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Research Article
Crataegus oxyacantha Extract Mitigates Diabetic Nephropathy via Oxidative Stress Regulation in Streptozotocin-Induced Zebrafish Model

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Abstract

Background and Objective: The global prevalence of diabetes mellitus is alarmingly rising. Diabetes mellitus has multi-systemic complications. One of the widespread complications is diabetic nephropathy, where patients have a progressive decline in renal function chiefly due to oxidative stress. The current study aims to counteract oxidative stress and explored the nephroprotective potential of *Crataegus oxyacantha* extracts in streptozotocin-induced diabetic nephropathy in Zebrafish Model. **Materials and Methods:** Intraperitoneal administration of STZ induced diabetic nephropathy in the Zebrafish Model. The hydroalcoholic *Crataegus oxyacantha* extract was supplemented and its activity was assessed by measuring blood parameters (blood glucose, serum urea and serum creatinine), kidney antioxidant enzyme and oxidative stress levels (GST, GSH, SOD and TBARS) and the kidney histology. **Results:** The supplementation of the plant extract successfully alleviated hypoglycemia and serum urea and creatinine levels. It also enhanced the activity of powerful antioxidants and reduced stress levels. The histological section of the kidney showed restoration of deregulated structure and anti-inflammatory effects. **Conclusion:** It was evident that the antidiabetic, nephroprotective and anti-inflammatory properties were attributed to the hydroalcoholic extracts of *Crataegus oxyacantha*.

Key words: Diabetic nephropathy, crataegus oxyacantha, streptozotocin, nephroprotective, antidiabetic

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic and endocrine disorder that is characterized by insulin deficiency. The prevalence of DM globally is alarmingly raising, it is estimated that, in 2045 about 783.2 million people will have diabetes[1]. A chronic condition that is commonly associated with diabetes mellitus is Diabetic Nephropathy (DN) or Diabetic Kidney Disease (DKD). It is distinguished by albuminuria and progressive decline in Glomerular Filtration Rate (GFR) and involves the deterioration of kidney function[2]. It is commonly associated with DM[3,4] and about 30-40% of diabetic patients develop diabetic nephropathy[5]. The DN is the predominant cause of morbidity and mortality as well as end-stage renal disease in patients with diabetes[6,7], requiring them to opt for treatment options such as kidney transplants and dialysis. Despite potent treatment options are available to treat diabetes, most of them only slow the disease progression and cause severe adverse effects. Therefore, utilizing drugs of natural origin like plant sources that cause minimal/no adverse effects and are capable of preventing the genesis of diabetic nephropathy is critical[8,9].

Medicinal plants have been utilized for decades to treat a variety of human diseases and have played a vital role in identifying and developing effective therapeutic agents. *Oxyacantha* is a common herb that has been explored as it has a wide range of therapeutically beneficial compounds such as flavonoids (hyperoside, vitexin and rutin), phenol carboxylic acids (chlorogenic acids and a variety of amines) and triterpenic acids (crataegolic, ursolic and oleanolic acids)[10]. These compounds exhibit hyperglycemic activity and can have potential antidiabetic effects. This plant also has anti-oxidative, anti-inflammatory, hypolipidemic, hypotensive, anti-hyperglycemic, immunomodulatory and antimicrobial effects[11-14]. Most of the plant compounds and their beneficial activities are known to subside diabetic effects. Due to the properties of *Crataegus oxyacantha* leaf extract, this study aim to investigate the remedial effects of the hydroalcoholic extract against streptozotocin-induced diabetic nephropathy in zebrafish (*Danio rerio*).

MATERIALS AND METHODS

Study area: This study was carried out at the Clinical Biochemistry Unit, Department of Pathology, College of Medicine, King Saud University, Riyadh, Saudi Arabia from October, 2021 to January, 2022.

Preparation of plant extracts: Dried *Crataegus oxyacantha* powder was obtained from the local markets of the Kingdom of Saudi Arabia. Twenty grams of this powder was soaked in 70% methanol for 48 hrs at room temperature with periodic stirring and shaking. After 48 hrs, the solvent was filtered using a Whatman filter paper. The methanolic solution was evaporated at 40°C and the dried powder was stored in a refrigerator for further use.

Maintenance of adult zebrafish: About 75 adult zebrafish weighing 270-300 mg were used in this experiment. The fishes were acclimatized in a 40 L tank filled with dechlorinated water and continuously aerated. The pH of the water was 7.2-7.5, the temperature was maintained at 25±2°C with a 14/10 hrs light/dark cycle and the fishes were fed twice a day with commercial food.

Toxicity test of the hydroalcoholic *Crataegus oxyacantha* extracts: Zebrafish toxicity test was performed based on the OECD guidelines. 6 batches were divided with 10 fishes each and added into the 5 L tanks to test the toxicity. The batch was as follows: Batch 1: Control (water without any extracts), batch 2: Water with 20 mg L^{-1} extract, batch 3: Water with 40 mg L^{-1} extract, batch 4: Water with 60 mg L^{-1} extract, batch 5: Water with 80 mg L^{-1}, batch 6: Water with 100 mg L^{-1}. The period of testing was 7 days and during this period the fishes were regularly observed to assess their swimming, movement and the presence of any deaths.

Experimental design: The 75 zebrafish were divided into 5 groups and placed in 5 L tanks. The hydroalcoholic extracts of *Crataegus oxyacantha* were added into the 5 L tanks with varying concentrations (5, 10 and 15 μg mL^{-1}). Streptozotocin (STZ) was used to induce diabetic nephropathy. The treatment was given daily for 7 days. By the end of the experiment, the fish were euthanized for further experiments. The experimental groups were as follows:

Group 1: Control group (normoglycemic fish)

Group 2: Induced group, intraperitoneal administration of 10 μL streptozotocin (STZ) for the induction of diabetic nephropathy

Group 3: STZ-induced diabetic fish administered with 5 μg mL^{-1} of *C. oxyacantha* extract

Group 4: STZ-induced diabetic fish administered with 10 μg mL^{-1} of *C. oxyacantha* extract

Group 5: STZ-induced diabetic fish administered with 15 μg mL^{-1} of *C. oxyacantha* extract

Estimation of biochemical parameters in blood: Biochemical parameters in the blood such as blood glucose, serum urea and serum creatinine levels were measured. Blood glucose was measured to confirm diabetes development. This was measured using a commercial glucometer OneTouch Select®. Glomerular damage markers, serum urea and creatinine were measured using a biochemical autoanalyzer (Roche Analytic Instruments, Nutley, NJ).

Estimation of kidney antioxidant enzyme and oxidative stress levels: Different antioxidant enzymes and oxidative stress parameters such as GSH, GST, SOD and TBARS levels were estimated.

Reduced glutathione (GSH) activity: Reduced glutathione was performed[15]. The 0.5 M potassium phosphate buffer was used to homogenize the tissue and 4% sulfosalicylic acid was employed to deproteinate the tissues. The principle behind this assay is Dithiobis 2-Nitrobenzoic acid reacts with reduced glutathione and produces yellow colour that can be measured at 412 nm. The GSH was measured in μM mg^{-1} of protein.

Glutathione-s-transferase (GST) activity: The GST assay was performed[15]. The 0.2 M potassium phosphate buffer and 1 mM GSH were used. Tissues were homogenized with 10 mM potassium phosphate buffer and 1-chloro-2,4-dinitrobenzene was used as the substrate. The GSH form conjugate with CDNB that is measured at 340 nm and the rate of increase in absorbance is directly proportional to the GST activity. The GST was measured in terms of nmols mg^{-1} of protein.

Superoxide dismutase (SOD) activity: This assay is performed[16]. One of the prominent scavengers of superoxide is superoxide dismutase. The scavenging activity in the tissues of zebrafish is determined by the ability of the superoxide in tissues to interact with Nitro Blue Tetrazolium reducing it to form a blue colour that is measured at 560 nm. The SOD was measured in U mg^{-1} of protein.

Thiobarbituric Acid Reactive Substance (TBARS) activity: Lipid peroxidation in the fish tissue is estimated by the thiobarbituric acid (TBA) assay[17]. Thiobarbituric acid measures malonaldehyde (MDA), a secondary product of lipid peroxidation. An MDA molecule reacts with 2 molecules of TBA in an acidic solution and the developed pink colour was measured by 532 nm. The TBARS was measured in nmols mg^{-1} of protein.

Statistical analysis: The results obtained in the current experiment were expressed as Mean±Standard deviation. A value of $p \leq 0.001$ was generally considered significant. GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) was used. The statistical analyses were performed using one-way ANOVA (Tukey multiple comparison tests) and the experiments were performed in triplicates.

RESULTS

Toxicity test of hydroalcoholic *Crataegus oxyacantha* extract in *Danio rerio* observation: Zebrafish has been widely employed as a successful model to screen and assess toxicity because of its striking genome similarity to humans, inexpensive, less time-consuming and smaller body size. *Crataegus oxyacantha* hydroalcoholic extract at tested concentration did not show any toxic effects. No observable effect was found between the control and the treated group. The survival rate of zebrafish in the extract was 100% (trail period = 7 days). With the results obtained, it can be inferred that the LC_{50} for this extract is greater than 100 mg L^{-1}.

Biochemical parameters in blood

Measurement of blood glucose level: The serum glucose level of the STZ-induced group (306.67±1.25 mg dL^{-1}) was significantly higher compared to the control (198.33±1.25 mg dL^{-1}). A significant reduction in the serum glucose level was observed in a dose-dependent manner when treated with hydroalcoholic extract of *C. oxyacantha* with mean values of 275.33±0.94, 208.33±1.25 and 168.33±1.25 mg dL^{-1} for doses 5, 10 and 15 μg mL^{-1}, respectively as illustrated in Fig. 1.

Attained serum urea level: The STZ-induced zebrafish showed an increase in the circulating levels of urea (9.63±0.12 mg dL^{-1}) when compared to the control group (5.6±0.16 mg dL^{-1}). Treatment groups (5, 10 and 15 μg mL^{-1}) with *C. oxyacantha* extract resulted in suppression of the serum urea levels (8.67±0.12, 7.43±0.05 and 6.2±0.08 mg dL^{-1}), respectively, in a dose-dependent manner as shown in Fig. 2.

Serum creatinine level observed: The serum creatinine levels were significantly elevated in the STZ-induced group (0.29±0.01 mg dL^{-1}) than the control group (0.05±0.01 mg dL^{-1}). *Crataegus oxyacantha* extract-treated groups (5, 10 and 15 μg mL^{-1}) caused a significant decrease in the creatinine level (0.19, 0.16±0.01 and 0.1±0.01 mg dL^{-1}), respectively as illustrated in Fig.3.

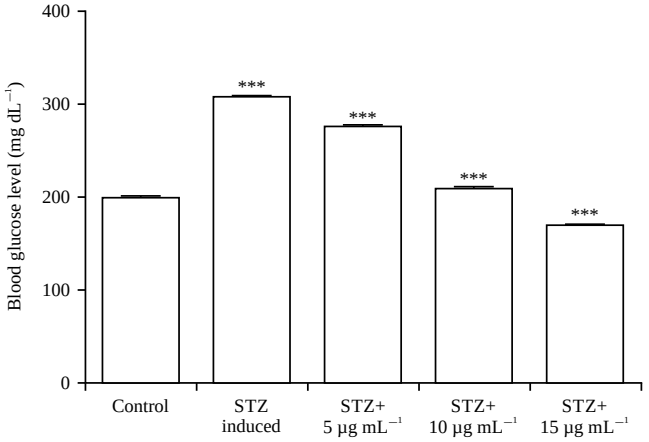

Fig. 1: Effect of *Crataegus oxyacantha* on blood glucose levels of control, induced and treated groups

Results are expressed as Mean±SD. Treated groups showed high significance compared to the induced model (p<0.0001)

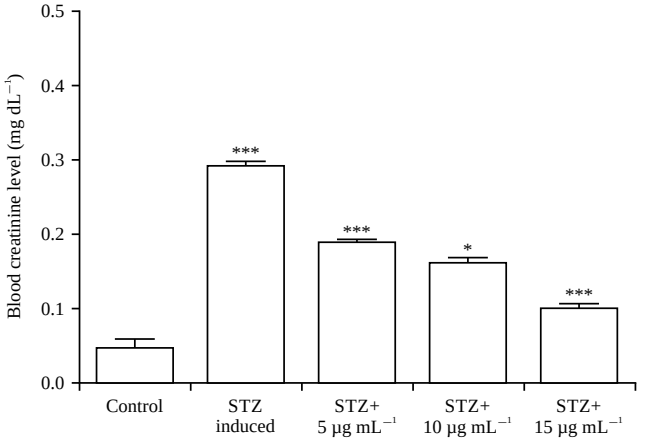

Fig. 3: Effect of *Crataegus oxyacantha* on serum creatine kinase level of control, induced and treated groups

Results are expressed as Mean±SD. Treated groups showed high significance compared to the induced group (p<0.0001)

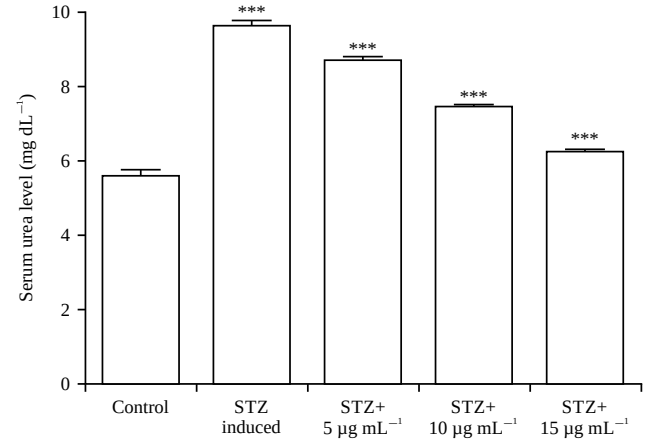

Fig. 2: Effect of *Crataegus oxyacantha* on serum urea level of control, induced and treated groups

Results are expressed as Mean±SD. Treated groups showed high significance compared to the induced group (p<0.0001)

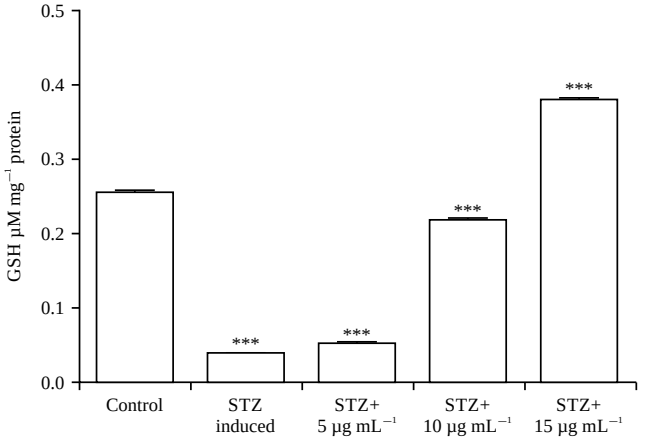

Fig. 4: Effect of *Crataegus oxyacantha* on GSH activity of control

Induced and treated results are expressed as Mean±SD. Treated groups showed high significance compared to the induced group (p<0.0001)

Kidney antioxidant and oxidative stress parameter

Reduced glutathione (GSH) activity measurement: The GSH activity in the STZ-induced group (0.038 µM mg^{-1} of protein) was significantly lower compared to the control group (0.254±0.001 µM mg^{-1} of protein). Administration of *C. oxyacantha* extract (5, 10 and 15 µg mL^{-1}) showed a dose-dependent increase in the GSH activity (0.050±0.001, 0.216±0.001 and 0.379±0.001 µM mg^{-1} of protein), respectively. It was noted that the GSH value of the dose 15 µg mL^{-1} was relatively higher than that of the control group as represented in Fig. 4.

Glutathione-s-transferase (GST) activity evaluation: The STZ-induced group showed suppressed GST activity (0.90 nmols mg^{-1} of protein) compared to the control group (1.45 nmols mg^{-1} of protein). An increase in the GST activity was observed when administered extracts of *C. oxyacantha*, in a dose-dependent manner with mean values of (1.16, 1.74±0.38 and 2.43 nmols mg^{-1} of protein) for doses (5, 10 and 15 µg mL^{-1}), respectively. The GST values of doses 10 µg mL^{-1} were mildly elevated and 15 µg mL^{-1} were significantly elevated than that of the control group as shown in Fig. 5.

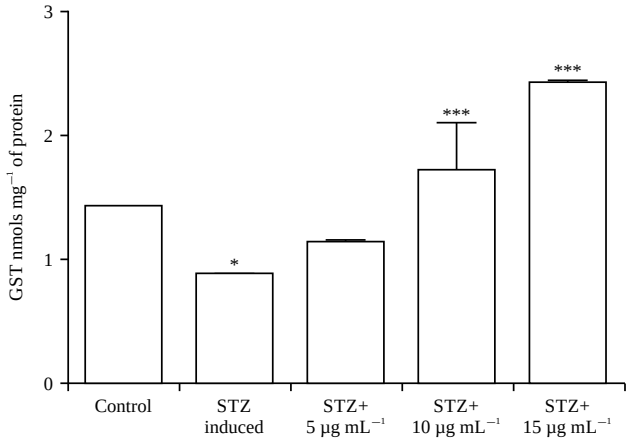

Fig. 5: Effect of *Crataegus oxyacantha* on GST level of control, induced and treated groups

Results are expressed as Mean±SD. Treated groups showed high significance compared to the induced group (p<0.0001)

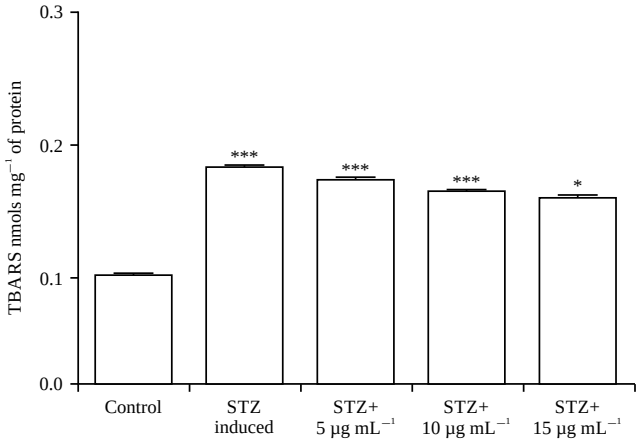

Fig. 6: Effect of *Crataegus oxyacantha* on TBARS level of control

Induced and treated results are expressed as Mean±SD. Treated groups showed high significance compared to the induced group (p<0.0001)

Achievements from Thiobarbituric Acid Reactive Substance (TBARS) activity: The STZ-induced group showed increased TBARS activity (0.18 nmol mg^{-1} of protein) as compared to the control group (0.10 nmoL mg^{-1} of protein). Treatment groups (5, 10 and 16 µg mL^{-1}) with *C. oxyacantha* extract showed a mild reduction in TBARS activity (0.17, 0.163 and 0.159 nmol mg^{-1} of protein, respectively). A higher dose of *C. oxyacantha* (15 µg mL^{-1}) showed a similar amount of TBARS activity as in dose 10 µg mL^{-1}. The results were illustrated in Fig. 6.

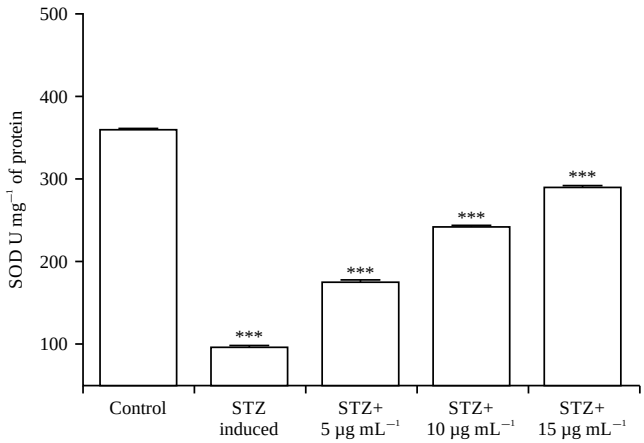

Fig. 7: Effect of *Crataegus oxyacantha* on SOD level of control, induced and treated

Results are expressed as Mean±SD. Treated groups showed high significance compared to the induced group (p<0.0001)

Superoxide dismutase (SOD) observation: The SOD activity was significantly inhibited in the STZ-induced group (94.72±0.70 U mg^{-1} of protein) in comparison with the control group (358.51±0.55 U mg^{-1} of protein). A dose of *C. oxyacantha* extract at 5, 10 and 15 µg mL^{-1} caused increased SOD activity than the STZ-induced group (174.71±0.95, 240.33±0.96 and 288.20±0.92 U mg^{-1} of protein, respectively) as depicted in Fig. 7.

Kidney histopathological measurement: Sections of kidneys from zebrafish of the control group showed normal histological features with normal renal tubules and glomerulus without any signs of inflammation and necrosis in Fig. 8a. The STZ-induced group had deregulated glomerulus structure with infiltration of mononuclear cells denoting inflammation and renal injury in Fig. 8b. At dose 5 µg mL^{-1} of *C. oxyacantha*, signs of mild tissue regeneration were observed in Fig. 8c. Treatment with 10 µg mL^{-1} showed better tissue regeneration and reduced infiltration of inflammatory cells in Fig. 8d. Attenuation of renal injury and complete regeneration was observed in the maximum dose of 15 µg mL^{-1} in Fig. 8e.

DISCUSSION

Despite the availability of potent therapeutic agents which slow down the progress of diabetic nephropathy, a growing interest to use natural plants and herbal products to prevent the origin of the complications associated with diabetes is crucial.

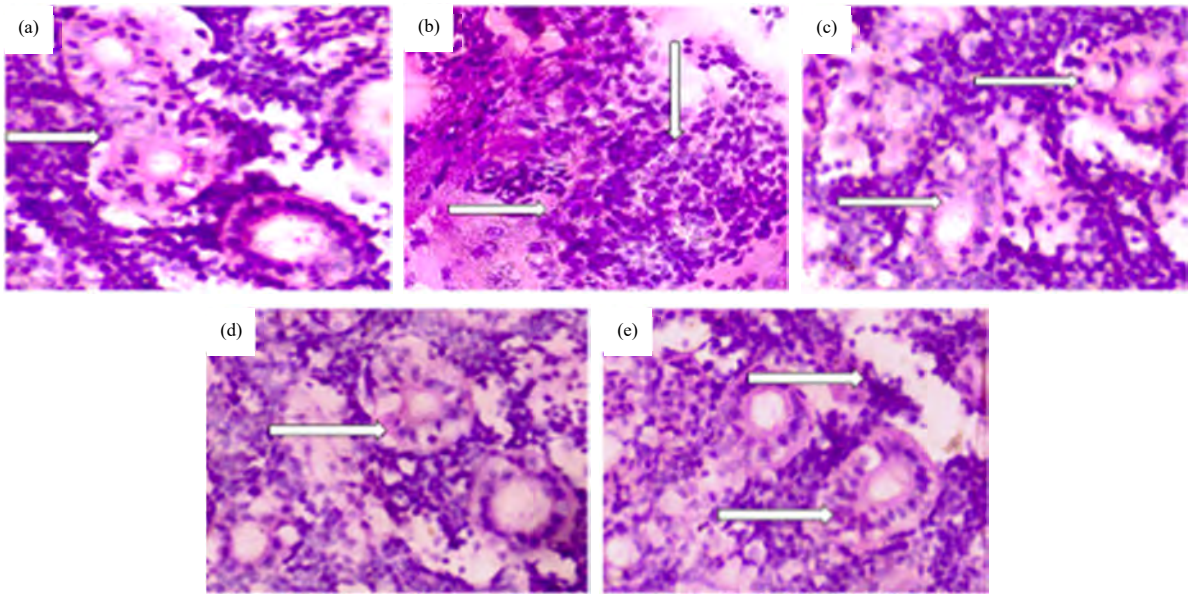

Fig. 8(a-e): Histopathological analysis of zebrafish kidney, (a) Control group showing normal kidney architecture, (b) STZ induced group showing high infiltration of immune cells and destruction of the glomerulus structure, (c) STZ-induced and treated with 5 µg mL^{-1} showing the slightly visible structure of Bowman's capsule, (d) STZ-induced and treated with 10 µg mL^{-1} showing better effects than 5 µg mL^{-1} and (e) STZ-induced and treated with 15 µg mL^{-1} showing signs of restoration of deregulated glomerulus and architecture similar to control can be observed

In this study, the potential of hydroalcoholic extract of *Crataegus oxyacantha* in eliminating the complication of diabetic nephropathy was evaluated. Streptozotocin was used to experimentally induce diabetic nephropathy because of its ability to selectively cause the destruction of pancreatic beta cells by necrosis which leads to hypoinsulinemia and hyperglycemia[18-20]. The administration of STZ in zebrafish caused a significant elevation in blood glucose levels due to the damage of beta cells. Hypoglycemic effect in the induced group was observed when treated with *C. oxyacantha* extract. This effect is not dependent on beta cells' insulin secretion, it may be due to suppression of liver glucose production and/or utilization of glucose by peripheral tissue. Several studies conducted with plant extracts having antidiabetic and hypoglycemic effects reported similar findings[21-23].

Elevated serum urea and creatinine profiles are due to a lack of kidney clearance that results from renal insufficiency and damaged kidneys[24]. Many studies suggest that higher insulin resistance and defective insulin secretion are due to increased urea levels[25,26]. The STZ-induced groups showed increased levels of serum urea and creatinine indication for kidney damage and dysfunction. Treatment with *C. oxyacantha* extracts ameliorated the elevated urea and creatinine. Therefore, it's reasonable to assume the extract prevented the decline in renal function and played a role in the recovery of kidney damage.

There is a strong relationship between hyperglycemia, oxidative stress and renal damage[27]. A persistent hyperglycemic environment stimulates numerous biochemical pathways that lead to necrosis of beta cells and major oxidative stress (OS)[28]. Excess glucose in this environment goes into the polyol pathway and induces the production of ROS[29]. The GSH is an important endogenous hydrophilic antioxidant. It protects cells from reactive species via non-enzymatic reduction. The GSH deficiency is associated with oxidative and nitrosative stress[30]. The STZ induced groups saw a decline in the amount of GSH compared to the control group indicating severe oxidative stress in the induced group. The extracts of *C. oxyacantha* upregulated the quantity of GSH indicating the extract has strong antioxidant potential.

The GSTs are known to play a vital role in detoxifying the byproducts of lipid peroxidation and have protective effects over oxidative stress[31]. The GST levels were low in the STZ-induced group. Administration of *C. oxyacantha* extract to the induced groups restored the GSTs levels indicating that the plant has cellular protective role against oxidative stress. Malondialdehyde (MDA) is a mutagenic product of lipid peroxidation. MDA is a biomarker of LP which can be detected

by thiobarbituric acid (TBARS) activity[32-34]. The STZ-induce group has a significant increase in TBARS activity than the control demonstrating oxidative stress. However, after treating the induced zebrafish with extracts of *C. oxyacantha*, a slight reduction in TBARS was seen. This suggests a higher dose may have a significant reduction in oxidative stress. Results suggested lipid peroxidation was controlled by the plant extract and has radical scavenging ability.

Superoxide dismutase enzymes (SODs) are the primary defence enzymes against injuries mediated by ROS. The STZ induction in zebrafish caused elevation in the superoxide free radicals and low SOD activity. When the induced groups were treated with *C. oxyacantha* extracts, an increase in SOD activity in a dose-dependent manner. This observation suggested the ability of the extract to convert free radicals and reduce cell and tissue damage caused by oxidative stress[35,36]. Studies conducted with plants that have nephroprotective were in agreement with current findings[9,37].

Histopathology examination of the zebrafish kidney section revealed structural abnormalities in the STZ induced groups. Infiltration of immune cells can be observed indicating inflammation. Hyperglycemic environment induces infiltration in the glomerulus and it has been correlated with disease progression[38-40]. The highest dose of *C. oxyacantha* extract administration (15 μg mL^{-1}) showed restoration of the deregulated glomerulus structure indicating recovery from renal inflammation and implying that *C. oxyacantha* has anti-inflammatory effects as shown by previous studies conducted by[10,41]. Current investigation indicated that hydroalcoholic extract of *C. oxyacantha* restores the activities of antioxidant enzymes like SOD, GST and GSH activities and has potent anti-diabetic and nephroprotective effects against STZ-induced diabetic nephropathy in zebrafish.

CONCLUSION

Although drugs exist to treat diabetes and its complications, they cause adverse effects. Administration of hydroalcoholic *C. oxyacantha* extracts regulated various blood parameters (blood glucose, serum urea and serum creatinine level), showed remedial activity in renal injury by its anti-inflammatory properties and ameliorated the damage caused by oxidative stress through the reduction in LPO and increased antioxidant enzymes. In conclusion, the effectiveness of C. *oxyacantha* extracts against diabetic nephropathy is well demonstrated.

SIGNIFICANCE STATEMENT

The ameliorative effects of *C. oxyacantha* of STZ-induced diabetic nephropathy are proven by this study. These results can help in identifying potential therapeutic bioactive compounds from this plant to treat complications of diabetes.

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