PHYTOCHEMICAL ANALYSIS, IN-VITRO AND IN-VIVO ANTIDIABETIC ACTIVITY OF *FLAVERIA TRINERVIA* ETHANOLIC EXTRACT

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Abstract

Background: Diabetes mellitus is a disease caused by a malfunction in the way carbohydrates are metabolized. It is frequently associated with abnormal insulin levels in the blood and organ insulin resistance.
Aim: Antidiabetic activity of *Flaveria trinervia* ethanolic extract.
Objective: Phytochemical analysis, In-vitro, and In-vivo activity antidiabetic activity of *F. trinervia* ethanolic extract.
Material and methods: *F. trinervia* were collected from Kovilpatti village, Thoothukudi district, Tamil Nadu, India in June 2021. The air-dried whole plant was crushed, and 500 gm of the powder, ethanol was used for continuous extraction by the Soxhlet device. Extract phytochemical analysis done by conventional and GC-MS analysis. The antidiabetic activity of *F. trinervia* ethanolic extract was investigated in Wistar albino rats.
Results: *F. trinervia* ethanolic extract were prepared by using the Soxhlet system. *F. trinervia* ethanolic extract showed α-amylase inhibitory activity with IC_{50} value 125 µg/mL when compared with the standard acarbose. *F. trinervia* ethanolic extract conducted glucose uptake assay in 3T3-L1 cell lines and showed efficient glucose uptake enhancement capability. In-vivo antidiabetic studies confirmed that *F. trinervia* ethanolic extract has good antidiabetic properties. *F. trinervia* phytoconstituents are responsible for antidiabetic activity.
Conclusion: The study suggested treating antidiabetic disease can be used in *F. trinervia* ethanolic extract. Further in detailed study need to understand the mechanism of action of antidiabetic activity.

Keywords: *F. trinervia*, Ethanolic extract, Phytochemical analysis, GC-MS, 3T3-L1, In-vitro, In-vivo antidiabetic activity.

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1. Introduction

Diabetes mellitus is a disease caused by a malfunction in how sugars are metabolized, and it is frequently associated with abnormal insulin levels in the blood and organs resistance to insulin. Hyperglycemia is also brought on by a diabetic patient's inability to control their blood sugar. A significant amount of mortality and morbidity in diabetics is brought on by microvascular and macrovascular complications. Furthermore, persistent hyperglycemia in diabetic patients increases oxidative stress due to the auto-oxidation of glucose. Estimates suggest there will be 592 million diabetics globally in 2035, with significant increases in developing countries. This disease is caused by environmental, behavioural, and hereditary factors. Increasing diabetes mellitus necessitates preventive measures. This condition is incurable. [1, 2, 3]

Diabetes is controlled with insulin. Long-term use causes insulin resistance. Oral hypoglycemic drugs and insulin are currently used in allopathic therapy to treat diabetic problems, but due to their complications, poor tolerance, high cost, and other adverse effects, they are not widely accepted. To avoid these complications, many people use herbal medicine to treat diabetes. The hunt for better and safer treatments for this disease is a global priority. It is thought that managing diabetes mellitus is a global issue. This may be the primary driver behind the current shift in the general populace away from allopathic medicine and toward Ayurveda [4, 5].

Health workers in the Indian use the *F. trinervia* for management of antidiabetic disorder, a C4 plant, as a promising treatment for antidiabetic activity abnormalities. Traditional uses for *F. trinervia* extract include the treatment of skin infections and jaundice. Some studies have noted its capacity for wound healing. Antibacterial, anthelmithic, CNS-depressant, and antinociceptive properties are just a few of the biological effects of *F. trinervia*. Only a few reports on the phytochemical properties of *F. trinervia* are currently available. *In-vivo* antioxidant and hepatoprotective efficacy of *F. trinervia* against alcoholic liver injury has not yet been reported, as far as we are aware. to support the ethnomedical assertions that *F. trinervia* is a highly effective herb for treating diabetes. For their *In-vitro* and *In-vivo* anti-diabetic effectiveness against streptozotocin-induced diabetes, *F. trinervia* were tested.

2. Material And Methods

2.1. Chemical and reagents

Ethanol (Sigma Aldrich Mumbai, India), *F. trinervia*, ethanolic extract, 3T3 L1, (DMEM) Dulbecco's Modified Eagle's Medium, Fetal bovine serum (FBS), Streptozotocin (Sigma Aldrich Mumbai, India), Distilled water and Animal feed were used for the study.

2.2. Plant collection and authentication

The F. trinervia were collected from Kovilpatti village, Thoothukudi district, Tamil Nadu, India in June 2021. Identification and authentication were performed at the Department of Botany, St. Xaviour's College, Palayamkotaai, Tamil Nadu, India. The collected materials were cleaned, shade dried, and powdered. These pulverized materials were used for further analysis.

2.3. F. trinervia ethanolic extract-Soxhlet

A "thimble" made of durable filter paper is used to hold ground crude powder in chamber E of the Soxhlet system. The extracting ethanolic solvent in flask A receives heat treatment, causing the vapours to condense in condenser D. [6] The crude medication is extracted by touch as the condensed extractant drips into the thimble. When the liquid level in chamber E reaches the top of syphon tube C, the contents of chamber E syphon into flask A. Repeat this process until a drop of the solvent in the syphon tube evaporates completely. The unprocessed ethanolic extract was then gathered and kept for phytochemical analysis. For additional research, the extract was kept in airtight plastic vials at 4°C. The ethanolic extract of F. trinervia was subjected to preliminary phytochemical examination, which revealed the presence of alkaloids, carbohydrates, glycosides, phenolic compounds, flavonoids, tannin, and proteins.

2.4. Amylase inhibitory assay using the DNSA method

The ethanolic extract of F. trinervia is available in quantities ranging from 50 to 500 µg/mL. 200 µL (2 U) of α-amylase solution were mixed with 200 µL of the F. trinervia ethanolic extract, and the mixture was then incubated for 10 min at 30°C. Each tube then received 200 µL of the starch solution, which was then incubated for 3 minutes. 200 µL of DNSA reagent were added to the reaction to stop it, and it then boiled for 10 min in a water bath at 85–90 °C. After being diluted with 5 mL of distilled water and chilled to room temperature, the liquid was tested for absorbance at 540 nm with a UV-Visible spectrophotometer. The plant extract was swapped out with 200 µL of buffer to create a blank with 100% enzyme activity. In the absence of the enzyme solution, a blank reaction was similarly constructed using the plant extract at each concentration. Acarbose (100 µg/mL-2 g/mL) was used to make a positive control sample, and the reaction was carried out in the same way as the previously reported reaction with plant extract. The percent inhibition used to express the-amylase inhibitory action. [7, 8]

$$\%\ \text{of inhibition} = \frac{(\mathrm{B} - \mathrm{A}) * 100}{(\mathrm{B} - \mathrm{C})}$$

C- Alpha amylase-free and with starch as a control.

B- Alpha amylase and starch constitute the control.
A- Test

2.5. Glucose up take assay-3T3 L1 cell lines

Ethanolic extract and marker chemicals stimulated glucose absorption in 3T3-L1 adipocytes using previously reported methodologies. Cells were cultured in 48-well plates with serum-free DMEM containing 0.2% BSA for 12 hrs. The cells were washed and cultured with various sample concentrations in low glucose medium with 10% FBS for 24 hrs. Insulin was standard. Using a commercial GO kit, glucose uptake was measured in 96-well plates. [9, 10, 11]

2.5. Acute toxicity study *F. trinervia* ethanolic extract

Wistar Albino female rats in good health, weighing between 150 and 200 grammes, were purchased from the Biogen animal facility in Bangalore, Karnataka, India. The Institutional Animal Ethical Committee (IAEC), which is recognised by the Committee for the Purpose of Control and Supervision of Experiments on Animals, gave its approval to the entire investigation (CPCSEA). The animals were kept in clean, dry polycarbonate cages in well-ventilated animal homes with a 12-hour light/12-hour dark cycle. Standard pellet food and unlimited water were provided for the animals. The animals were fasted overnight for experimental purposes but were given free access to water.

2.5.1. Acute toxicity class method

According to OECD guideline 423, acute oral toxicity was evaluated. A single dosage of the ethanolic extract was delivered using a tuberculin syringe. Animals are fasted 3 hrs prior to dosage (food was withheld for 3 h but not water) (food was withheld for 3 h but not water). Following the interval of fasting animals was weighed and test drug was delivered orally at a dose of 500 mg, 1000 mg, and 2000 mg/kg. Animals are monitored individually at least once during the first 30 minutes, periodically over the first 24 hours, with specific care paid to the initial four hours, and then daily for a total of 14 days.

***2.7.* In-Vivo anti-diabetes activity of *F. trinervia* ethanolic extract**

After overnight fasting, by administering a single injection intra peritoneally of STZ (40 mg / kg b.w) in the freshly prepared citrate buffer solution with the pH 4.5 to the animals were induced diabetic. Animals injected with STZ provided with a 20% glucose water overnight in order to combat death due to hypoglycaemic caused by medications. The same amount of isotonic saline was injected into the test rats. Diabetes state of rats were proved by observing level of blood glucose in fasting condition after the time period of 72 hrs after the administration of STZ. The rats with glucose levels more than 250 mg/dl were helpful in this research. [12, 13]

2.7.1. Experimental design

Experimental Wistar rats were grouped into five groups, each group containing six animals. The plant extract was administered intra peritoneally for 21 days.

Group 1: "Control rats"
Group 2: "Diabetic control rats"
Group 3: "Diabetic + Plant extract (500 mg/kg b.w)"
Group 4: "Diabetic + Plant extract (250 mg/kg b.w)"
Group 5: "Diabetic + Glibenclamide (5 mg/kg b.w)

Animals were kept for fasting overnight, at the end of the experimental phase. They were anaesthetized with the help of ketamine at the dose of 24 mg/kg b.w. administered intramuscularly and sacrificed was done by decapitation. Blood samples were obtained in test tube containing EDTA for analysis of inflammatory markers, blood glucose, non-enzyme antioxidants and insulin. Pancreatic tissues were then taken out and they are homogenized using Tris-Hydro chloride buffer having the pH of about 7.5 and the homogenates were helpful in the determination of enzymic or nonenzymic anti-oxidants, lipid peroxidative and inflammatory markers. For the sake of histological analysis, small part of the pancreatic tissues was removed and it is fixed in 10 % neutral formalin buffered solution.

2.8. Biochemical analysis

Measurement of the blood glucose and insulin were determined spectrophotometrically with the help of a commercial diagnostic kit which is described by the method of Trinder. ELISA kit technique was used for plasma insulin assay.

2.9. Statistical analysis

Statistical analysis was done via graph pad prism software version 8.0.1. Comparisons were tested by one-way analysis of variance (ANOVA) subsequently Duncan's test. The significance was measured at *P* values less than 0.05.

3. Results And Discussion

3.1. DNSA-based test for measuring the inhibitor of amylase

In-vitro amylase inhibitory studies were conducted on F. trinervia ethanolic extract and acarbose. F. trinervia ethanolic extract showed the inhibitory effect on α-amylase with an IC_{50} value 125 µg/mL. Acarbose (500 µg/mL) showed the 98.12 % inhibitory activity (figure 1).

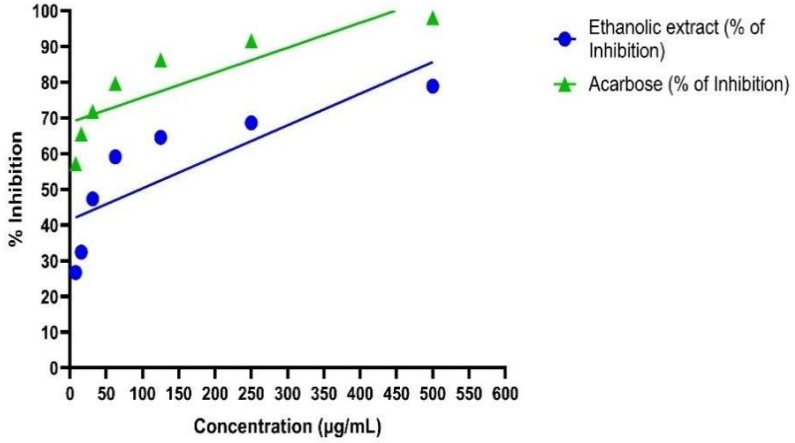

Figure 1: α-amylase inhibitory effect of *F. trinervia* ethanolic extract and acarbose.

3.2. Glucose up take assay-3T3 L1 cell lines

Studies on the 3T3-L1 cell lines consumption of glucose were done *In-vitro*. Glucose was added to 3T3-L1 cells, and cells were treated for 48 hrs with *F. trinervia* ethanolic extract (25 µM/mL, 50 µM/mL), both with and without insulin. According to experimental findings, insulin uptake stimulation is enhanced by 50 µM/mL when compared to control. According to the findings of the current investigation, *F. trinervia* ethanolic extract increases glucose absorption in *In-vitro* analysis (figure 2).

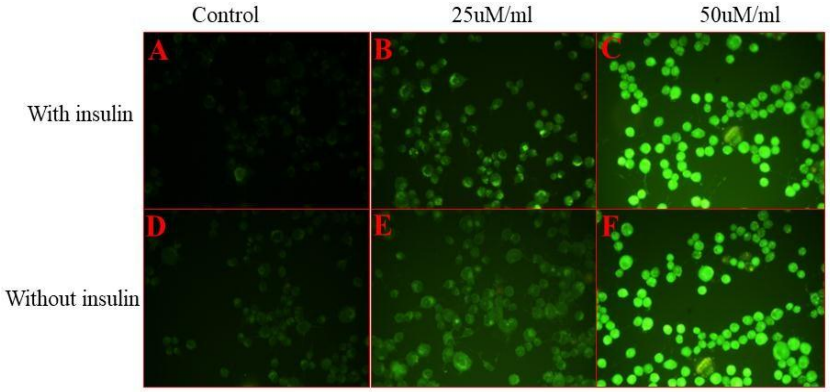

Figure 2: F. trinervia ethanolic extract enhance the glucose uptake was observed by using fluorescence microscope under 40X magnification by using the AO/ED staining. A &D: Control, B&E: 25 µM/mL F. trinervia ethanolic extract, C&F: 50 µM/mL F. trinervia ethanolic extract.

***3.3.* In-Vivo anti-diabetes activity of *F. trinervia* ethanolic extract**

In-vivo anti-diabetes study of F. trinervia ethanolic extract studied by using STZ induced method on Wistar albino rats.

3.1. Effect of *F. trinervia* ethanolic extract on body weight changes

The effect of *F. trinervia* ethanolic extract on body weight alterations in normal and diabetic hypertensive rats is shown in table 1. Before the HFD, all groups had similar body weights. The body weight increased considerably after the HFD diet was used to induce diabetic hypertension. Treatment of diabetic hypertensive rats with ethanolic extract resulted in a considerable reduction in body weight (table 1).

Table 1: Analysis of body weight (gm) in normal and experimental rats

Group	Before Induction of STZ on body weight	Body weight on diabetes animals treated with standard and extract			
		Body weight on 1st week	Body weight on 2nd week	Body weight on 3rd week	Body weight on 4th week

Control	156±1.2	162±1.61	167±1.45	170±1.45	174±1.36
STZ+Nicotinamide 120 mg/kg	156±1.82	94.2±29.9	102±32.3	102±32.5	105±33.2
STZ+Nicotinamide 120 mg/kg + Glibenclamide 10mg/kg	155±1.21	112±22.6	119±23.9	126±25.3	136±27.2
STZ+Nicotinamide 120 mg/kg + 250 mg/kg extract	156±1.89	96.7±30.7	100±31.8	106±33.4	112±35.3
STZ+Nicotinamide 120 mg/kg + extract 500 mg/kg	153±0.989	120±24	124±24.9	129±25.8	137±27.4

3.2. Acute toxicity studies

A basic toxicity analysis was carried out to show the correct and the safest dose range which can be applied for the further studies instead of providing full extract toxicity results. Acute toxicity tests have confirmed that ethanolic extract of *F. trinervia* up to the dosage of 2000 mg/kg was administered. *F. trinervia* made no significant changes in animal behaviour. No death up to the 2000 mg/kg body weight dose was observed. Mice were externally active. In the experimental duration (14 days), these results were found.

3.3. Effect *F. trinervia* ethanolic extract on blood glucose levels

The impact of a *F. trinervia* ethanolic extract blood glucose levels in experimental and control rats (table 2).

Table 2: Estimation of blood glucose in normal and experimental rats (gm/dl)

Group	Before Induction of STZ	After Induction of STZ		Fasting blood sugar level on diabetes animals treated with standard and extract	
	Initial fasting blood sugar	Fasting blood sugar on 72hr	Fasting blood sugar on 10th day	Fasting blood sugar on 15th day	Fasting blood sugar on 28th day
Control	94.5±2.4	87.3±3.07	82.2±2.37	72.7±2.09	74.8±2.64
STZ+Nicotinamide 120 mg/kg	85.8±3.88	588±52.8***	452±83.2ns	380±107*	350±84.8**
STZ+Nicotinamide 120 mg/kg + Glibenclamide 10mg/kg	80.1±6.9	345±58.2**	270±64.9^{ns}	255±56.1^{ns}	120±25.4^{ns}
STZ+Nicotinamide 120 mg/kg + 250 mg/kg extract	83.7±4.36	373±48.7**	242±85.7^{ns}	192±64^{ns}	165±35**
STZ+Nicotinamide 120 mg/kg + extract 500 mg/kg	81.5±3.31	348±57.5**	305±67.1*	248±51.9^{ns}	145±30.63^{ns}

3.4. Effect of *F. trinervia* ethanolic extract on insulin level in normal and experimental rats

The impact of a *F. trinervia* ethanolic extract on regulation of insulin levels on experimental and control rats (table 3).

Table 3: Estimation of insulin levels in normal and experimental rats (gm/dl)

Parameters	Control	STZ+ Nicotinamide 120mg/kg	STZ + Nicotinamide 120mg/kg +Glibenclamide 10mg/kg	STZ + Nicotinamide 120mg/kg+ extract 250mg/kg	STZ + Nicotinamide 120mg/kg+ extract 500mg/kg
Insulin (IU/ml)	0.99±0.185	1.88±0.0549***	0.717±0.0219^{ns}	1.18±0.127^{ns}	0.883±0.0549^{ns}

3.5. Effect of *F. trinervia* ethanolic extract on lipid profile in normal and experimental rats

Effects of an *F. trinervia* ethanolic extract on rats' normal and experimental lipid profiles (Table 4).

Table 4: The effect of an ethanolic extract of *F. trinervia* on the lipid profile of normal and experimental rats

Parameters	Control	STZ+ Nicotinamide 120mg/kg	STZ + Nicotinamide 120mg/kg +Glibenclamide 10mg/kg	STZ + Nicotinamide 120mg/kg +extract 250mg/kg	STZ + Nicotinamide 120mg/kg +extract 500mg/kg
Total Cholesterol (mg/dl)	176±4.46	257±13.6***	175±9.39^{ns}	222±5.92*	167±5.5^{ns}
Triglycerides (mg/dl)	180±7.45	265±12.8***	175±6.49^{ns}	216±7.9*	186±3.81^{ns}
HDL cholesterol (mg/dl)	38.1±0.889	65±1.46***	40.5±0.379^{ns}	58.9±3.49***	41.1±1.4^{ns}

3.6. Effect of F. trinervia ethanolic extract on liver glycogen level in normal and experimental rats

The impact of a F. trinervia ethanolic extract on rats with normal and experimental hepatic glycogen levels (table 5).

Table 5: The effect of an ethanolic extract of *F. trinervia* on liver glycogen levels in normal and experimental rats

Group	Control	STZ+ Nicotinamide 120mg/kg	STZ + Nicotinamide 120mg/kg +Glibenclamide 10mg/kg	STZ + Nicotinamide 120mg/kg +extract 250mg/kg	STZ + Nicotinamide 120mg/kg +extract 500mg/kg
Liver Glycogen (gm/dl)	0.149±0.0232	0.454±0.0177***	0.208±0.017^{ns}	0.266±0.037*	0.198±0.0113^{ns}

3.7. Effect of *F. trinervia* ethanolic extract on haematological levels in normal and experimental rats

The impact of an *F. trinervia* ethanolic extract on haematological parameters in experimental and normal rats (table 6).

Table 6: The effect of an ethanolic extract of *F. trinervia* on haematological levels in normal and experimental rats

Group	Control	STZ+ Nicotinamide 120mg/kg	STZ + Nicotinamide 120mg/kg +Glibenclamide 10mg/kg	STZ + Nicotinamide 120mg/kg +extract 250mg/kg	STZ + Nicotinamide 120mg/kg +extract 500mg/kg
RBC (X10 ^ $^{6/\mu L}$)	5.44±0.134	4.65±0.273^{ns}	5.68±0.226^{ns}	5.54±0.297^{ns}	4.71±0.2^{ns}
WBC (X10 ^ $^{3/\mu L}$)	10.3±0.757	10.4±1.54^{ns}	10.8±0.666^{ns}	11.3±0.437^{ns}	13±0.2^{ns}
Total Haemoglobin (g/dl)	13.5±0.404	10.9±0.367*	13.8±0.664^{ns}	14.2±0.867^{ns}	11.2±0.633^{ns}
Lympocytes (%)	91±2.52	86±1.5^{ns}	86.3±2.96^{ns}	88.3±2.4^{ns}	86.7±2.4^{ns}

3.8. Effect of *F. trinervia* ethanolic extract on enzymatic, non-enzymatic and lipid peroxidation levels in normal and experimental rats

Ethanolic extract of *F. trinervia* effect on enzymatic, non-enzymatic, and lipid peroxidation levels in normal and experimental rats (table 7).

Table 7: The effect of an ethanolic extract of *F. trinervia* on enzymatic, non-enzymatic, and lipid peroxidation levels in normal and experimental rats.

Group	Control	STZ+ Nicotinamide 120mg/kg	STZ + Nicotinamide 120mg/kg +Glibenclamide 10mg/kg	STZ + Nicotinamide 120mg/kg +extract 250mg/kg	STZ + Nicotinamide 120mg/kg +extract 500mg/kg
Total Protein (mg/dl)	0.75±0.0359	1.09±0.173^{ns}	0.785±0.056^{ns}	0.847±0.0427^{ns}	0.706±0.0725^{ns}
SOD (unit/min/ Mg protein)	0.422±0.0199	0.888±0.0618***	0.428±0.0303^{ns}	0.493±0.0542^{ns}	0.393±0.039^{ns}
Catalysis (µmole h202/min/mg protein)	0.332±0.0228	0.764±0.06**	0.426±0.0207^{ns}	0.624±0.109*	0.316±0.0263^{ns}
GPX (µmoles of glutathione oxidized/min/mg protein)	0.733±0.0382	1.22±0.12**	0.473±0.067^{ns}	0.84±0.0763^{ns}	0.447±0.0663^{ns}
GSH (µg/mg protein)	1.011±0.140	1.88±0.0648**	0.7557±0.094^{ns}	0.871±0.246^{ns}	0.7233±0.0880^{ns}
LPO (µmol/mg of MDA nmol/gm)	0.826±0.1968	1.669±0.1738	0.844±0.0617	0.984±0.145	0.619±0.144

3.9. Effect of ethanolic extract on carbohydrate metabolizing enzyme levels in normal and experimental rats.

Effect of ethanolic extract of *F. trinervia* on carbohydrate-metabolizing enzyme levels in experimental and normal rats (table 8).

Table 8: The effect of ethanolic extract of *F. trinervia* on the levels of carbohydrate metabolising enzymes in normal and experimental rats.

GPS	Glucose-6-phosphatase (unit/min/mg protein)	Fructose 1.6 bis phosphatase (unit/min/mg protein)	Glucokinase (unit/min/mg protein)	Hexokinase (unit/min/mg protein)
Control	0.209±0.0024	0.394±0.0393	0.246±0.0462	4.34±0.127
STZ+ Nicotinamide 120mg/kg	0.342±0.019*	0.642±0.0389***	0.554±0.0367**	2.65±0.0664***
STZ + Nicotinamide 120mg/kg +Glibenclamide 10mg/kg	0.255±0.0289^{ns}	0.34±0.0311^{ns}	0.198±0.00643^{ns}	4.07±0.185^{ns}
STZ + Nicotinamide 120mg/kg +extract 250mg/kg	0.295±0.0424^{ns}	0.41±0.00617^{ns}	0.457±0.0836^{ns}	3.84±0.0384^{ns}
STZ + Nicotinamide 120mg/kg +extract 500mg/kg	0.207±0.0142^{ns}	0.336±0.0335^{ns}	0.298±0.0539^{ns}	3.9±0.178^{ns}

3.10. *F. trinervia* ethanolic extract on oral glucose tolerance test (OGTT) in normal rats after treatment with extract.

The effect of *F. trinervia* ethanolic extract on experimental animal rats on glucose regulation (figure 3).

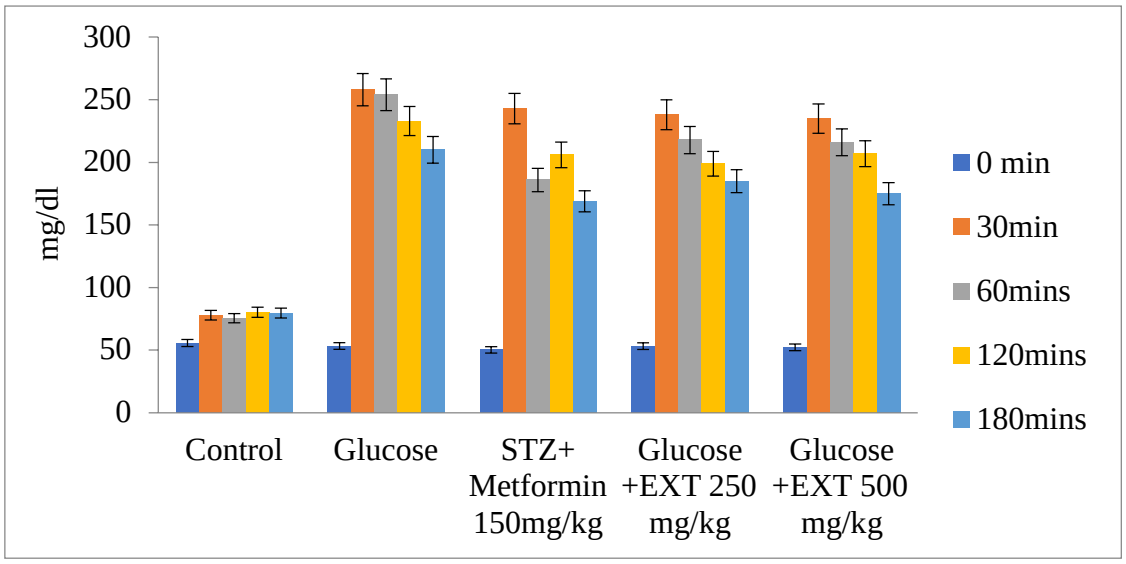

Figure 3: Effect of *F. trinervia* ethanolic extract on oral glucose tolerance test (OGTT) in normal rats after treatment with extract.

3.12. The histopathological analysis showed a defensive effect of STZ-induced *F. trinervia* ethanolic extract given on substantial alteration in pancreas of rats which are diabetic.

The standard rat pancreas is seen in fig.4-A and displays a normal pattern of islets. fig. 4-B demonstrates a diabetic rat's pancreas, and demonstrates all pancreatic acini and shrunken islet cells. fig. 4-C shows a diabetic rats pancreas handled with ethyl acetate extract, revealing regenerated islets of the pancreas. The pancreas of a diabetic mouse treated with Glibenclamide, demonstrating regeneration of the pancreatic islets, is described in fig.4-D.

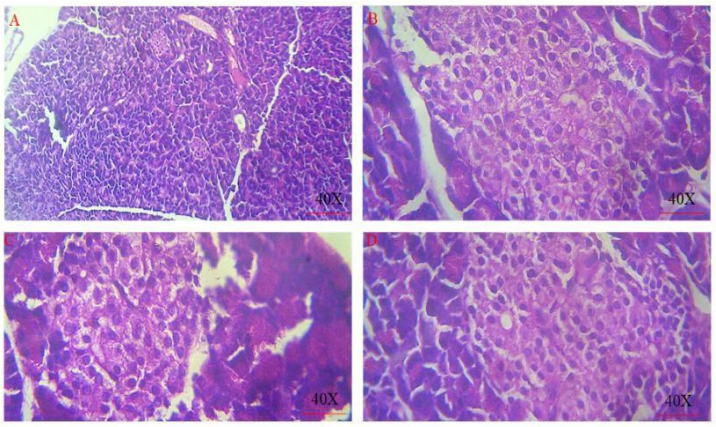

Figure 4: Histopathological analysis showed a defensive effect of STZ-induced *F. trinervia* ethanolic extract given on substantial alteration in pancreas of rats which are diabetic.

3.13. Statistical analysis

Each group (n=6), each value represents Mean ± SEM. One way ANOVA, followed by Dunnett comparison was performed. ***(***P<0.001)*** control group was compared with Only Glucose group-II. ***(***P<0.001-**P<0.01, *P<0.05)*** treated groups III, IV and V was compared with group I.

4. Conclusion

The medicinal plant named *F. trinervia* was taken for the present study. *F. trinervia* ethanolic extract conducted *In-vitro* and *In-vivo* diabetic study. The powdered plant was subjected to successive hot continuous extraction process using Soxhlet apparatus. The ethanolic extract was taken for the further isolation of phyto-constituents. The ethanolic extract showed significant *In-vitro* and *In-vivo* anti-

diabetic activity. *F. trinervia* phytoconstituents are responsible for the antidiabetic activity and further in detail study need to understand the mechanism of action of antidiabetic activity.

Conflicts Of Interest: Nil

Institutional Animal Ethical Certificate: Kmcret/Rere/Ph.D/56/2022

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