free radicals, contribute to increased β-cell apoptotic destruction in diabetic condition [21]. Chronic exposure of pancreatic β cells to IL-

1β induces the expression of proinflammatory genes that results in β cell cytotoxicity, mainly through induction of iNOS and NO production [22]. The role of iNOS in the cascade of events culminating in β-cell death is well established and NO is one of the important mediators used by the immune system to destroy the β-cells [23]. In our experiment, the glucose lowering ability of EtSF-BM might be independent of preventing pancreatic β-cell damage. To assess the effect of EtSF-BM on pancreatic β-cell protection, the levels of insulin, MPO and MDA in the pancreatic samples were estimated. The treatment with EtSF-BM significantly lowered the levels of MPO and MDA levels were lowered while the level of pancreatic insulin content was increased in the samples. These evidences indicated the reduction in inflammation and damage in the islets.

Studies have shown that each injection of STZ will result in damage of a fraction of β-cells eventually leading to a local inflammation and infiltration by mononuclear cells, which culminates in widespread β-cell destruction and diabetes [24]. Thus, we have examined the histology of the islets to evaluate the effect of the treatment with EtSF-BM on insulitis. The results clearly showed the dose-dependent reduction with EtSF-BM treatment in the mononuclear cell infiltration and islet damage and it was in line with the biochemical assays carried out in pancreatic samples.

Previous reports indicated the beneficial effects of anthocyanins in diabetic condition. Cyanidin 3-*O*-glucoside significantly suppressed TNF-α induced insulin resistance in 3T3-L1 adipocytes [25, 26]. Pretreatment of the cells with cyanidin-3-*O*-glycoside caused dose-dependent inhibition of LPS-induced nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at both the mRNA and protein levels together with a decrease in nitric oxide and PGE_2 production [27]. Our results suggested that EtSF-BM might have pancreatic β-cell protective effect possibly through alleviating the inflammation in islets and the anthocyanins might be the active ingredients.

Conclusion

In our experiment, the treatment with EtSF-BM reduced the MPO and MDA levels in the pancreas. The anthocyanins present in the active fraction might be the active constituent that ameliorated β-cell death in diabetic animals. However, further experiments on the characterization of anthocyanins in EtSF-BM and mechanistic based antidiabetic activity are required to assess potential application of *B. malabarica* as a food supplement and remedy for diabetes.

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