ASSESSMENT OF CARBOHYDRATE-METABOLIZING ENZYMES AND HISTOPATHOLOGICAL CHANGES FOLLOWING CTAF TREATMENT IN EXPERIMENTAL DIABETIC RATS

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Abstract:

Cyanotis tuberosa (Roxb.) Schult. &Schult. f. is an ethnomedicinal plant that has traditionally been used to treat diabetes, liver diseases, ulcers, and other diseases. The present study was aimed to analyse bioactive compounds present in the *Cyanotis tuberosa* active fraction by GCMS analysis and also its long-term effect on carbohydrate metabolising enzymes and histopathological studies in experimental group of rats after long-term treatment. The CTAF was subjected to GCMS analysis for the qualitative analysis of bioactive compounds. Biochemical analyses and histological examinations were performed were carried out in experimental group of rats treated with CTAF for 30 days. And GCMS analysis of CTAF for the identification of bio active phytochemicals responsible for therapeutic effects were evaluated. GCMS analysis of CTAF revealed the presence of 14 bioactive compounds**.** Diabetic rats treated with CTAF had shown improved activities of carbohydrate metabolizing enzymes. Normal morphology of liver, pancreas, and kidney tissues were identified in CTAF-treated diabetic rats. Our findings have shown that the isolated active fraction of *C. tuberosa* (CTAF) positively controlled glucose homeostasis by improving the function of carbohydrate metabolising enzymes and improved the normal tissue architecture of the liver, pancreas and kidney tissues in STZ induced diabetic rats.

Key words: *Cyanotis tuberosa*, Diabetic rats, carbohydrate enzymes, Hyperglycemia, antidiabetic activity.

1. Introduction

According to the International Diabetes Federation, 425 million people worldwide have diabetes, with the number anticipated to increase to 629 million by 2045[1]. Diabetes mellitus (DM) is an illness characterized by elevated blood glucose levels caused by a breakdown in glucose homeostasis caused by a partial or complete lack of insulin and/or insulin action. Another condition called Hyperglycemia is also caused by changes in carbohydrate, lipid, and protein metabolisms in target tissues such as the liver, kidney, skeletal muscle, and adipose tissues. Both type 1 and type 2 diabetes are characterized by insulin resistance and oxidative damage, which are both brought on by persistent hyperglycemia.

Hyperglycemia-induced oxidative stress is caused by a number of metabolic processes, including glucose autooxidation, protein kinase C activation, methylglyoxal production, glycation, hexosamine metabolism, sorbitol generation, and oxidative phosphorylation. Many research studies [2], including one by our research group, have been published the connection between oxidative stress and diabetic complications.

Pharmaceutical drugs and insulin used to treat diabetes are scarce, expensive, and have significant adverse effects in developing countries (particularly rural areas). Treatment costs are high and costly in developing countries, which is a major obstacle to DM treatment. As a result, phytotherapy and the usage of natural anti-diabetic substances are frequently used as first-line treatments and care [3]. Herbal therapy has the potential to mitigate the detrimental effects of synthetic medications. Medicinal herbs have been used to treat diabetes [4]. Due to their availability, low side effects, and cost-effectiveness, bioactive chemicals are the basic necessary component of modern therapies, particularly in rural areas [3].

Cyanotis tuberosa (Roxb.) Schult. &Schult.f., is one of such plant that belongs to Commelinaceae family which is also known as "Eggobull gadda" in Telugu, "Sahyadri dew grass," or "Greater cat ears" in English. Tribal people in North-Eastern India eat this plant as a leafy vegetable [5]. The tribal tribes of eastern India utilize the root sections of *C. tuberosa* to treat fevers and worm infestation in livestock [6]. The Santal tribe uses two spoonfuls of *C. tuberosa* root paste twice daily for 15 days to treat liver problems, menstrual disorders, and diabetes [7]. It also works well as an antipyretic and laxative [8]. The antidiabetic and anti-hyperglycemic activity of CTAF were studied [9]. Hence, in this study for the first time we have evaluated the effect of CTAF on carbohydrate metabolising enzymes in STZ induced diabetic rats.

2. Materials and Methods

2.1 Collection of plant material and preparation of active fraction of *Cyanotis tuberosa*

Cyanotis tuberosa root tubers were collected in and around the Tirumala-Tirupati area and had done the procedure to collect the active fraction described in the previous study [9]. To summarize, the hexane extract was obtained by immersing powdered root tubers of *Cyanotis tuberosa* in a glass container for 48 hours at ambient temperature, followed by solvent collection through filtration. This process was repeated three to four times until the filtrate became devoid of color. The filtrate in the Buchi Rotavapor R-200 was subjected to concentration under reduced pressure prior to freeze-drying. The hexane extract was stored in airtight containers at a temperature of 0°C until it is required. As stated earlier [9], the hexane extract of *Cyanotis tuberosa* was subjected to bioassay-guided fractionation in order to produce CTAF.

2.2 GC-MS analysis of CTAF

The GC-MS analysis of CTAF was performed using JEOL GCMATE II GC-MS (Agilent Technologies 6890N Network GC system for gas chromatography), equipped with sea condary electron multiplier. The column (HP5) used was fused silica 29.3 m x 0.25 mm I.D. Analysis conditions were 20 minutes at 100°C, 3 minutes at 235°C for column temperature, 240°C for injector temperature, helium as the carrier gas and split ratio was 5:4. One micro litre sample was evaporated in a split less injector at 300°C with a run time of 30 minutes. The compounds were identified by gas chromatography coupled with mass spectrometry. The molecular weight and structure of the compounds of test materials were ascertained by interpretation on mass spectrum of GC-MS using the database of National Institute Standard and Technology (NIST).

2.3 Induction of Diabetes

Albino Wistar male rats aged 3–4 months and weighing around 180–200 g was selected for the induction of Diabetes. Diabetes was induced in overnight-fasted rats by a single intraperitoneal injection of freshly produced STZ in ice-cold citrate buffer (45 mg/kg. b.w) as described by Vedasree et al., 2022 [9]. STZ can cause deadly hypoglycemia as a result of excessive pancreatic insulin release caused by the death of pancreatic β cells, the rats were given a 5 percent glucose solution six hours after STZ injection to prevent hypoglycemia. Fasting blood glucose (FBG) levels were utilized to diagnose diabetes, and rats with FBG levels of ≥250 mg/dl, were marked as diabetic and selected for experimental studies.

2.4 Evaluation of Carbohydrate metabolizing enzymes

Enzyme assays of hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PDH), glucose6-phosphatase (G6Pase), and fructose-1,6-bisphosphatase (FBPase) were carried out according to Brandstrup et al., [12], Langdon et al., method [13], King [14] and, Gancedo and Gancedo [15] respectively. Inorganic phosphate was estimated by the Fiske Subbarow method [16].

2.5 Histological studies

Following treatment, the pancreas, liver, and kidney tissues were processed, preserved, and paraffin blocks were created. Serial sections of 5m thickness was cut with a rotary microtome and stained with hematoxylin and eosin according to established techniques [17].

2.6 Statistical Analysis

All the data were analyzed with Prism 5.0 version. The statistical variance was analyzed by using one-way ANOVA and Tukey's test was used for multiple comparisons between groups.

3. RESULTS

3.1. GC-MS analysis of CTAF

Fourteen compounds were detected and identified in CTAF by GC-MS analysis (Table 8). The representative mass spectrogram of CTAF is shown in fig.1. These compounds were identified by their fragmentation pattern

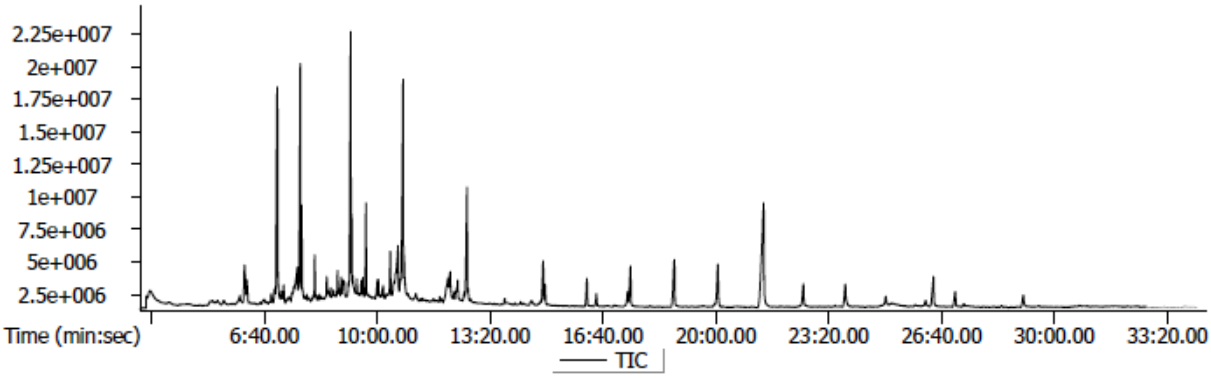

Figure 1. GC-MS chromatogram of CTAF

Table.1. The list of compounds present in the CTAF with their Retention times, molecular weights, and molecular formulas

S.no	Compound name	Retention time	Molecular weight	Molecular formula
1	*Thymol*	5.12	150.104	$C_{10}H_{14}O$
2	*2', 6'-Dimethoxyacetophenone*	6.47	180.078	$C_{10}H_{12}O_3$
3	*"(-) Quinic acid"*	8.14	192.0634	$C_7H_{12}O_6$
4	*2-Ethyl-1-Pentamethyldisilyloxyhexane*	9.22	260.1992	$C_{13}H_{32}OSi_2$
6	*Eicosane,*	10.01	282.328	$C_{20}H_{42}$

7	*n.-Hexadecanoic acid*	10.36	256.240	$C_{16}H_{32}O_2$
8	*Oleamide*	12.02	281.2719	$C_{18}H_{35}NO$
9	*Phenylglyoxylic acid*	12.03	150.0317	$C_8H_6O_3$
10	*9-Hexadecenoic acid"*	12.09	254.224	$C_{16}H_{30}O_2$
11	*Piperazine, 1-(5-chloro-2-methoxybenzenesulfonyl)-4-(2-fluorophenyl)-*	12.11	384.071	$C_{17}H_{18}ClFN_2O_3S$
12	*Eicosanoic acid"*	12.22	312.3028	$C_{20}H_{40}O_2$
13	*n-Tetracosanol-1*	14.54	354.3862	$C_{24}H_{50}O$
14	*(+)-à-Tocopherol"*	23.51	430.3811	$C_{29}H_{50}O_2$

3.2 Effect of CTAF on hepatic and renal carbohydrate metabolizing enzymes

The activities of carbohydrate metabolizing enzymes in hepatic and renal tissue of the different groups of rats are presented in Fig.2 and Fig.3 respectively. In hepatic and renal tissues of diabetic control rats, activities of HK and G6PDH were decreased significantly, conversely the activities of gluconeogenic enzymes G6Pase and F1-6-BPase were increased. But, treatment with CTAF has significantly decreased the activities of gluconeogenic enzymes G6Pase and F1-6-BPase with an increase in glycolytic enzymes HK and G6PDH activities in hepatic and renal tissues of diabetic treated rats. No significant changes in the activities of both glycolytic and gluconeogenic enzymes were observed in normal control and normal treated rats. In diabetic rats treated with glibenclamide, the activities of the glycolytic enzymes were increased and the decreased activities of gluconeogenic enzymes were observed but the effect was less than that of CTAF.

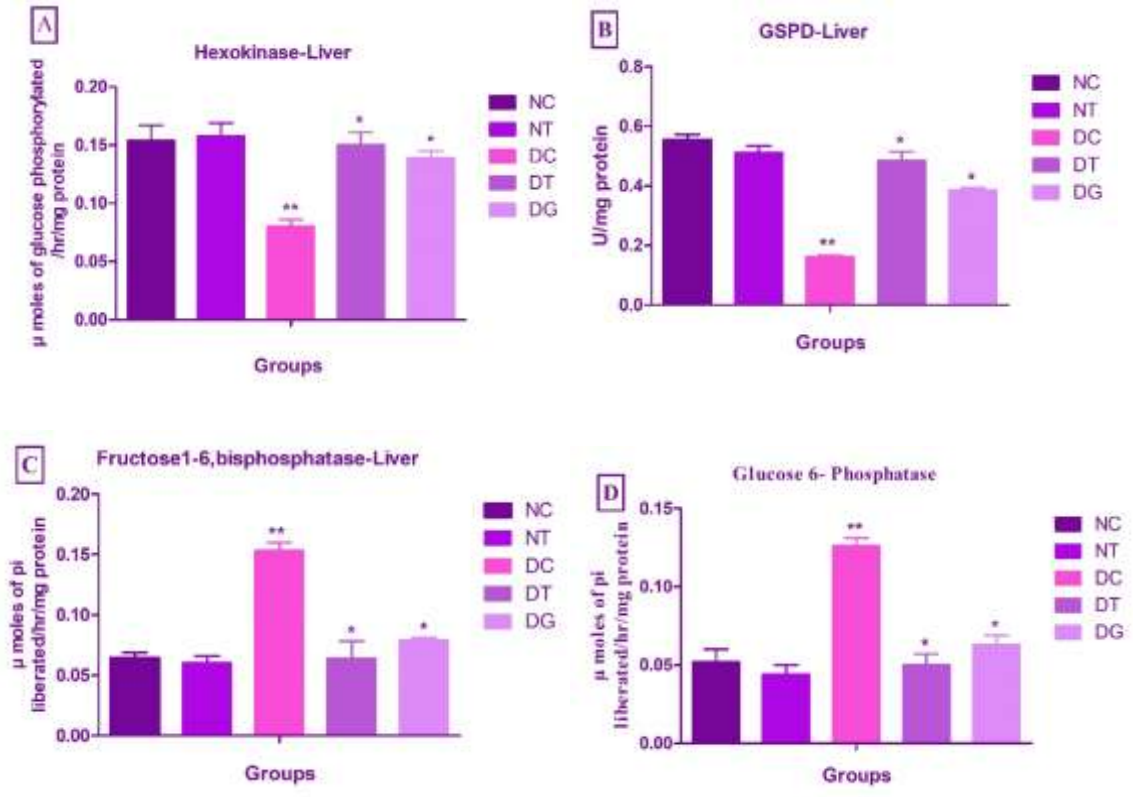

Figure: 2 Effect of the CTAF on carbohydrate metabolic enzymes activities in liver

NC-Normal control rats; NT: Normal treated rats; DC-Diabetic control rats; DT Diabetic treated rats (75 mg/kg b.w of CTAF) and DG-Diabetic glibenclamide treated rats (20 mg/kg b.w).
Values are given as mean ± S.D (n = 6 six in each group).
**p < 0.01 compared to normal control rats.
*p < 0.05 compared to normal control rats.
*p < 0.05compared to diabetic control rats.

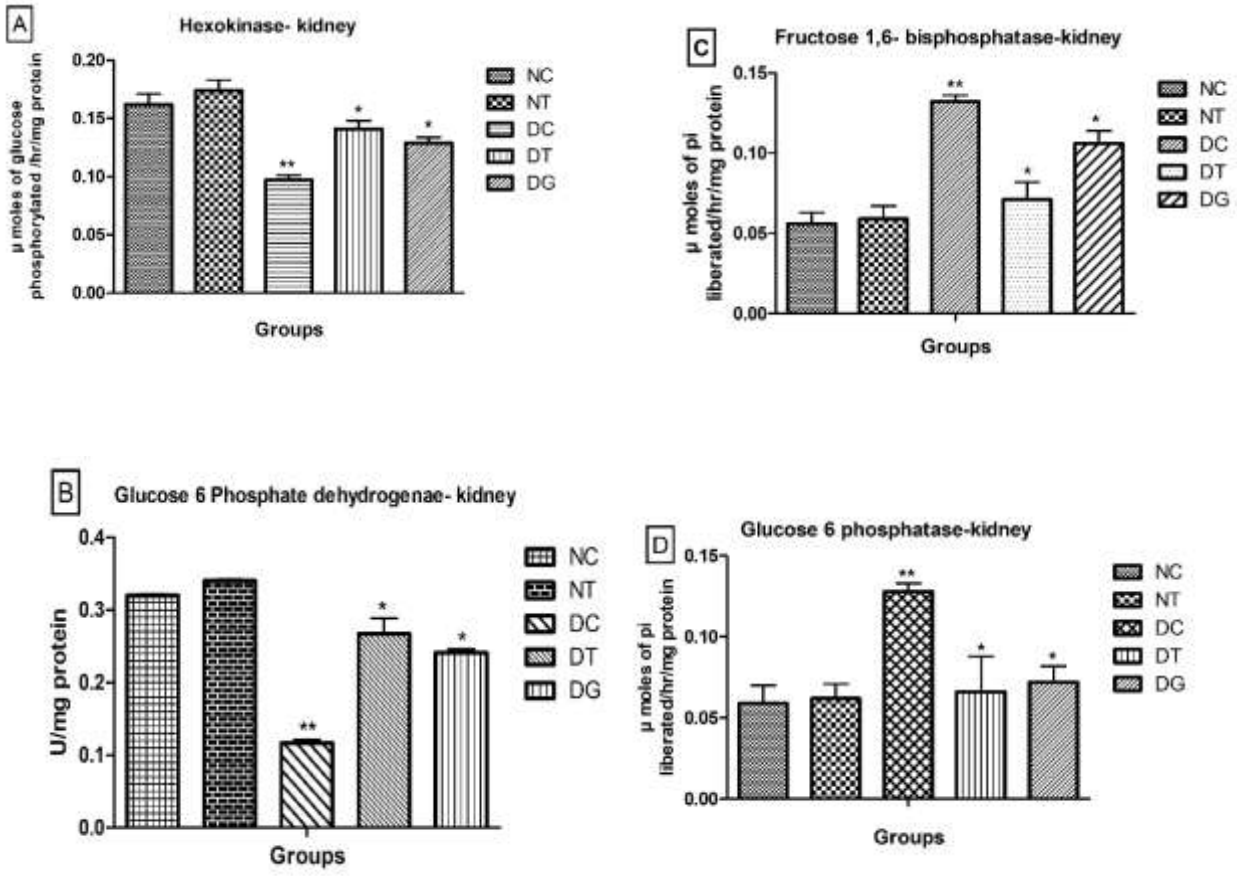

Figure: 3 Effect of the CTAF on carbohydrate metabolic enzymes activities in Kidney

NC-Normal control rats; NT: Normal treated rats; DC-Diabetic control rats; DT Diabetic treated rats (75 mg/kg b.w of CTAF) and DG-Diabetic glibenclamide treated rats (20 mg/kg b.w).
Values are given as mean ± S.D (n = 6 six in each group).
**p < 0.01 compared to normal control rats.
*p < 0.05 compared to normal control rats.
*p < 0.05compared to diabetic control rats.

3.3 Effect of CTAF on glycogen and protein levels of normal and experimental diabetic rats.

In the diabetic control group of rats, the levels of hepatic and muscle glycogen and protein levels in plasma, liver, and kidney were significantly lower than in normal control and normal treated rats with CTAF. In diabetic rats treated with CTAF, there was a significant increase in glycogen levels in both liver and kidney and also a rise in protein levels in plasma, liver, and kidney. Similar results were observed in the group of diabetic rats treated with glibenclamide. The results are shown in table (2).

Table. 2 Effect of long-term treatment with the CTAF on glycogen levels in Liver and Muscle & Protein levels in Plasma, Liver and Kidney of Normal and Diabetic Rats

Groups	Glycogen mg glucose equivalents/g wet tissue		Protein		
	Liver	Muscle	Plasma (mg/dL)	Liver (mg/g.wet tissue)	Kidney (mg/g.wet tissue)
NC	13.6±2.26[a]	7.9±0.71[a]	6.45±3.05[a]	146.8±4.07[a]	123.5±5.61[a]
NT	12.4±1.28[a]	7.5±0.75[a]	6.71±0.15[a]	148.5±5.89[a]	115±8.56[a]
DC	8.2±1.05[d]	3.26±0.41[d]	3.12±0.208[c]	96±2[d]	71±3.60[d]
DT	12.10±1.38[b]	6.8±0.82[b]	5.60±1.17[b]	130. 5±4.17[b]	107. 8±4.49[b]
DG	10.4±1.26[c]	5.2±0.84[c]	5.0±0.200[b]	109±6.32[c]	98±4.18[c]
F-Value	18.45	95.44	735.4	80.42	2456.58
Significance	0.00	0.00	0.00	0.00	0.00

Values are given as Mean ± S.D. from six rats in each group.
Values not sharing a common superscript letter differ significantly at $p<0.05$

3.4 Histology Studies

We performed histology experiments in the pancreas, liver, and renal tissues to investigate the effects of the CTAF on morphological changes in these tissues. Figure 5 depicts the histological alterations in the pancreas, liver, and kidney. Insulitis with lymphocytic infiltrations, atrophy, and death of β-cells were seen in the pancreas of diabetic control rats (top panel, A–D). CTAF therapy resulted in the regeneration of -β-cells in the pancreas of diabetic rats, with enhanced shape and quantity of β-cells. The liver of diabetic controls showed tissue degradation, with severe congestion of the central vein, haemorrhages in the sinusoidal regions, and granular appearance of the hepatocytes in the current investigation. When compared to normal control rats (middle panel, E–H), the degenerative alteration is associated with cloudy swelling (hazy nucleus). The normal architecture was observed in CTAF-treated diabetic rat livers, with mild congestions in the central vein, normal sinusoidal gaps, and normal hepatocytes.

The kidneys of diabetic control rats (bottom panel, I–L) showed glomerular shrinkage, necrotic tubular epithelial cells, dark pyknotic nuclei, and bleeding in the bowman's gap. Normal glomeruli, normal intertubular vessels, and tubular epithelial cells were found in the kidneys of CTAF-treated diabetic rats, showing regenerative protective alterations.

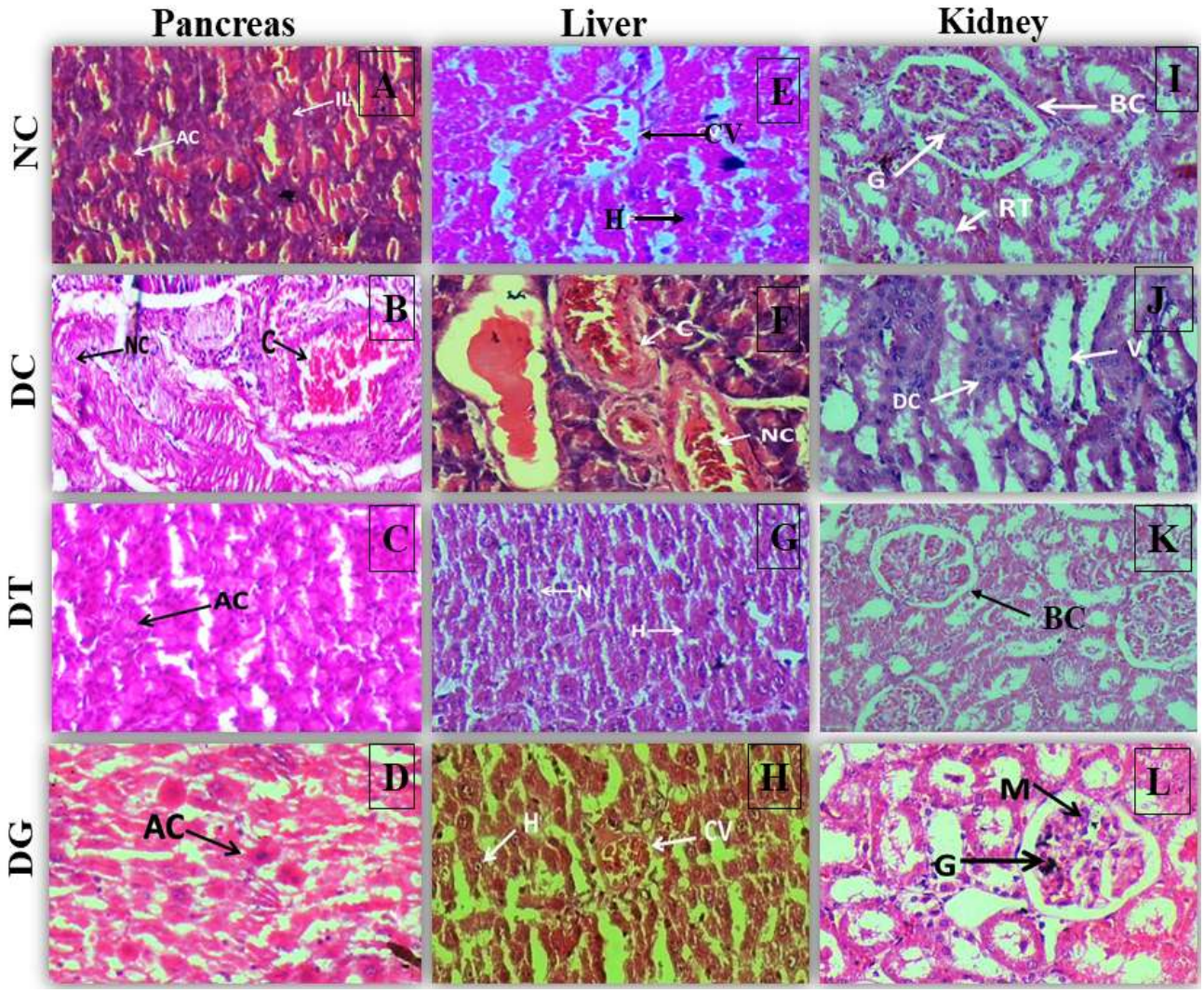

Figure: 4. Effect of long-term treatment with CTAF on histology of pancreas, liver, and kidney in normal and experimental diabetic rats. A-D) Histology of pancreas – A) Normal, B) Diabetic, C) Diabetic + CTAF, D) Diabetic + Glibenclamide. E-H) Histology of liver E) Normal, F) Diabetic, G) Diabetic + CTAF, H) Diabetic + Glibenclamide. I-L) Histology of kidney I) Normal, J) Diabetic, K) Diabetic + CTAF, L) Diabetic + Glibenclamide. NC: Normal control, DC: Diabetic control, DT: Diabetic rats treated with 75 mg/kg. b.w CTAF, DG: Diabetic rats treated with 20 mg/kg. b.w Glibenclamide. Il-Islets of Langerhans, AC-Acinar Cells, NC-Necrotic change, C-Congestion, CV-Central Vein, SS-Sinusoids Space, H-Hepatocyte, V-Vacuolization, BC-Bowman's capsule, RT- Renal tubule, G-Glomeruli, DBC- Distractive Bowman's Capsule, DC- Degenerative changes.

4. DISCUSSION

The antidiabetic efficacy and beneficial effect of the active fraction of root tubers of *Cyanotis tuberosa* in experimental-induced diabetic rats were evaluated in our previous studies [9]. This study examined the effect of CTAF on carbohydrate metabolizing enzymes, histopathological studies of liver, pancreas, and kidney and identification of active Phyto-compounds responsible for the efficient antidiabetic activity of *Cyanotis tuberosa* by GC-MS analysis.

Diabetes in humans and rodents is caused by partial or total insulin insufficiency, which changes glucose homeostasis and ultimately reduces hexokinase and glucokinase enzyme activity, which in turn downregulates GLUT-2 expression [18]. Insulin also plays a major role in the synthesis of hepatic glycogen by mobilising glucose and producing the substrate for glycogenesis and glycolysis. Additionally, by inhibiting two crucial gluconeogenic enzymes, G6Pase and FBPase, it lowers hepatic glucose production. These results imply that

by returning glucose levels to normal, hexokinase/glucokinase activity restoration enhances diabetes treatment [19]. Oral administration of the active fraction of *C. tuberosa* increased the hexokinase activity in hepatic and renal tissues and upregulated the hepatic glucokinase expression by encouraging the pancreatic β-cells or regenerated β-cells to secrete more insulin, which in turn promoted glycolysis and glycogenesis and inhibited gluconeogenesis. These findings support the preliminary findings that compounds derived from medicinal plants have a beneficial impact on the activity of enzymes involved in the metabolism of glucose (20).

Hexose monophosphate shunt's rate-limiting phase is catalyzed by the enzyme glucose-6-phosphate dehydrogenase. In comparison to normal rats, the hepatic and renal tissues of STZ-induced diabetic rats showed a marked decrease in G6PD activity. It has been found that insulin increases glucose 6-phosphate dehydrogenase activity in a dose-dependent way [21]. In our study, the administration of CTAF significantly raised the activity of glucose 6-phosphate dehydrogenase, most likely due to an increase in insulin secretion. These findings are consistent with earlier research that found that diabetic rats treated with medicinal plants or substances derived from them had higher body weights, protein, and glycogen contents in their hepatic and renal organs. [22,23].

GC-MS analysis of hexane extract of root tubers of CT has shown the presence of various bioactive compounds: fatty acids, hydrocarbons, and saturated and unsaturated fatty acids. Many of which have been reported to possess various medicinal effects including anti-diabetic activity (24). In this study we reported for the first time, the presence of 9-hexadecanoic acid (palmitoleic acid), 10-heneicosane, Eicosanoic acid, (+)-à-Tocopherol, Oleamide in the hexane extract of *Cyanotis tuberosa*. Palmitoleic acid has a beneficial effect on the reduction of hyperglycemia, insulin resistance, and hepatic lipid accumulation [25]. Oleamide also known as oleic acid is present in CTAF improves insulin production, insulin-stimulated glucose transport, and reduces insulin resistance [26, 27]. Taken together the result of GC-MS analysis the antidiabetic activity of the *C. tuberosa* might be due the presence of the above compounds.

The decreased insulin levels in the STZ-induced diabetic rats that we recently described may be mostly due to necrosis of the islets of Langerhans, as revealed by the histology of pancreatic tissue.[28]. In the current work, CTAF treatment in DT rats corrected the aberrant architecture of pancreatic tissue in diabetic rats. This might be because the phytochemicals in CTAF have the ability to regenerate the pancreas. Because of decreased glycogen levels and fat droplet formation, chronic hyperglycemia and hyperlipidaemia caused degenerative alterations in the liver's histology, including aberrant hepatocytic nuclei infiltration and localisation [29]. The hepatic tissue of the diabetic rats treated with CTAF exhibited regenerative alterations in our study, which may be connected to elevated antioxidant mechanisms and lowered tissue lipid profiles. Renal lesions, cell debris scattered in tubular lumina, thicker tubular epithelial cells, massive cellular infiltration, interstitial tissue haemorrhage, and degeneration with thickened Bowman's capsule were among the significant progressive degenerative changes observed in the kidneys of STZ-induced diabetic rats. However, treatment with CTAF alleviated these changes in the kidney of diabetic rats confirming its renoprotective activity during hyperlipidemia [9] and hyperglycemia. Previous studies with different plant extracts [30] had shown similar results as *Cyanotis tuberosa*.

Based on our earlier and present findings, we conclude that the active fraction of C. tuberosa (CTAF) significantly affects glycaemic control and the glucose-lowering effect.

5. CONCLUSION

In our studies, rats used in experiments had their pathophysiological abnormalities brought on by streptozotocin-induced diabetes mellitus, lessened after oral administration of the active fraction of *C. tuberosa* (CTAF). The results of our studies conclude that the CTAF has beneficial effects on pancreas, liver and kidney tissues and activities of carbohydrate metabolizing enzymes.

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