Original Article

Protective Effects of *Ficus benghalensis* in Streptozotocin (STZ) Induced Diabetic Zebrafish (*Danio rerio*) Model

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ABSTRACT

Background: Diabetes Mellitus (DM) is a chronic metabolic disorder that is characterized by hyperglycemia. Severe complications such as cardiovascular disease, neuropathy, nephropathy, and retinopathy are associated with DM. **Objectives:** To explore a safer treatment option with no/less adverse effects, we evaluated the potential of *Ficus benghalensis* to alleviate certain diabetic conditions. **Materials and Methods:** Administering streptozotocin to zebrafish intraperitoneally induced diabetes. After induction, *Ficus benghalensis* hydroethanolic bark extract was administered to zebrafish in different concentrations (5, 10, and 15 μg/mL). Biochemical blood parameters such as Blood Glucose, total cholesterol levels, triglycerides, and liver enzymes such as alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase were analyzed. **Results:** Treating the streptozotocin-induced group with *Ficus benghalensis* hydroethanolic bark had a hypoglycemic effect and reduced the cholesterol, triglycerides, and liver enzymes. The histopathological evaluation also showed improvement in the damaged pancreatic tissues. **Conclusion:** Our results suggest that *Ficus benghalensis* hydroethanolic bark extract can be used as a potent therapeutic candidate for treating diabetes.

Keywords: Diabetes mellitus, *Ficus benghalensis*, Alkaline phosphatase, Alanine aminotransferase, Aspartate aminotransferase, Zebrafish.

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INTRODUCTION

Diabetes mellitus (DM) is an endocrine disorder that is a widespread public health problem. In DM, either insulin production by pancreatic beta cells isn't sufficient (Type-1 DM) or the produced insulin by peripheral tissues is defective (Type-2 DM) resulting in glucose elevation in the blood that interferes with macronutrient metabolism.[1] The main cause of mortality and morbidity in diabetic patients are vascular complications, which include micro and macrovascular systems (Cardiovascular system, diabetic neuropathy and retinopathy, and diabetic kidney disease).[2] The prevalence of DM is increasing and it's estimated that more than 10.5% adult population has this condition.[3] The most common treatment options that are currently available include insulin and oral antidiabetic drugs like biguanides, glinides, and sulfonylureas. These drugs have limitations due to their adverse effects,[4-5] the spike in the prevalence and the adverse effects of the existing drugs have resulted in the ongoing pursuit of drugs that will allow for more effective remedies. As many plants have vast phytochemicals of therapeutic importance, they can be immensely useful in treating various disorders. Many traditional plants have been studied and have been reported

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for better anti-diabetic potential with no adverse effects.[6] *Ficus benghalensis*, commonly known as the banyan tree or Figure is from the Moraceae family and it is a large tree high as 20-30 m.[7] Different parts of this plant such as aerial roots, leaf, bark, and fruit are well documented to have different therapeutic properties such as antioxidant, antimicrobial, anti-inflammatory, antimutagenic, anticancer, and antistress.[8-10] Its rich source of phytochemical constituents contribute to the properties mentioned above. Because of its various pharmacological properties, we designed the present experiment to determine the anti-diabetic activity of ethanolic *Ficus benghalensis* bark extract in *Danio rerio*.

MATERIALS AND METHODS

Plant Extract Preparation

Powdered bark of *Ficus benghalensis* was weighed and 30 g was mixed with 70% ethanol for 24 hr with occasional stirring. After stirring, the hydroethanolic solvent with the bark extract was filtered using a filter paper (Whatman - 125 mm). The ethanolic filtrate was dried at 40°C and the obtained powder was stored and used for further experiments.

Maintenance of Adult *Danio rerio*

Adult zebrafish with body size 2.0-3.0 cm weighing 250-300 mg were selected and used. These fishes were accustomed for 10 days in a 30 L tank of dechlorinated water with continuous aeration at 25±2°C. The fishes were maintained with a 14hr light/10hr dark photoperiod with the pH of dechlorinated water ranging from 7.0 - 7.4. Fishes were given commercial food twice a day.

Toxicity test of *Ficus benghalensis* Hydroethanolic Bark Extract

A toxicity test of *Ficus benghalensis* hydroethanolic bark (FBHB) extract was carried out based on the recommendations of OECD guidelines. 55 fishes were randomly divided into 11 batches; each batch had 5 fishes in 5 L tanks. The first batch was the control batch which did not have any extract and the following 10 batches had various concentrations ranging from 10 µg/mL to 100 µg/mL FBHB extract. The testing period was for a week and during this time, the fishes were monitored regularly to analyze their swimming pattern, gills movements, and the presence of deaths.

Streptozotocin-induced Diabetes and Experimental Design

5 Groups of disease-free zebrafish (n = 10) were placed in 5 L tanks. FBHB extracts were given in concentrations 5, 10 and 15 µg/mL. Streptozotocin (STZ) was utilized to induce diabetes. The rapid cooling ice-cold method was employed to anesthetize and then 10 µl of streptozotocin was intraperitoneally (i.p.) administered using an insulin syringe. After administrating STZ, the fishes were kept in 2% sucrose for 24 hr, diabetes was induced and the fishes were transferred to the respective groups (Group 02 - 05) for the experiment

The treatment period was 7 days during which the fish were treated daily and after that, the zebrafish were euthanized and used for analysis. The experimental groups were as follows:

- Group 01: Control (Normal glucose level).
- Group 02: Induced group, Intraperitoneal administration of STZ.
- Group 03: Treated group - 1, STZ-induced diabetes + 5 µg/mL of FBHB extract
- Group 04: Treated group - 2, STZ-induced diabetes + 10 µg/mL of FBHB extract
- Group 05: Treated group - 3, STZ-induced diabetes + 15 µg/mL of FBHB extract

Estimation of Biochemical Blood Parameters

Parameters such as Blood Glucose, total cholesterol levels, and triglycerides levels were analyzed. The blood glucose levels were measured to confirm diabetes induction. Fishes' blood was collected and measured using a OneTouch Select® commercially available glucometer. Total cholesterol and triglycerides were measured using an autoanalyzer.

Liver Homogenate Preparation

The fishes were dissected and the liver was isolated. The isolated liver was kept in ice-cold sucrose solution (0.25 M) and homogenized using a micropestle. The homogenized tissues were centrifuged at 7500 rpm at 4°C for 20 min. The supernatant was transferred to a new tube and used for analyzing liver enzymes such as alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase.

Alkaline Phosphatase (ALP) Assay

ALP assay was performed by the method reported by Vaidyanathan *et al.*[11] Briefly, 1.5 ml of carbonate-bicarbonate buffer along with 200 µl magnesium chloride and 1.5 ml disodium phenyl phosphate were mixed and incubated at 37°C for 5 min. 100 µl of the liver homogenate was added to the mixture and incubated for 20 min at 37°C. 1 ml trichloroacetic acid (10%) was added and the mixture was centrifuged at 5000 rpm for 10 min. 1 ml of Folin's phenol reagent, 2 ml of supernatant, and 2 ml of sodium carbonate (15%) were added and mixed. A blue color was formed

and it was measured spectrophotometrically at 620 nm. ALP activity was expressed in µmol of phenol liberated/mg of protein/min.

Alanine Aminotransferase (ALT) Assay

ALT assay was performed by following the protocol mentioned by Reitman and Franke *et al.*[12] Briefly, 200 µl of liver homogenate was added to 1 ml buffered substrate (a-Ketoglutarate (2 mM) and dl-alanine (200 mM)) and incubated for 1 hr at 37°C. After incubation, 1 ml of 2,4 -Dinitro Phenyl Hydrazine reagent was added to the mixture and incubated for 20 min at room temperature. 10 ml of NaOH (0.4 N) was added and kept for 5 min to stop the reaction. The absorbance was measured spectrophotometrically at 540 nm. ALT activity was expressed in µmoles of pyruvate liberated/hr/mg protein.

Aspartate Aminotransferase (AST) Assay

ALT assay was performed by following the protocol mentioned by Reitman and Franke *et al.*[12] Briefly, 200 µl of liver homogenate was added to 1 ml buffered substrate (a-Ketoglutarate (2 mM) and dl-aspartate, (200 mM)) and incubated for 1 hr at 37°C. After incubation, 1 ml of 2,4 -Dinitro Phenyl Hydrazine reagent was added to the mixture and incubated for 20 min at room temperature. 10 ml of NaOH (0.4 N) was added and kept for 5 min to stop the reaction. The absorbance was measured spectrophotometrically at 540 nm. ALT activity was expressed in µmoles of pyruvate liberated/hr/mg protein.

Statistical Analysis

The experiments performed in the work were repeated in triplicates and the results were reported as mean ± SD. A *P-value* less than 0.001 was considered to be significant. Statistical analysis (one-way ANOVA - Tukey multiple comparison tests) and the graphs were done using GraphPad Prism 5 software.

RESULTS

Toxicity Test

Ficus benghalensis hydroethanolic bark extract was tested and no toxic effects were observed in all the 10 concentrations given. When compared to the control group, the tested groups showed no observable effects. The toxicity test period was a week and the survival rate of adult zebrafish was 100% in FBHB extract. From our results, we conclude that the LC_{50} for FBHB extract is greater than 100mg/L.

Blood Glucose Levels

The serum glucose levels of STZ-induced zebrafish were 149.67 ± 0.47 mg/dL which is comparatively higher than the control group (125 ± 0.82 mg/dL). However, on treatment with FBHB extracts the glucose levels of zebrafishes showed a dose-dependent reduction with the mean values of 113.67 ± 0.94, 67 ± 0.82, and 62.33 ± 0.94 mg/dL for doses 5, 10, and 15 µg/mL as shown in Figure 1.

Total Cholesterol Levels

The fish in the control group had a cholesterol level of 39.17 ± 0.62 mg/dL. Intraperitoneal administration of STZ caused a significant elevation of cholesterol levels to 81.8 ± 0.71 mg/dL. But, on treating the induced groups with FBHB extracts suppressed cholesterol levels to 56.43 ± 0.42, 54.27 ± 0.29, and 46.23 ± 0.76 mg/dL for doses 5, 10, and 15 µg/mL in a dose-dependent fashion and it is depicted in Figure 2.

Blood Triglycerides Levels

The FBHB extracts significantly alleviated triglycerides levels in the treated groups compared to the induced group. The triglyceride levels of the induced group was 158.67 ± 0.47 mg/dL, whereas in the control group it was 136.33 ± 0.47 mg/dL and at doses 5, 10, and 15 µg/mL it was 106.33 ± 0.94, 96.5 ± 0.41, and 88.83 ± 0.57 mg/dL. (Figure 3).

Alkaline Phosphatase Activity

ALP activity in the liver homogenate of the fishes in the induced group was 66.17 ± 1.84 µmol/mg protein/min which was significantly high compared to the control group (15.43 ± 0.37 µmol/mg protein/min). A dose-dependent suppression was seen when the induced

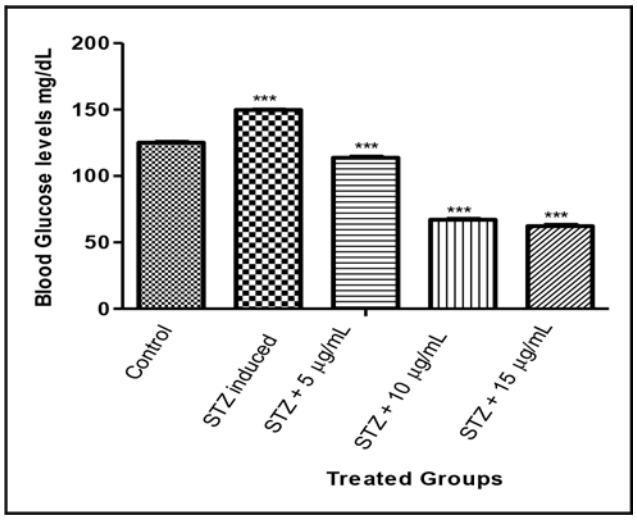

Figure 1: Effect of *Ficus benghalensis* hydroalcoholic bark extract on blood glucose level.

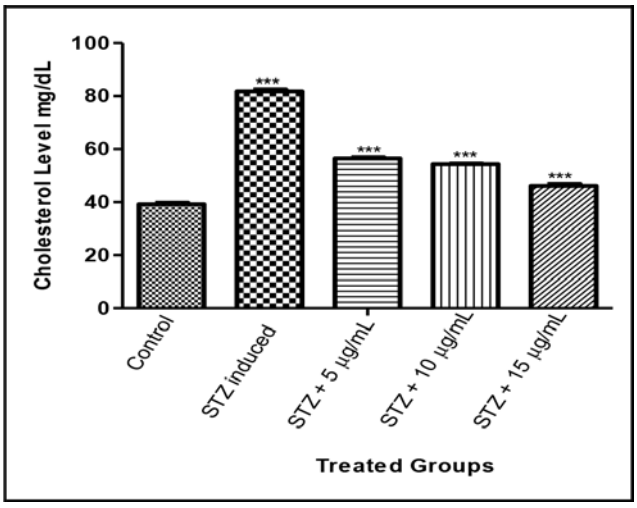

Figure 2: The changes in total cholesterol levels in the induced and *Ficus benghalensis* hydroalcoholic bark extract treated groups compared to the control group.

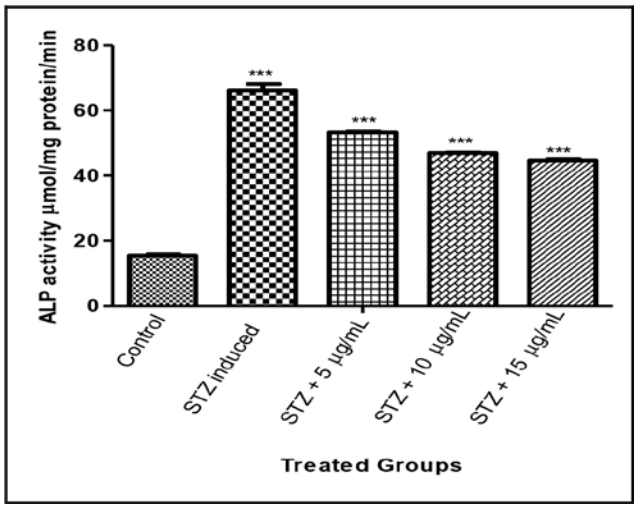

Figure 4: Effect of *Ficus benghalensis* hydroalcoholic bark extract on alkaline phosphatase (ALP).

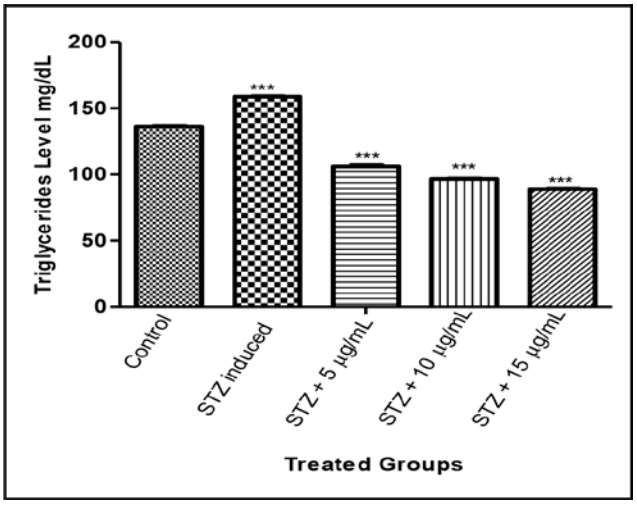

Figure 3: Effect of *Ficus benghalensis* hydroalcoholic bark extract on blood triglycerides level.

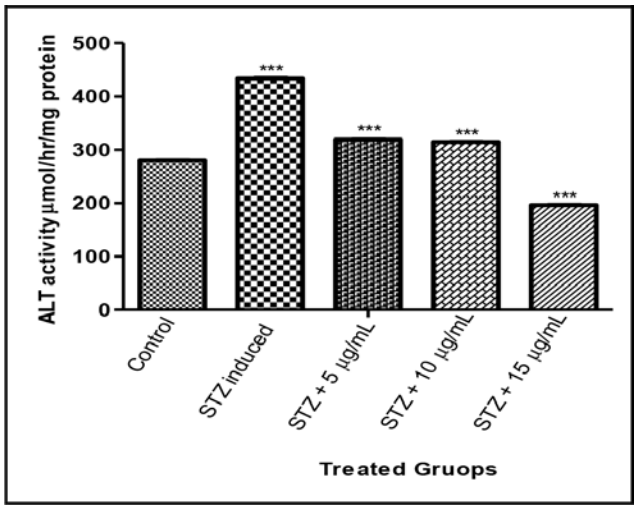

Figure 5: Effect of *Ficus benghalensis* hydroalcoholic bark extract on alanine aminotransferase (ALT).

groups were supplemented with the FBHB extracts. The ALP activity were 53.23 ± 0.29, 46.97 ± 0.12, and 44.57 ± 0.37 µmol/mg protein/min for doses 5, 10, and 15 µg/mL respectively. (Figure 4).

Alanine Aminotransferase Activity

STZ significantly elevated ALT activity (Control group: 280 ± 0.82; Induced group: 434 ± 0.82 µmol/hr/mg protein). Treating the induced group with *Ficus benghalensis* hydroethanolic bark extract had a significant reduction in the ALT activity with mean values of 319.33 ± 0.47, 314.67 ± 0.47, and 196.5 ± 0.41 µmol/hr/mg protein for doses 5, 10, and 15 µg/mL respectively as illustrated in Figure 5.

Aspartate aminotransferase activity

The fishes in the induced group received STZ which caused a strong elevation in AST activity (119.53 ± 0.45 µmol/hr/mg protein) compared to the control group (93.73 ± 0.49 µmol/hr/mg protein). When treated with FBHB extract, the AST activity was significantly reduced. The AST activity was 107.07 ± 0.49, 84.7 ± 0.36, and 40.1 ± 0.65 µmol/hr/mg protein for doses 5, 10, and 15 µg/mL respectively (Figure 6).

Histopathological Evaluation of Pancrease

The section of the pancreas from the zebrafish - control group showed normal histological features with a preserved architecture and intact epithelium (Figure 7A). STZ-induced groups had a severe loss in the structure and there is abrasion of epithelium (Figure 7B). A

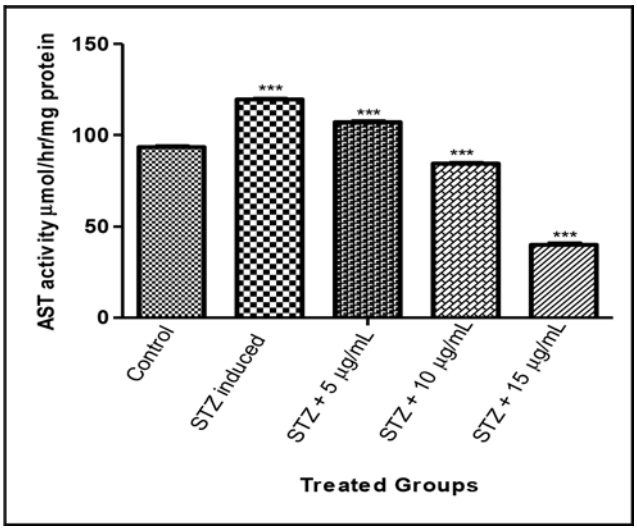

Figure 6: Effect of *Ficus benghalensis* hydroalcoholic bark extract on aspartate aminotransferase (AST).

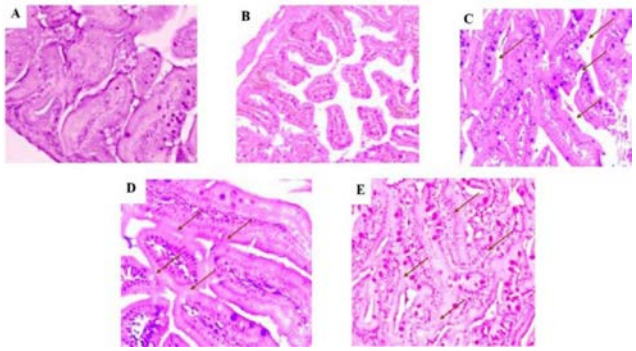

Figure 7: Histopathological analysis of zebrafish from different groups.

A – Control group (Normal glucose levels), B – STZ-induced group, C – Induced group treated with 5 µg/mL FBHB extract, D - Induced group treated with 10 µg/mL FBHB extract, E - Induced group treated with 15 µg/mL FBHB extract.

gradual restorement of the pancreatic structure is seen as the dosage of FBHB increases. Infiltration of immune cells can be clearly visualized in the induced groups and a reduction of infiltrated immune cells is seen in higher doses of FBHB. Attenuation of the pancreatic tissue injury is visualized in the highest FBHB extract dose - 15 µg/mL. (Figure 7 C-E).

DISCUSSION

Several drugs that are used to treat diabetes have adverse effects. Plant-based drugs are widely preferred and they tend to have no or fewer adverse effect than synthetic drugs.[13] The major objective of the current study was to evaluate the remedial effects of *Ficus benghalensis* hydroethanolic bark extract against diabetes mellitus and some of the complications surrounding it. We preferred Zebrafish as it is a classic experimental model to study metabolic disorders due to the functional similarity in pancreas structure, lipid metabolism, glucose homeostasis, and adipose biology.[14] Streptozotocin (STZ), a diabetogenic drug was our choice to induce diabetes as it is effective in the ablation of pancreatic β-cell that eventually leads to elevated blood glucose levels and suppresses insulin levels.[15-16] Intraperitoneal administration of STZ caused a spike in the blood glucose levels indicating diabetic conditions were induced. After treating the induced group with FBHB extract, hypoglycemic effects were observed. This observation may be because of better peripheral glucose uptake, restricted production of glucose by the liver, or the ability of the extract to restore the damaged pancreatic β-cell that causes the production of insulin. Dyslipidemia is characterized by increased cholesterol levels in the blood. Insulin resistance plays a critical role in the development of diabetic dyslipidemia.[17] This increase in cholesterol levels is harmful to diabetic patients as they are at risk of cardiovascular disease.[18] Cholesterol is vital for the proper functioning and survival of β-cells. If cholesterol irregularly accumulates in β-cells because of upregulated LDL-R expression, it can lead to cell dysfunction, the onset of many conditions such as insulin resistance.[19-20] Therefore suppressing cholesterol levels may reverse insulin resistance and control the development of diabetes. Previous studies conducted with *Ficus benghalensis* suggest that the cholesterol-lowering ability may be due to the presence of beta-glucan, a soluble polysaccharide.[21] Similar to cholesterol, hypertriglyceridemia manifests itself as patients develop resistance to insulin. The risk of cardiovascular disease in Diabetic patients with hypertriglyceridemia is estimated to be 2-10 fold higher than the patients without diabetes.[22-23] Higher levels can lead to triglyceride-induced pancreatitis which will lead to acidosis and hyperglycemia.[24] Moderate elevation of triglycerides is a risk factor that is modifiable and triglycerides are a growing target in diabetes care.[25] Hypotriglyceridemic and hypocholesterolemic effects can be seen when treating the induced groups with *Ficus benghalensis* hydroethanolic bark extract. This may be attributed to the induction of lipoprotein lipase enzymes. Alkaline phosphatase is widely used as a clinical marker to diagnose bone or hepatic disease.[26] Several studies show elevated ALP levels in diabetic patients.[27] ALP is a key contribution to vascular calcification and endothelial dysfunction which is linked to insulin resistance eventually leading to the development of diabetes.[28] ALP causes glomerular hyperfiltration and plays a role in the progression of diabetic complications such as diabetic nephropathy and end-stage renal

disease.[29-30] The ALP activity was reduced mildly at the highest dose suggesting that *Ficus benghalensis* bark extract at a much higher dose might greatly reduce the ALP levels. Liver dysfunction is one of the factors that cause the development of diabetes. Aminotransferases are generally considered biomarkers for hepatocellular health.[31] ALT is predominantly found in the liver and is associated with the accumulation of liver fat. The liver is the site of insulin clearance[32] and if insulin losses its ability to suppress glucose production in the liver, it causes a surge in liver glucose production leading to diabetic progression.[33] Diabetes is most likely to cause non-alcoholic fatty liver disease (NAFLD). ALT is a classic marker for NAFLD.[34] Like ALT, Aspartate aminotransferase is also a live enzyme and abnormal levels indicate cholestatic or liver damage.[35] An increase in ALT and AST in the streptozotocin-induced groups suggest hepatic damage and treatment with FBHB extracts alleviates the increased aminotransferase levels and indicates its hepatoprotective roles even in a diabetic environment. Histopathological evaluation of Zebrafish kidneys was performed to evaluate the effect of streptozotocin and FBHB extracts. In the STZ-induced groups, the destruction in cellularity of the endocrine islet can be clearly visualized indicating a severed pancreas. Administering a dose of 10 µg/mL of FBHB extracts reduced the infiltration of immune cells compared to a dose of 5 µg/mL. At the highest dose (15 µg/mL), the pancreatic cells seem to be recovering well.

CONCLUSION

Induction of diabetes using streptozotocin was confirmed by the rise in blood glucose. The hydroethanolic bark extract of *Ficus benghalensis* has hypoglycemic effects as well as suppresses dyslipidemia and hypertriglyceridemia that is caused due to diabetes. Hepatic enzymes such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST) are biomarkers of liver damage were also reduced in the FBHB extract-treated group. Overall, FBHB extract can act as a therapeutic agent and could be used for effectively managing diabetes mellitus. However, in order to understand the compound responsible for these effects and the mechanism of action, further studies are required.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

FBHB: *Ficus benghalensis* Hydroethanolic Bark; **STZ:** Streptozotocin; **i.p:** Intraperitoneal; **ALP:** Alkaline phosphatase; **ALT:** Alanine aminotransferase; **NaOH:** Sodium Hydroxide; **AST:** Aspartate aminotransferase; **LDL-R:** Low Density Lipoprotein receptor; **ANOVA:** Analysis of Variance; **NAFLD:** Non-alcoholic fatty liver disease.

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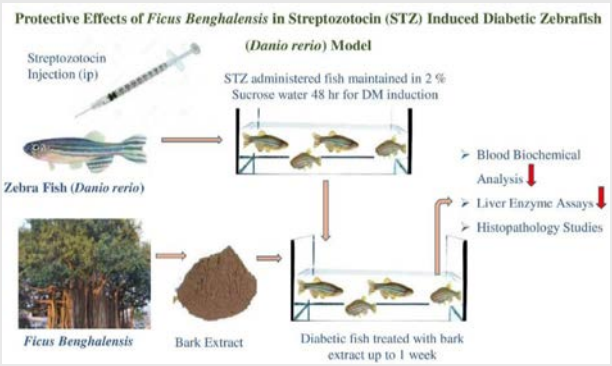
PICTORIAL ABSTRACT



SUMMARY

- *Ficus benghalensis* have positive effects in alleviating experimentally induced diabetes.
- FBHB extracts has hypoglycemic, hypotriglyceridemic and hypocholesterolemic potency in STZ-induced diabetes.
- FBHB extracts reduced hepatocellular health biomarkers - ALP, AST, and ALT indicating hepatoprotective effects in diabetic mellitus.

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