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Anti-diabetic analysis and insulin expression study of a wild leafy vegetable *Acalypha alnifolia* Klein ex wild. on streptozotocin-induced diabetic rats

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Abstract

Assessing the anti-diabetic potential of *Acalypha alnifolia* is the main objective of the present study. Two doses of leaf extracts were used for 21 days to produce streptozotocin-induced diabetic rats. The glucose level, insulin level, plasma lipids, total protein, electrolytes and plasma enzymes were monitored. The acetone extract of 400 mg/kg had potentiated to diminish the glucose and lipid level as well as other diabetic complications. Internal structures of pancreas were studied by histopathological sections and insulin expression was also observed by immunohistochemical study. Though the extracts having good anti-oxidant capability, it might be able to reduce the beta cell destruction or helps to repair it. The result of the toxicity analysis, histopathology and insulin expression supports the acetone extract. The present study attesting the anti-diabetic properties of *A. alnifolia* hence this plant would be focused and taken for cultivation leads to add in human diet for getting therapeutic benefit.

Introduction

Many medicinal plants have been evidenced for having anti-diabetic activity with lipid lowering feature. Ethnobotanical information indicates that more than 800 plants are used as traditional remedies for the treatment of diabetes throughout the world. The plant *Acalypha alnifolia* which is also known as *A. capitata* belongs to the family Euphorbiaceae, is traditionally used as a leafy vegetable (Sasi and Rajendran, 2012), for skin problem (Revathi et al., 2013b) and diabetes (Balakrishnan et al., 2009). Evidently the use of *A. capitata* in Southern Nigeria local communities as vegetable and it is used in the treatment of hypertension (Johnkennedy et al., 2011) are also the reason to focus on this plant for diabetes evaluation.

The plant *A. alnifolia* having significant anti-oxidant property which is coincidentally comparable with quantity of phenolic compounds present in it. Hence it should be have a good pharmacological potential (Ponnusamy and Thangaraj, 2014). *In vivo* anti-oxidant activity showed that *A. alnifolia* was effective in restoring activities of the oxidant stress to normal levels (Evanjelene and Natarajan, 2012). The previous reports proved their hypolipidemic ability on high cholesterol rats (Johnkennedy et al., 2011). As a result the research is needed to explore the plant potential against diabetes. Hence the study has undertaken to fulfill the objectives to reveal the anti-diabetic potential which includes the refurbish of diabetic complications and to evaluate the insulin secretion-inducing capacity on diabetes induced rats.

Materials and Methods

Extraction

The leaves were separated from the plant, washed in running tap water and shade dried which were powdered and packed in small thimbles and extracted

successively with different solvents such as petroleum ether, chloroform, acetone, methanol and hot water (maceration), in the increasing order of polarity using soxhlet apparatus each for 72 hours at 30°C. The extract obtained was dried and used for further assessments.

The yield percentage was measured by the weight obtained at each solvent extraction. Methanol (16%) was yielded higher than acetone (9.7%). Petroleum ether (3.3%) and chloroform (2%) yield was very least where both were low polar solvents whereas, hot water yields 7.5%. Besides, acetone and methanol extracts have potent anti-oxidant capacity with good quantity of secondary metabolites (Revathi et al., 2013a). Hence, these extracts were selected for the pharmacological study.

Animals used

Adult Wister albino rats weighing between 150-200 g (for anti-diabetic experiment) and adult Swiss albino mice weighing between 25-30 g (for toxicity experiment only) of either sex were used for the studies. The animals were maintained under normal laboratory condition and kept in standard polypropylene cages at room temperature and 60 to 65% relative humidity and provided with standard diet and water *ad libitum*. The study was carried out by the methodology described in the OECD guidelines with consent from Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA) and Institutional Animal Ethics Committee (IAEC), Proposal number NCP/IAEC/No. 03/2013-14.

Acute toxicity

The animals were divided into control and test groups containing six animals each. The test groups got marked doses of acetone and methanol extracts orally up to 2000 mg/kg gradually and was observed for mortality, muscle tensile, changes in body secretion and behavioral changes till 48 hours. There were no observable changes and no mortality hence the doses were fixed as 1/10th (200 mg/kg) and 1/5th (400 mg/kg) of the higher dose as per OECD Guideline 423. Each extract were dissolved in vehicle 0.6% carboxy methyl cellulose (CMC), for experimental study. In all the experiments CMC have given for control group.

Effects on normal and glucose loaded rats

A combined methodology was used to determine the effect of the extract on normal and glucose-loaded animals (2005; Orhan et al., 2006). Male Wistar-albino rats were divided into five groups of 6 rats each. Fasting blood glucose levels were measured after which doses of 400 and 200 mg/kg of the acetone and methanol leaf extracts of *A. alnifolia* were administered (p.o.) and vehicle was administered (p.o.) to control group. Blood glucose levels were monitored at 30 and 60, 120, 180 and 240 min for observe the hypoglycemic nature of the extracts. Then the oral glucose tolerance test was performed. The rats were orally loaded with 2 g/kg glucose and observance of blood glucose levels were continued to determine the anti-hyperglycemic effect of extracts like previous.

Diabetes induction

Streptozocin (STZ) was administered in a single intraperitoneal injection at the dose of 55 mg/kg (dissolved in 0.1 M citrate buffer pH 4.5) to 16 hours fasted rats (Sezik et al., 2005). Fasting blood glucose levels were measured after 5 days, animal with blood glucose concentration level above 250 mg/dL was considered to be with diabetes. To overcome the hypoglycemia which occurred during the first 24 hours following the STZ administration, diabetic rats were orally given 5% glucose solution.

Treatment protocol

The diabetic animals were divided into six groups, each containing six animals and one group of normal (non diabetic) animals. The acetone and methanol extracts of *A. alnifolia* were given at the doses of 200 and 400 mg/kg, orally for a period of 21 days as a suspension dissolved in CMC to different groups of diabetic animals.

Group I: Normal animals received vehicle; Group II: Diabetic animals received vehicle; Group III: Diabetic animals received standard anti-diabetic drug glibenclamide (5 mg/kg, p.o.); Group IV: Diabetic animals received acetone extract (200 mg/kg, p.o.); Group V: Diabetic animals received acetone extract (400 mg/kg, p.o); Group VI: Diabetic animals received methanol extract (200 mg/kg, p.o); Group VII: Diabetic animals received methanol extract (400 mg/kg, p.o.).

At the end of the experimental period the animals were fasted overnight and blood was taken from the retino orbital plexus under mild ether anesthesia, serum was separated out and blood sugar level was evaluated by the method of glucose oxidase-peroxides method using span diagnostic kits. The lipids like triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) were also examined. Blood serum electrolytes, urea, creatinine, protein and enzymes like serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were observed. The pancreas was collected for further observation.

Histopathology and immunohistochemistry

Collected pancreas from the treated animals was preserved in 10% formalin which was undertaken for histopathology study. Then representative blocks of pancreas was taken and possessed for paraffin embeds

by using the standard microtechnique (Galighor and Kozloff, 1976). Sections (5 µm) of tissues stained with hemotoxylin and eosin were observed microscopically for histopathological studies. The pancreatic tissues were taken for immunohistochemistry. Insulin expressions of pancreatic beta cells were evaluated by using immunohistochemistry technique. The technique can be used on frozen and paraffin embedded tissue sections and on cytological samples. The immunohistochemically stained sample is analyzed with light microscope and find the location of insulin in pancreatic tissues. Generally this method is used for the analysis of protein localization in tissues.

Statistics

Results obtained from the data have been expressed as mean ± SEM and were compared with the corresponding control group by applying one-way analysis of variance (ANOVA). Significance of each and comparison with control was assessed by Dunnet's t-test.

Results

Effect of A. alnifolia leaf extracts on normal and glucose loaded rats

The effect on normal and glucose loaded rats has been used as a prediction experiment before doing the anti-diabetic experiment with induced animals. The two doses of each extract were administered to normal rats and the glucose levels were recorded. The glucose levels of extract treated rats got reduced during the course of time which was measured up to 4 hours. The untreated rats have no such decline in their glucose level. The results were expressed in mg/dL. Out of two doses of two extracts, higher doses (400 mg/kg) of acetone extract exhibiting good hypoglycemic effect in this experiment. The glucose level of acetone extract treated animal underwent till 54 mg/dL (average) in the fourth hour from normal range.

The oral glucose tolerance test (OGTT) is a widely used procedure that was originally developed to classify carbohydrate tolerance (WHO). The extract treated glucose loaded rats showed diminished glucose level and the values were at odds with glucose loaded control. The glucose level of treated animals was evaluated for 4 hours which is decreasing with time. The result of OGTT confirms the anti-hyperglycemic effect of extracts and like previous where the acetone extract decreased glucose level.

Effects of A. alnifolia leaf extracts on STZ induced rats

Insulin, glucose levels and lipid profile

Diabetes in the experimental rats was confirmed by the presence of high blood glucose level. Glucose level of the rats before and after STZ induction up to 21 days with treatment was monitored and tabulated (Table I).

The glucose level was gradually reduced while experimental period reaches 21st day. The 400 mg/kg acetone extract has the potential to reduce the glucose level (169 mg/dL) excellently than another dose and extract. The level of insulin confirms the diabetic condition of vehicle treated animals. Regeneration of pancreatic beta cells might leads to improvement in insulin secretion making this evaluation as positivity towards the desired result. The range of insulin and lipids is shown in Table II. The lipid components such as TC, TG, LDL and VLDL were reduced and HDL was increased when compare to diabetic control group animals.

Electrolytes

It has solidly supports to methanol and then acetone extract when compared to diabetic control. Na+, Cl- and HCO^{3-} are increased in methanol extract treated followed by acetone extract treated animals and K+ is well increased in acetone extract treated animals.

Table I

Blood glucose level of each group during the experiment

Names	Glucose level (mg/dL; mean ± SEM)			
	Day 0	Day 7	Day 14	Day 21
Normal control	129.6 ± 1.4	122.8 ± 2.3	137.5 ± 1.6	123.4 ± 2.9
Diabetic control	393.3 ± 2.2	387.3 ± 2.0	382.0 ± 1.3	375.8 ± 5.3
Glibenclamide (5 mg/kg)	376.1 ± 1.6	225.2 ± 2.4	187.3 ± 0.9	152.4 ± 1.8[a]
A. alnifolia (acetone extract; 200 mg/kg)	392.4 ± 1.3	297.8 ± 0.5	219.4 ± 2.4	181.5 ± 2.2
A. alnifolia (acetone extract; 400 mg/kg)	354.9 ± 1.9	237.8 ± 1.8	214.3 ± 0.8	169.2 ± 1.7[a]
A. alnifolia (methanol extract; 200 mg/kg)	322.2 ± 1.3	242.5 ± 1.4	289.2 ± 0.6	198.8 ± 1.4
A. alnifolia (methanol extract; 400 mg/kg)	345.3 ± 0.8	258.3 ± 2.1	228.5 ± 2.8	176.8 ± 2.5

[a]$p<0.05$

Table II

Insulin and lipid profile of animals at the end of the experiment

Names	Insulin (mIU/mL)	TC (mg/dL)	TG (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
Normal control	7.1 ±1.2	93.3 ± 2.2	103.3 ± 1.6	68.8 ± 2.4	44.5 ± 0.9	22.4 ± 2.8
Diabetic control	2.1 ± 0.4	160.1 ± 1.8	195.3 ± 1.4	20.1 ± 3.7	73.6 ± 2.3	39.2 ± 1.9
Glibenclamide (5 mg/kg)	6.7 ± 0.9[a]	98.3 ± 0.5[a]	100.3 ± 1.8[a]	62.3 ± 1.7[a]	46.2 ± 1.6[a]	21.7 ± 1.1[a]
A. alnifolia (acetone extract; 200 mg/kg)	3.2 ± 2.4	144.5 ± 1.3	139.3 ± 2.5	53.4 ± 2.7	64.7 ± 2.9	35.2 ± 3.3
A. alnifolia (acetone extract; 400 mg/kg)	4.9 ± 1.7	115.0 ± 2.6[a]	118.3 ± 3.3[a]	72.8 ± 1.9[a]	47.3 ± 1.3[a]	27.8 ± 2.9
A. alnifolia (methanol extract; 200 mg/kg)	2.8 ± 1.41	158.2 ± 1.8	158.0 ± 2.4	37.9 ± 2.7	58.4 ± 1.7	40.5 ± 0.6
A. alnifolia (methanol extract; 400 mg/kg)	3.5 ± 0.3	132.3 ± 1.3	135.3 ± 1.6	49.4 ± 1.1	31.3 ± 2.3	31.8 ± 1.3

[a]p<0.05

Urea, creatinine, albumin, protein and plasma enzymes

Blood urea and creatinine is widely accepted to assess the renal functions. The present study demonstrate that diabetes mellitus produced a significant increase in serum creatinine level compared with the control group (36.2 mg/dL) and showed which were significantly lower in the diabetic group receiving glibenclamide (18.2 mg/dL) on 21st day. There is considerably low in 400 mg/kg acetone extract treated group (27.8 mg/dL) compared with the respective diabetic group and other extract treated groups. Albumin and total protein level of diabetic groups were diminished and those are better in extract treated especially 400 mg/kg acetone extract (5.6 and 4.7 mg/dL respectively) and standard treated group. Desirably, the SGOT (44.4 U/L) and SGPT (37.2 U/L) levels were reduced in acetone extract treated groups whereas in standard treated group the values were 37.3 and 22.4 U/L respectively.

Histopathology of pancreas

Based on blood parameters the higher doses of each extracts (400 mg/kg) have been undertaken for histopathology study. The dissec-ted pancreas was fixed in 10% formalin. The islets of β-cells in diabetic rats are reduced in number and size with focal cytoplasmic vaculations and necrosis have been observed more in diabetic animals and which was well differentiated from treated animals (Figure 1). Pancreatic damage is far worse in diabetic animals when compared with standard treated and extract treated groups. The acetone extract treated pancreas showed minimal cell death which supports the extract potential is helps to regenerate the dead tissues.

Histopathology sections shows cytoplasmic vaculation have been observed in diabetic control animal and it is reduced in extract and glibenclamide treated rats

Immunohistochemistry

The insulin secretions from pancreas are articulated in

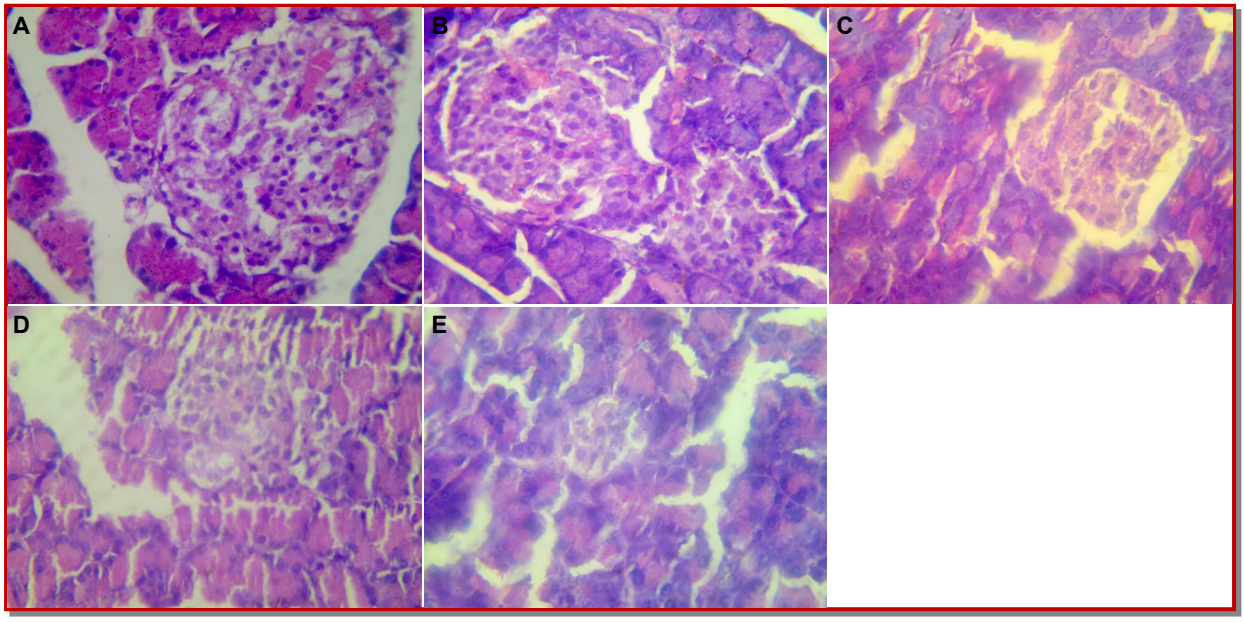

Figure 1: Histology of treated animal pancreas. (A) Normal control rat; (B) Glibenclamide treated rat pancreas; (C) STZ treated rat pancreas; (D) *A. alnifolia* acetone extract treated; (E) *A. alnifolia* methnol extract treated rat pancreas

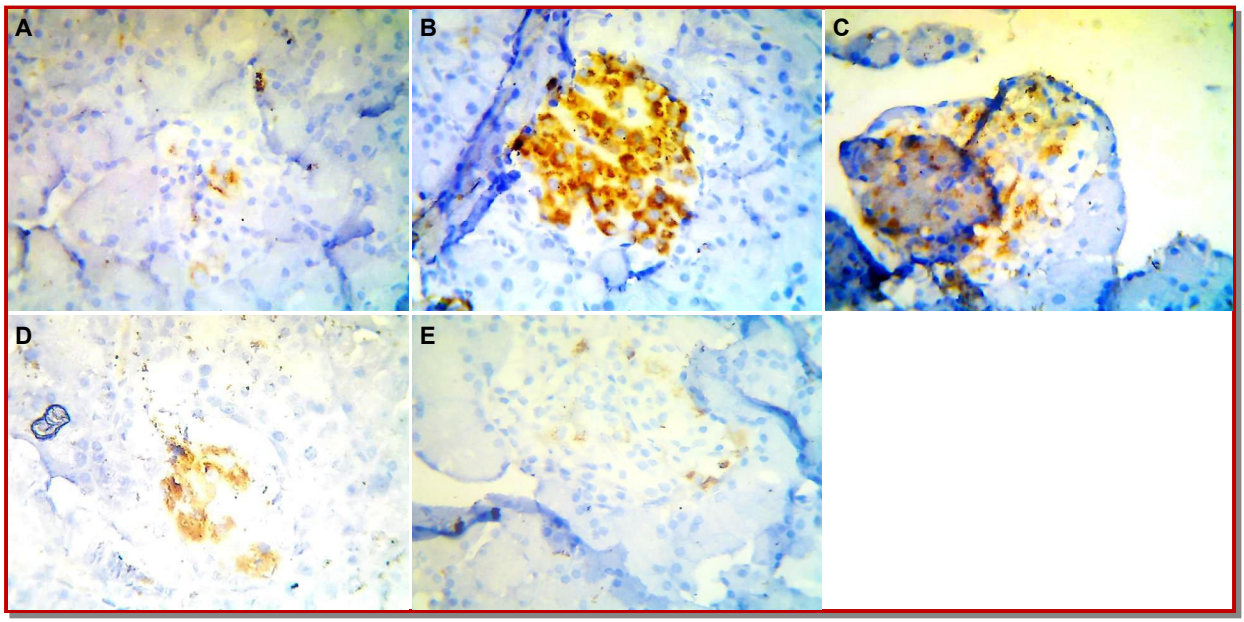

Figure 2: Insulin expressions of treated animal pancreas in immunohistochemistry study. (A) Diabetic control rat; (B) Normal control rat; (C) Glibenclamide treated; (D) *A. alnifolia* acetone extract treated; (E) *A. alnifolia* methanol extract treated

yellowish brown color (Figure 2) which retain on insulin-insulin antibody complex during destaining process. It is clearly indicates the insulin location on the tissue. The insulin expressions are much better in normal control and standard treated animals followed by acetone extract treated animal pancreas.

Discussion

The toxicity study denotes there were no general behavioral changes even at higher doses of extracts. However, the OGTT remains the most commonly performed test to examine glucose tolerance by which the hypoglycemic effect of extracts has been predicted while given to normal and glucose loaded rats. The diabetes inducing agent STZ is specifically induces DNA strand breakage in β-cells causing diabetes mellitus. Now-a-days, clinical treatment of diabetes targets both insulin deficiency and resistance and more recently the prevention of pancreatic β-cell function decline (Hansotia and Drucker, 2005). Since the ethnobotany reports are strong, the factual potency of *A. alnifolia* has revealed where the extracts might be taking better role in the repair of chromosomal and protoplasmic damages.

In normal metabolism insulin activates the enzyme lipoprotein lipase which hydrolyses triglycerides and the deficiency of insulin results inactivation of these enzymes thereby causing hypertriglyceridemia (Maruthapandian and Mohan, 2011). Besides, the excess of fat diet increased the TG level (XU et al., 2005) and VLDL production (Kesavalu et al., 2001) which is the causes for hardening of arteries. The present study results showing the extracts decreased the TG, TC and LDL levels and increased the level of HDL desirably which will definitely reduce the diabetic complications.

The sodium, potassium, chloride and bicarbonate ions present in treated groups were analyzed with the blood samples. The plant *A. alnifolia* extracts facilitate to reduce the electrolyte imbalance where it helps to adjust the rate of electrolyte retention to keep serum electrolyte levels constant. Good control of blood glucose level is absolute requirement to prevent progressive renal impairment (Shrestha et al., 2008). In present study the extracts assist to prevent the renal damage by reducing urea and serum creatinine. Keep hold of total protein and albumin near to normal metabolic serum in acetone extract treated animals obviously.

The administration of aqueous leaf extract of *A. capitata (A. alnifolia)* produced a hypolipidemic effect (Johnkennedy et al., 2011). In addition to that, the present study accomplished the extract being good in metabolism and helps to regain from diabetic complications. The β-cell necrosis was reduced in treated groups noticeably. The insulin expression confirms the β-cell proliferation capacity of extracts and it might helps to prevent from cell damage. The screened diabetic complications are reduced to a great extent in acetone extract. The effect is might be due to the anti-oxidant and secondary metabolites (Revathi et al., 2013a) were better in *A. alnifolia* acetone extract.

Conclusion

The present study results concluded that the acetone extract having ability to increasing the insulin secretion as well as mitigating the diabetic complications which is supporting with the previous reports and this study confirms the therapeutic effect of *A. alnifolia*.

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