IJPSR (2021), Volume 12, Issue 1 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 06 January 2020; received in revised form, 07 April 2020; accepted, 11 April 2020; published 01 January 2021

SCREENING AND EVALUATION OF ANTIDIABETIC POTENCY OF *VERNONIA ARBOREA* USING ZEBRAFISH MODEL

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Keywords:

Vernonia arborea, Anti-diabetic, Zebrafish, Leaf extract, Liver enzymes, Blood glucose

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ABSTRACT: Introduction: *Vernonia arborea*, belonging to the Asteraceae family, found in the Western Ghats of India and Sri Lanka, was traditionally used in wound healing. It is proven that some of the plants of this genus have anti-diabetic properties. *e.g., V. amygdalina*. So this study was done to screen and to evaluate the anti-diabetic potency of the leaf extract of *Vernonia arborea* in diabetic Zebrafish models. **Materials and Methods:** Extraction was done by cold percolation method. The toxicity of the extract was evaluated, and the concentrations of 25 mg/l and 50 mg/l of tank water were taken for treatment as higher concentrations were found to be toxic. The Zefrafish were divided into the respective control and treatment groups. The different groups were induced with diabetes by high-glucose feed for 30 days. The standard group was treated with metformin and the test groups with the chosen concentrations of the extract for 7 days. The blood glucose level was checked on alternate days for 7 days. On the 7th day, the liver enzyme levels, triglyceride & cholesterol levels were checked, the pancreas was studied histopathologically, and diabetic retinopathy effects were studied. **Results:** A reduction in the blood glucose, liver enzymes, triglyceride & cholesterol levels and an improvement in the histopathology of the pancreas and the thickness of the retina were observed in the 25 mg/dl extract-treated group. **Conclusion:** The leaf extract had a positive effect at 25mg/dl on diabetic Zebrafish models. Extensive studies can be done on the leaf extract to study the compound responsible for the anti-diabetic activity.

INTRODUCTION: Diabetes is one of the major metabolic disorders for which many drugs have been discovered in the past few decades. Though the drugs have been effective enough, more new drugs are discovered for more effective use and reduced adverse effects due to the complications. In recent years, natural drugs are preferred in order to reduce the adverse effects.

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DOI: 10.13040/IJPSR.0975-8232.12(1).385-94

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(1).385-94

There are many medicinal plants that have been implicated in the management of diabetes in various parts of the world.

Vernonia arborea is one such medicinal plant that has been used in the treatment of various diseases. It is an evergreen shrub or a tree that can grow up to a height of 15-26 meters. It belongs to the family Asteraceae. It is distributed in the coastal districts of Karnataka State, especially in the South Canara district. The plant has many medicinal properties *viz*., leaf juice is used to treat worms, infusion of roots, or decoction of bark in fever. In Southern Sumatra, the bark is chewed at the first sign of sprue. This plant contains sesquiterpene 'Zaluzanin D', which is a potent antifungal agent [1].

Tribal groups residing in the Western Ghats region of Chikmagalur district, Karnataka State, use the leaf extract for septic wounds [2], in treating jaundice fever and rheumatic pains [3]. It is proven that some of the plants of this genus have anti-diabetic property. *e.g. V. amygdalina, V. anthelmintica, V. cinerea* [4].

The leaf extract was screened and evaluated for anti-diabetic potency using Zebrafish model. The Zebrafish model system is one of the most widely used animal models for developmental research and it is now becoming an attractive model for drug discovery and toxicological screening. The utility of the Zebrafish as a model organism is further enhanced by the completion of sequencing of the Zebrafish genome and the availability of full-length cDNAs and DNA microarrays for expression analysis [5]. In addition, techniques for generating transgenic lines, targeted mutation, and nuclear transfer have made the Zebrafish model even more useful to researchers. Hyperglycemia in type II diabetes results from an ability of insulin to control gluconeogenesis. When glucose is available in the diet, insulin is produced by the pancreas, and gluconeogenesis is inhibited through the downregulation of genes involved in the pathway.

In the absence of glucose in the bloodstream, gluconeogenesis is induced by the action of glucagon. Insulin and glucagon are produced in the β-cells and α-cells of the pancreas, respectively. As in mammals, the Zebrafish pancreas is also comprised of two types of glandular tissues, each of which carries out essential physiological functions [6]. The endocrine tissue is critical for the regulation of glucose metabolism through the secretion of insulin, somatostatin, and glucagon directly into the bloodstream. The exocrine portion of the pancreas produces digestive enzymes, including trypsin and amylase, which are carried to the digestive tract. Hyperglycemia in type II diabetes has a complex etiology resulting from defects in insulin production and signaling, changes in insulin sensitivity of target tissues, and increased hepatic gluconeogenesis. The genes of Zebrafish insulin and glucagon and other important proteins in the regulation of glucose metabolism, have been identified and demonstrate similar regulation patterns and activity as seen in mammalian counterparts [7].

The leaf extract can be studied further, and the active compounds responsible for the anti-diabetic activity can be isolated and formulated as a drug.

MATERIALS AND METHODS:

Year of Experimentation: 2018 - 2019

Site of Experimentation: Zebrafish Facility, Department of Biomedical Sciences, Sri Ramachandra Institute of Higher Education and Research

Maintenance of Zebrafish Stock: A stock of wild type adult Zebrafish was purchased and maintained in the laboratory. They were put in tanks in suitable numbers. Overcrowding was avoided in the tanks. The tanks were filled with tap water, and the pH was 6.8. Oxygen pumps were fit in the tanks, and the tank was cleaned up once in three days. Methylene blue was added to the water to avoid the growth of fungus. The temperature was maintained at 25 °C. A natural light cycle was maintained. The fish were fed twice a day with freeze-dried tubefex worms and a variety of fish feed pellets. If fish are overfed, they die. The feeding and tank cleaning schedules were maintained.

Preparation of Leaf Extract: The leaf extract was prepared by the cold percolation method using hexane [8].

Toxicity Testing: Dimethyl sulphoxide (DMSO) was used as the dissolving agent for the leaf extract. 4 fish were exposed to 100% DMSO for 24 h to check the tolerance level.

The leaf extract was taken in 4 different concentrations- 25 mg/l, 50 mg/l, 100 mg/l, and 200 mg/l. 4 fish were taken for each group, and the fish were exposed to extract-treated water for 24 hours, and the fatality was checked.

Genotoxicity of the leaf extract was studied using the comet assay [9].

Blood Collection: The fish was anesthetized in ice. The tail of the fish was cut right above the caudal fin. A drop of heparin was put on the cut end, and the fish was placed in an Eppendorf tube with a collecting tube below it. A drop of heparin was put in the collecting tube as well. The fish were centrifuged at 300 rpm for 5 min.

Sample Preparation: 10µl of the collected blood was taken and 1000µl of PBS (Phosphate Buffer Saline) was added to it (1:1000 dilution). The tubes were centrifuged at 4000rpm for 10 mins. The supernatant was removed and the pellet was stored in the refrigerator.

Preparation of Slides: 1% NMPA (Normal Melting Point Agarose) was prepared. The slides were dipped in NMPA for about 45-60 sec. The slides were left to dry for an hour.

Sample Casting: The gel-coated slides were kept ready. 300 µl of 5% LMPA (Low Melting Point Agarose) was added to the pellet. 75 µl of the sample was cast on the slides, and a coverslip was placed. Each group had duplicates.

The slides were incubated at 4 °C for 30 min. The coverslips were removed, and they were incubated in lysis solution for 60 min at 4 °C in the dark. The slides were left to denature in the electrophoresis buffer for 10 min.

Electrophoresis: Electrophoresis was run for 15 min at 25V and 300 mA. The slides were immersed in a neutralization buffer for 15 min and then immersed in 100% methanol for 5 min. The bottom of the slides was wiped and left to air dry.

Slide Staining: The slides were stained with Ethidium Bromide. 80 µl was added to the slides, and a coverslip was placed and left for 5 min.

Image Analysis: The slides were viewed under a fluorescent microscope and checked for the presence of comets.

Hyperglycemia Induction: Hyperglycemia was induced through diet [10]. The fish were treated with 1% glucose on alternate days with fish feed on a regular basis for about 30 days.

Grouping of the Zebrafish: The following groups were separated with 30 fish under each group:

- Normal group
- Diabetic group
- Metformin-treated group-standard
- Extract-treated group-25mg/l concentration.
- Extract-treated group- 50mg/l concentration.

Treatment with Metformin: Metformin was dissolved in fish water to a final concentration of 20mM [11]. The metformin solution was freshly prepared, and the fish were exposed to metformin for 3 hours daily for a week. The blood glucose was tested every alternate day during the treatment week.

Treatment with Extract: Two groups were treated with 25 mg/l and 50 mg/l concentrations of the extract dissolved in fish water, respectively. The fish were exposed to the respective extracts for 3 h daily for a week. The blood glucose was tested on alternate days during the treatment week.

Blood Glucose Testing: The fish was anesthetized in ice. The fishtail was cut right above the caudal fin. The glucose test strip was held at a 45° angle, and the blood glucose level was checked using the glucometer.

Liver Enzyme Assays:

Preparation of Liver Homogenate: The livers were collected using the following protocol [12]. 10 fish were dissected, and the contents of the abdominal cavity were taken out. The liver was identified by its large size, lobed morphology, tannish color, and extensive vascularization. The livers were collected in a microcentrifuge tube and kept in ice. The livers were homogenized [13] with a mortar and pestle with 0.25M sucrose solution. The homogenate was centrifuged at 900xg for 10 min. The supernatant was taken and centrifuged at 10000 rpm for 20 min. The supernatant was collected and stored. The supernatant served as the enzyme source.

Alkaline Phosphatase (ALP): ALP was assayed using the protocol of King and King [14]. 1.5 ml of carbonate-bicarbonate buffer of pH 10.4 was taken in the ‘test’ and ‘control’ tubes. To this, 1.5 ml of substrate (Disodium Phenyl Phosphate) was added followed by 0.2 ml of magnesium chloride. The contents were mixed well and preincubated at 37 °C for 5 mins. 0.1ml of the enzyme was added to the ‘test’ tube and mixed well. The contents were incubated at 37 °C for 20 min. After incubation 1ml of 10% trichloroacetic acid was added to all the test tubes. 0.1ml of the enzyme was added to the ‘control’ tube. The contents were centrifuged for 10 min. 2 ml of the supernatant was taken and 1ml of

Folin's phenol reagent was added followed by 2ml of 15% sodium carbonate. The contents were mixed well and the intensity of blue color was read at 620 nm using a spectrophotometer against a water blank. The ALP activities were expressed as µmol of phenol liberated/mg of protein/min.

Alanine Aminotransferase (ALT): ALT was assayed using the following protocol [15]. 1ml buffered substrate was pipetted out into 'test' and 'control' tubes. 0.2 ml of the enzyme source was Added to the 'test' and the tubes were incubated at 37°C for 60 min. After incubation, 0.2 ml enzyme was added to the control. 1ml of 2,4 -Dinitro Phenyl Hydrazine (DNPH) reagent was added and kept at room temperature for 20 min. The reaction was stopped by the addition of 10 ml of 0.4 N NaOH, vortexed and kept at room temperature for 5 min. The absorbance was measured at 540 nm in a spectrophotometer against a reagent blank. The ALT activities were expressed as µmoles of pyruvate liberated/hr/mg protein.

Aspartate Aminotransferase (AST): AST was assayed using the following protocol [16]. 1ml buffered substrate was pipetted out into 'test' and 'control' tubes. 0.2 ml of the enzyme source was added to the 'test' and incubated the tubes at 37 °C for 60 min. After incubation, 0.2 ml enzyme was added to the control. One ml of 2,4 -DNPH reagent was added and kept at room temperature for 20 min. The reaction was stopped by the addition of 10 ml of 0.4 N NaOH, vortexed, and kept at room temperature for 5 min. The absorbance was measured at 540 nm in a spectrophotometer against a reagent blank. The AST activities were expressed as µmoles of pyruvate liberated/hr/ mg protein.

Cholesterol Assay: The total cholesterol was estimated by the following protocol [17]. To 0.1 ml of the lipid extract, 9.9 ml of ferric chloride-acetic acid reagent was added and allowed to stand for 15 min and then centrifuged. To 5.0 ml of the supernatant, 3.0 ml of concentrated H_2SO_4 was added. The color developed was read after 20 minutes at 560 nm against a reagent blank. A set of standards was also performed in a similar manner. Values were expressed as mg/100 gm of wet tissue.

Triglyceride Assay: Triglyceride was estimated by the method of Rice [18]. 0.2 ml of tissue lipid extract was mixed with 9.8 ml of the chloroform-methanol mixture (2:1) and shaken vigorously, and allowed to stand for 30 minutes. This was centrifuged, and 4.0 ml of lipid extract was added to tubes containing 8.0 ml of saturated saline solution. The tubes were stoppered, shaken vigorously and allowed to stand for 1 h, and centrifuged. The upper aqueous layer was discarded, and the chloroform layer containing the lipids was filtered into a dry tube. 200 mg of activated silicic acid was added to the filtered lipid extract. The mixture was shaken gently, allowed to stand for 1 h and then centrifuged. 0.5 ml of the supernatant was taken in a test tube and dried at 70 °C. Standard solutions of tripalmitin (10-50 µg) were taken in test tubes and similarly evaporated together with a blank containing the solvent alone.

After cooling, 0.5 ml of alcoholic potassium hydroxide was added to all the tubes, and the mixture was saponified at 60-70°C in a water bath for 20 minutes. 0.5 ml of 0.2N H_2SO_4 was then added and placed in a boiling water bath for 10 min, cooled and then, 0.1 ml of sodium meta periodate and sodium meta arsenite was added. 5.0 ml of chromotropic acid was added to each tube, mixed and kept in a boiling water bath for 30 minutes. After cooling, 0.5 ml of thiourea solution was added. The color developed was read at 570 nm against a reagent blank. The values were expressed as mg/100 mg of wet tissue.

Histopathological Studies: At the end of the 7 days treatment, the fish were euthanized and fixed in 10% neutral buffered formalin. The fish were processed further by dehydration, clearing, and wax infiltration. Sectioning of the fish was done, stained using haematoxylin and eosin stains, and observed under a light microscope for histo-pathological changes [19].

Diabetic Retinopathy Testing: The fish that were sectioned for histopathological studies were also observed for changes in the thickness of the retinal layer, indicating diabetic retinopathy.

RESULTS:

Toxicity Testing: The following observation **Table 1** was made after the exposure to the different concentrations of leaf extract and DMSO.

TABLE 1: TOXICITY OF DMSO AND DIFFERENT CONCENTRATIONS OF THE LEAF EXTRACT

S. no.	Treatment solution	No. of fish taken	No. of dead fish after 4.5 h	No. of dead fish after 5.5 h	No. of dead fish after 6 h
1	DMSO	4	-	-	-
2	25mg/l extract	4	-	-	-
3	50mg/l extract	4	-	-	-
4	100mg/l	4	1	1	2
5	200mg/l	4	4	-	-

DMSO, which was used as a dissolving agent for the extract, was not toxic to the fish. The extract concentrations of 100mg/l and 200mg/l were found to be toxic to the fish, and the extract concentrations of 25mg/l and 50mg/l were not toxic to the fish and were chosen for treatment.

Comet Assay Results: For the comet assay, 2 fish were taken from each group. All the fish that were exposed to 100mg/l and 200mg/l leaf extract died. So comet assay was done only with the normal fish, DMSO-treated fish, and fish exposed to 100mg/l extract and 200mg/l extract.

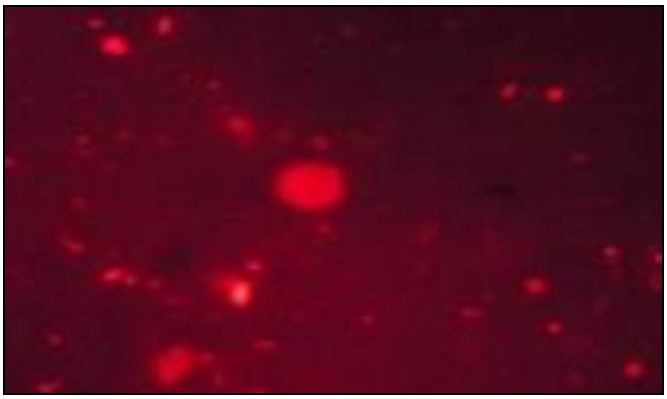

FIG. 1A: NORMAL GROUP

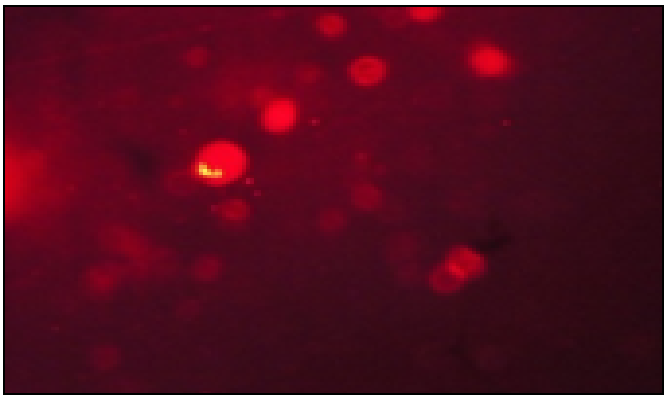

FIG. 1B: 100% DMSO TREATED GROUP

The results of the comet assay were interpreted from the pictures **Fig. 1A** and **1B** taken from the normal group, and the 100% DMSO treated group. The formation of comets shows the presence of genotoxicity, whereas condensed heads with no tails show the absence of genotoxicity. The fish treated with 100% DMSO (solubilizing agent for extract) do not show genotoxicity after 1 h exposure to the maximum amount of DMSO.

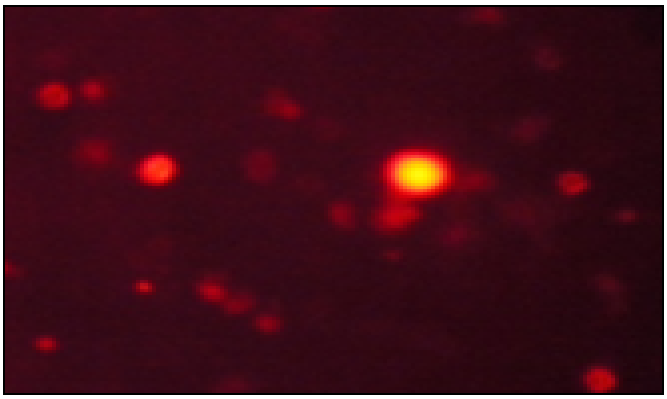

FIG. 2A TREATED WITH EXTRACT – 25mg/l

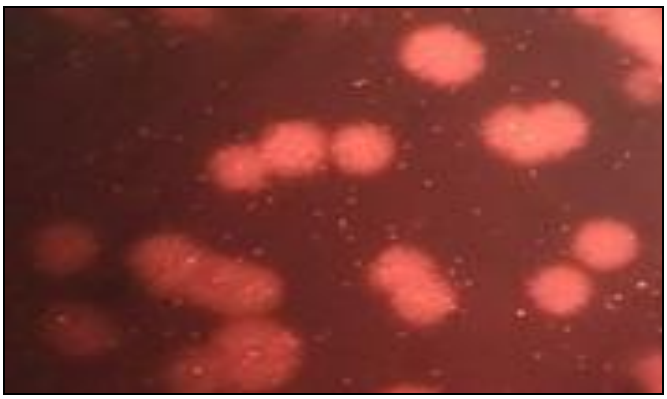

FIG. 2B: TREATED WITH EXTRACT - 50mg/l

The fish treated with 100mg/l and 200mg/l of the extract died within 24 h. So these concentrations were considered to be fatal. Comet assay was performed for the fish that survived in 25mg/l and 50mg/l concentration. The results for comet assay were interpreted from the pictures **Fig. 2A,** and **2B** was taken from the group treated with 25mg/l and 50mg/l. No comets were observed in both 25mg/l, and 50mg/l extract treated groups. This proves that the extract is not genotoxic at 25mg/l and 50mg/l concentrations to the fish.

The toxicity and the genotoxicity testing were done for 24 h and the fish survived at 25mg/l and 50mg/l concentrations of the extract treatment.

But during the treatment period of 7 days, the fish did not survive in the 50mg/l concentration of the extract treatment. So, the results of the 50mg/l treatment group were not considered in further analysis.

Blood Glucose Levels: The blood glucose levels of the treatment groups were observed on alternate days, and the following results, **Table 2** were obtained. Sample no. = 3 per day

TABLE 2: BLOOD GLUCOSE LEVELS OF ALL THE TREATMENT GROUPS

Day	Normal group	Diabetic group	Metformin treated group	25mg/l extract-treated group	50mg/l extract-treated group
2	58.66mg/dl	220mg/dl	59mg/dl	142.33mg/dl	101.66mg/dl
4	59mg/dl	238.33mg/dl	59.33mg/dl	93.33mg/dl	111.66mg/dl
6	60.33mg/dl	184mg/dl	61.33mg/dl	97.33mg/dl	90.66mg/dl

The blood glucose levels were compared. Exposure to 25mg/l and 50mg/l concentrations of leaf extract has reduced blood glucose levels as compared to the standard Metformin treatment.

Liver Enzyme Assays: All the biochemical assays were done only in the group exposed to 25mg/l extract concentration as the fish did not survive the higher concentrations studied.

The liver enzyme levels and activities are generally elevated due to the induction of diabetes when compared to the normal group. The metformin treatment has been shown to reduce the levels of the liver enzymes to the normal range.

Alkaline Phosphatase: As seen in **Fig. 3**, the diabetic group has elevated activity of alkaline phosphatase. Metformin treatment has been shown to decrease the alkaline phosphatase level to the normal range. The group exposed to 25mg/l concentration of the extract has also shown to reduce the activity of alkaline phosphatase to the normal range.

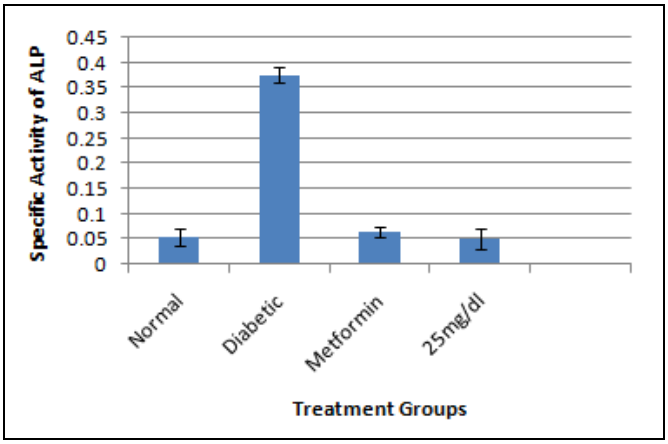

FIG. 3: SPECIFIC ACTIVITY OF ALP IN DIFFERENT GROUPS

Alanine Transferase: Increased activity of trans-aminases, which are active in the absence of insulin because of increased availability of amino acids in diabetes, is believed to be responsible for the increased gluconeogenesis and ketogenesis observed in the disease. The extract treatment has not shown a reduction in the ALT levels when compared to the metformin treatment, as seen in **Fig. 4**.

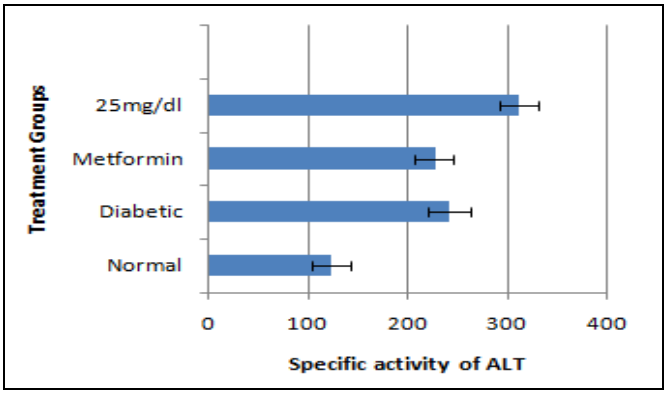

FIG. 4: SPECIFIC ACTIVITY OF ALT IN DIFFERENT GROUPS

Aspartate Transferase: According to **Fig. 5**, the extract treatment has not shown a reduction in the AST levels compared to the metformin treatment.

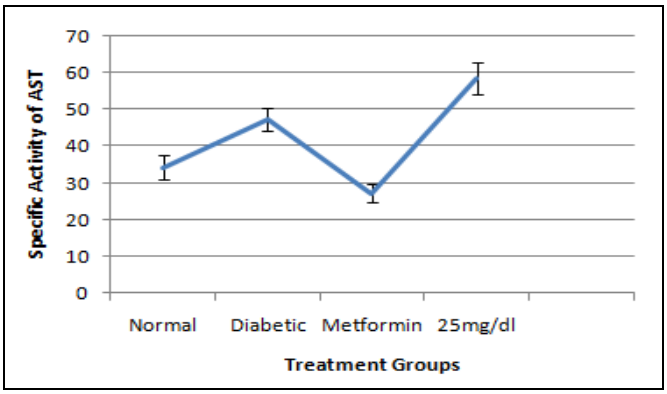

FIG. 5: SPECIFIC ACTIVITY OF AST IN DIFFERENT GROUPS

Cholesterol Assay: The total cholesterol level, which was increased in comparison to the normal control due to the induced diabetes, was decreased to significant levels with the administration of the leaf extract **Fig. 6**.

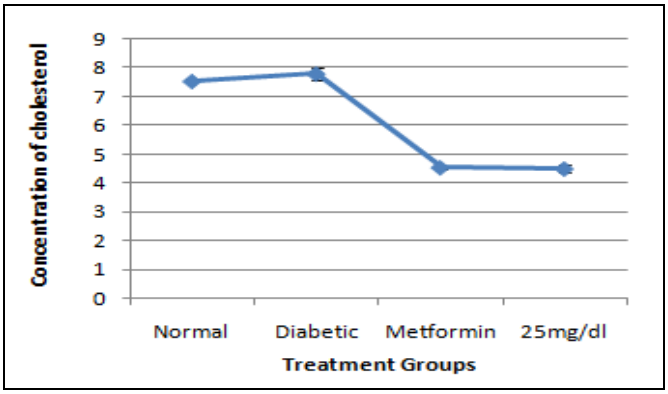

FIG. 6: CHOLESTEROL CONCENTRATION IN DIFFERENT GROUPS

Triglycerides Assay: The total triglyceride level, which is generally increased in the diabetic group, is reduced by the metformin and extract administration **Fig. 7**.

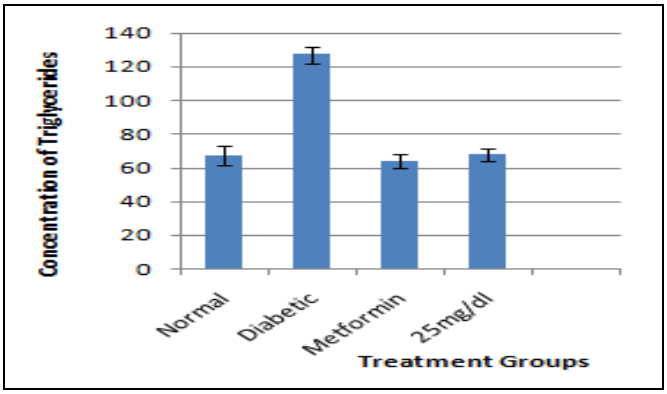

FIG. 7: TRIGLYCERIDE CONCENTRATION IN DIFFERENT GROUPS

Histopathological Studies: The pancreatic cells are distorted in the diabetic group **Fig. 8.2** when compared to the normal group **Fig. 8.1**.

The metformin group **Fig. 8.3** and the extract-treated group **Fig. 8.4** show regeneration of cells in the pancreas when compared with the diabetic group.

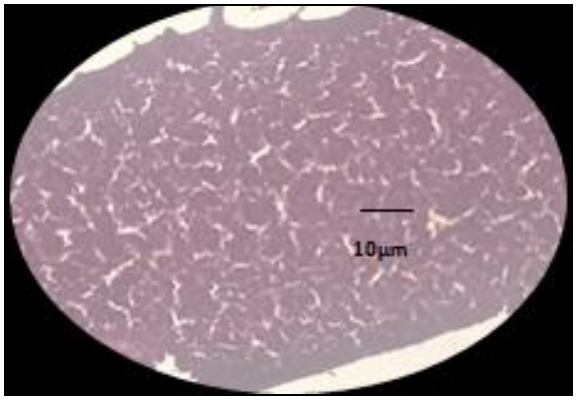

FIG. 8A: NORMAL-10X

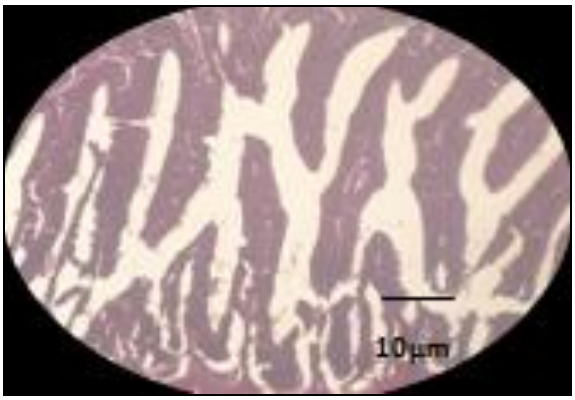

FIG. 8B: DIABETIC-10X

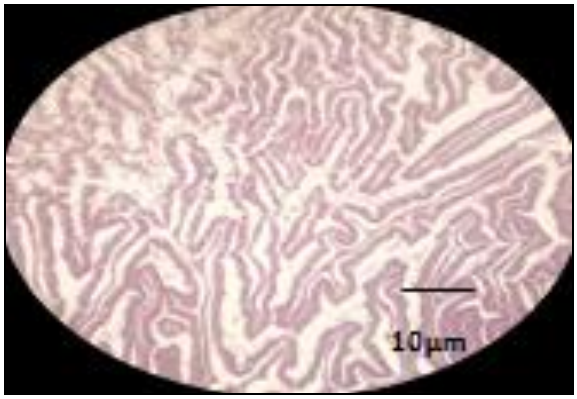

FIG. 8C: METFORMIN-TREATED-10X

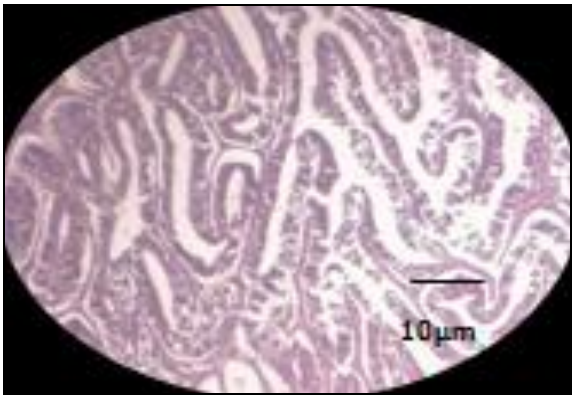

FIG. 8D: 25mg/l EXTRACT-TREATED -10X

Diabetic Retinopathy Testing: The thickness of the retinal layer is considerably reduced in the diabetic fish **Fig. 9B,** and it is distorted compared to the normal ones **Fig. 9A**. The metformin group **Fig. 9C** and extract-treated group **Fig. 9D** show an improvement in the thickness of the retinal layer.

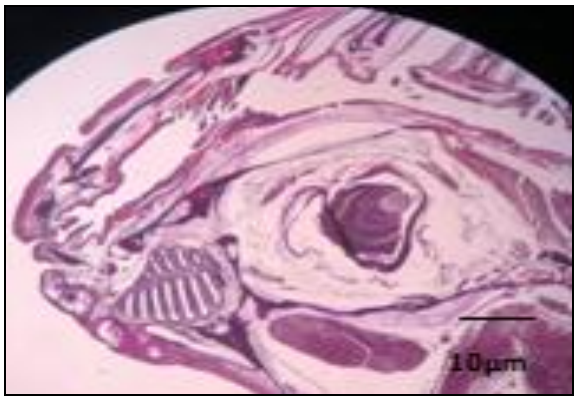

FIG. 9A: NORMAL-10X

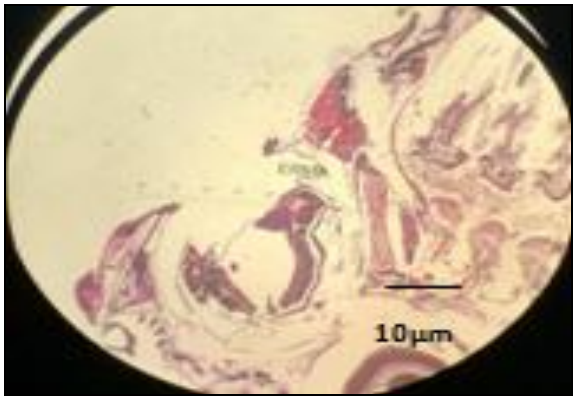

FIG. 9B: DIABETIC-10X

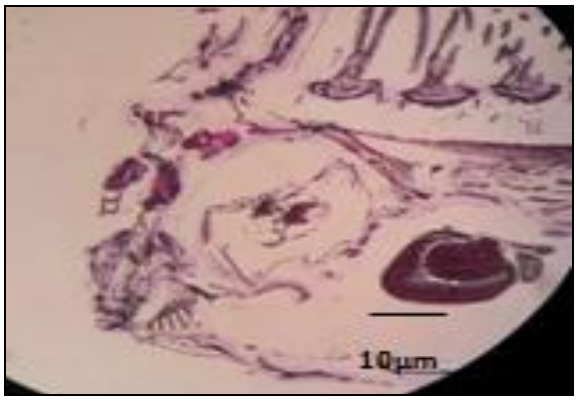

FIG. 9C: METFORMIN-10X

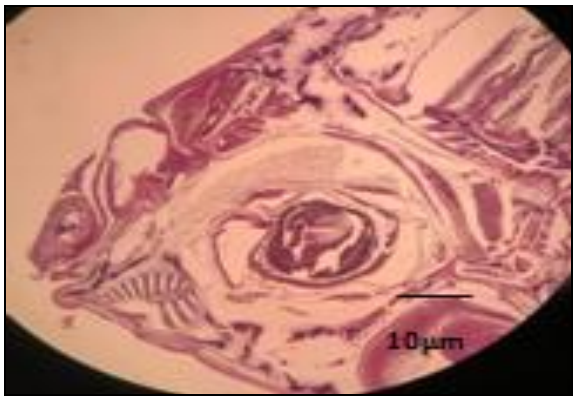

FIG. 8D: 25mg/l EXTRACT-TREATED -10X

DISCUSSION: Diabetes is one of the major metabolic disorders of glucose homeostasis for which natural drugs are preferred for treatment. Zebrafish is one of the suitable experimental models for diabetic study as diabetes can be induced through a high glucose diet.

The hexane leaf extract was administered in diabetic Zebrafish to check the presence of anti-diabetic compound(s). A negligible amount of DMSO was required to dissolve the water-insoluble hexane extract. The toxicity and the genotoxicity of the extract and the maximum amount of DMSO were tested, and the treatment level was fixed to be 25mg/l of tank water **Table 1**. The extract concentrations below 25mg/l were considered to be non–toxic.

The diabetic fish have shown a reduction in their blood glucose levels on extract treatment, but the extract doesn't have an effect as that of metformin **Table 2**. The active compound(s) responsible for the blood glucose reduction can be found on fractionating the extract.

Diabetic fish have an increase in their liver enzyme levels. This is characterized by a failure of insulin to signal an increase in insulin receptor substrate-2. Upregulation of sterol regulatory element-binding protein 1c (SREBP-1c) also occurs, leading to increased lipogenesis. Despite downregulation of the insulin receptor substrate-2–mediated insulin signaling pathway in insulin-resistant states, the up-regulation of SREBP-1c and subsequent stimulation of de novo lipogenesis in the liver leads to increased intracellular availability of triglycerides, promoting fatty liver. This also increases Very Low-density Lipoprotein (VLDL) assembly and secretion. Thus, hyperinsulinemia might directly lead to hepatic insulin resistance with associated fatty changes.

Other potential explanations for elevated transaminases in insulin-resistant states include oxidant stress from reactive lipid peroxidation, peroxisomal beta-oxidation, and recruited inflammatory cells. The insulin-resistant state is also characterized by an increase in pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), which may also contribute to hepatocellular injury. In preliminary studies, an increased frequency of specific TNF-α–promoter polymorphism was found in nonalcoholic steatohepatitis (NASH) patients, suggesting a possible genetic link or predisposition to fatty liver

found in insulin-resistant states [20]. The extract treatment has reduced the ALP, cholesterol, and triglyceride levels but has not reduced the ALT and AST levels **Fig. 3**, **Fig. 4**, **Fig. 5**, **Fig. 6** & **Fig. 7**.

Histopathological studies were done to microscopically examine the destruction of cells in the liver. The extract treatment showed regeneration of cells in the liver **Fig. 8A, 8B, 8C,** and **8D**.

It is shown that hyperglycemia leads to degradation and thinning of the retina, which occurs under diabetic conditions in humans and other mammals[21]. The extract treatment has increased the thickness and structure of the retina of Zebrafish **Fig. 9A, 9B, 9C,** and **9D**. Further isolation of the bioactive compound from the leaf extract is essential to reveal the synergistic/individual contribution of the phytoconstituents towards the studied anti-hyperglycemic activity

CONCLUSION: The 25mg/l concentration of the leaf extract of *Vernonia arborea* has shown anti-diabetic activity in the Zebrafish model by the reduction in the blood glucose levels, reduction in the alkaline phosphatase liver enzyme, reduction in the cholesterol and triglyceride levels, regeneration of the distorted pancreatic cells and the restoration of the thickness of the retinal layer. The effect of the extract is as good as the effect of the standard anti- diabetic drug, metformin. From this, it can be concluded that the lower concentrations of the extract will also show an anti-diabetic effect after a longer treatment period. The active compound responsible for this property can be identified by further research.

ACKNOWLEDGEMENT: The authors are thankful to the Chancellor, Sri Ramachandra Institute of Higher Education and Research, for the Summer research fellowship that funded the study.

COMPETING INTEREST: Authors have declared that no competing interests exist.

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How to cite this article:
Deebiga TH and Vaidyanathan L: Screening and evaluation of antidiabetic potency of *Vernonia arborea* using zebrafish model. Int J Pharm Sci & Res 2021; 12(1): 385-94. doi: 10.13040/IJPSR.0975-8232.12(1).385-94.