

Evaluation of Acute toxicity, *In-vitro*, *In-vivo* Antidiabetic Potential of the Flavonoid Fraction of the plant *Chenopodium album* L.

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ABSTRACT

Background: The *Chenopodium album* L. commonly recognized as *Bathua*, is widely distributed globally and contains various phytoconstituents that help treat several diseases. However, until now, aerial parts' antidiabetic potential and the plant's acute toxicity at fraction level have never been established. **Objectives:** To investigate the acute toxicity, the *in-vitro*, *in-vivo* antidiabetic potential of the plant at fraction level. **Materials and Methods:** The aerial parts of the plant were fractionated into different fractions, i.e., flavonoid fraction (CAFF), tannin fraction (CATF), alkaloid fraction (CAAF), saponin fraction (CASF), and were analyzed for *in-vitro* alpha-amylase inhibition assay. The CAFF, CATF, and CAAF were selected based on *in-vitro* alpha-amylase inhibition assay results and were further screened for its acute toxicity and *in vivo* antidiabetic activity using a high-fat diet and streptozotocin-induced diabetes model. The CAFF was characterized by LC-MS, and a molecular docking study was carried out. **Results:** The *in-vitro* alpha-amylase inhibition assay revealed that CAFF was found to be more potent than standard Acarbose having IC₅₀ values 122.18 ± 1.15 and 812.83 ± 1.07 µg/ml, respectively. The CAFF fraction was found to possess potent antidiabetic activity in a dose-dependent manner in both *in vitro* and *in vivo* diabetic models and did not produce any sign of severe toxicity. Furthermore, the bioactive CAFF fraction was characterized by LC-MS, showed the presence of quercetin 3-O-(2",6"-di-O-rhamnosyl) glucoside (QRG) or quercetin 3-O-(2",6"-di-O-rhamnosyl) galactoside (QRGa) and quercetin 3-O-rutinoside (rutin) (QR). It is predicted from the molecular docking study that the CAFF fraction primarily acts as an alpha-amylase inhibitor. **Conclusion:** The CAFF fraction was found to poses dose-dependent potent antidiabetic activity and did not produce any sign of severe toxicity and primarily act as an alpha-amylase inhibitor.

Key words: *Chenopodium album*, Alpha-amylase, acute toxicity, antidiabetic activity, LC-MS, Molecular docking.

INTRODUCTION

Diabetes is one of the foremost causes of death across the globe¹. In 2017, around 425 million adults in the age group of 20–79 years were reported to be affected by diabetes, and it is estimated to rise to 629 million by 2045². Type 2 diabetes is a metabolic disorder characterized by hyperglycemia³⁻⁵ and also associated with the risk of cardiovascular disease and obesity^{6,7}. Clinical evidence showed that improving lifestyle by maintaining healthy body weight and modest physical exercise can avoid Type 2 diabetes mellitus. Moreover, in a short time, lifestyle modification fails to exert its effects on diabetes, and it is tough to maintain the modified lifestyle^{8,9}. Nowadays, combined therapy of several oral hypoglycemic agents exhibits an efficient treatment for glycemic control in the management of diabetes. However, the available combinational therapies possess numerous side effects¹⁰. The inclusion of phytoconstituents in combination therapy provides more efficient treatment with significantly decreased side effects in the management of diabetes¹¹. Phytochemicals are

considered less toxic compared with their synthetic counterparts¹². Therefore, it is the need of the hour to explore phytochemicals for drug intervention that can help prevent and treat Type 2 diabetes mellitus with fewer side effects.

The *Chenopodium album* L. commonly known as *Bathua* in Hindi, is classified into the Chenopodiaceae family, widely grown globally, and contains various bioactive constituents that possess various therapeutic activities like anticancer hepatoprotective, antioxidant, antinociceptive activity, etc.^{13,14}. The methanolic roots extract was earlier reported for the treatment of diabetes when evaluated using streptozotocin (STZ)-induced diabetes in Wistar rats¹⁵, and the aqueous extract of the aerial part of the *C. album* was showed considerable α-amylase inhibition assay of 98.72% at 3mg/ml of concentration¹⁶. However, the aerial parts' Bioactivity-guided fractionation was not evaluated for antidiabetic potential and acute toxicity study. This study pertains to investigate the potential fraction against the high-fat diet and STZ induced diabetic rat model and *in-silico* prediction of the possible mechanism of the bioactive fraction.

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MATERIALS AND METHODS

Chemicals

STZ was purchased from (CDH, New Delhi, India). Ingredients of feed such as cholesterol and casein from (CDH, New Delhi), formalin, sodium carboxymethylcellulose and L-cysteine (Loba Chemicals, Mumbai), yeast powder (Molychem, Mumbai), mineral and vitamin mix (Sarabhai chemicals, Baroda, India), Ghee (Patanjali, Haridwar), Acarbose (Sisco Research Laboratories Pvt. Ltd.).

Collection and authentication of plant material

Air-dried aerial parts of *Chenopodium album* L. was procured from the native areas of Ludhiana (Punjab), India, and was authenticated by Dr. Sunita Garg, Emeritus Scientist, Department of Raw Material Herbarium & Museum, National Institute of Sciences Communication and Information Resources, New Delhi with the voucher specimen number NISCAIR/RHMD/Consult/2018/3227/28-1.

Processing of the plant material

The aerial part (3000 g) of *C. album* was collected on maturity, adequately cleaned, and was further air-dried at room temperature to prevent microbial growth, and after drying, the aerial parts were powdered using a mechanical grinder and preserved till further use in an airtight container.

Fractionation of total tannins and flavonoids

The powdered aerial parts were defatted using petroleum ether (40–60 °C), followed by successive extraction with chloroform and ethyl acetate using microwave-assisted extraction. The dried ethyl acetate extract, which was then dissolved in the aqueous phase and 10% NaCl solution, was added to the aqueous solution, centrifuge the solution to precipitate tannins (CATF), and further, the supernatant liquid was partitioned with ethyl acetate. The ethyl acetate layer was evaporated to dryness under reduced pressure to achieve the total flavonoids (CAFF)¹⁷.

Fractionation of total alkaloids

The aerial part of the plant was mixed with NH₄OH (25%) to form a slurry, further extracted with ethyl acetate using microwave-assisted extraction. The dried ethyl acetate extract, dissolved in water acidified with sulfuric acid to maintain pH 3-4, was further extracted with petroleum ether (40–60 °C) followed by diethyl ether eliminate the lipophilic, acidic, and neutral impurities. The solution was basified to pH 9-10 with NH₄OH. The resulting solution was further extracted with chloroform and washed with distilled water, concentrated to dryness under reduced pressure to obtain total alkaloids (CAAF)¹⁸.

Fractionation of total saponin

Defatting of the powdered aerial part was done as mentioned earlier, followed by successive extraction using chloroform and methanol as a solvent by microwave-assisted extraction. The dried methanolic extract was suspended in water, partitioning was done using diethyl ether and saturated n-butanol to isolate the total saponin (CASF)¹⁹.

In-vitro alpha-amylase inhibitory activity assay

The alpha-amylase inhibitory assay was carried out using the iodine-starch method. Acarbose was used as a standard. The assay depends on developing an iodine and starch complex, i.e., blue, and exhibits maximum absorbance at 580 nm. The positive control solution was prepared using alpha-amylase enzyme and starch in the absence of an inhibitor to achieving 100% enzymatic activity with minimum absorbance value. Whereas the negative control solution contains the starch that converts into the dark green colored complex after the

addition of iodine solution having maximum absorbance due to the absence of inhibitor and alpha-amylase and possess no enzymatic activity. However, the test solution absorbance and color intensity should lie in the middle of positive and negative control absorbance^{20,21}.

Preparation of stock sample solution

The stock solution 1000 ppm concentration was prepared for each fraction (CAFF, CATE, CAAF, and CASF) by dissolving 10 mg in 10 ml methanol.

Preparation of sample solution

The various sample concentrations were prepared by withdrawing 0.5, 1, 1.5, 2, and 2.5 ml of the stock solution into 10 ml of different volumetric flasks. Volume was prepared using methanol and labeled the sample solutions 50, 100, 150, 200, and 250 µg/ml.

% Inhibition and enzyme activity were calculated using the formula mentioned below:

$$\text{Enzyme activity} = \frac{(\text{Abs. of negative control} - \text{Abs. of positive control}) - \text{Abs. of test sample}}{(\text{Abs. of negative control} - \text{Abs. of positive control})} * 100$$

% Inhibition = 100 - enzyme activity.

Inhibition concentration (IC₅₀): The drug concentration where 50% enzyme inhibition takes place.

Experimental animals

Female albino mice were procured from the central animal facility from the Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, for acute toxicity study. Sprague-Dawley (SD) rats of either sex procured from National Institutes of Pharmaceutical Education and Research, Mohali, for *in-vivo* antidiabetic study. The animals were kept in standard polypropylene cages and maintained under controlled conditions, i.e., room temperature (22±2 °C) and relative humidity (55±5%) with 12:12 h light and dark cycle, and the experiments were conducted in the light cycle. All the rats and mice were supplied commercially accessible rat regular pellet diet (NPD) and water ad libitum before the dietary management. The animal experiments were conducted after approval from the Institutional Animal Ethics Committee of Lovely Professional University (Approval No. LPU/IAEC/2019/53), and experiments were conducted as per the guidelines of CPSCEA (Govt. of India). For the acute toxicity, the female mice were selected as per the OECD test guidelines 425, and for the antidiabetic activity, SD rats were selected for HFD and STZ induced diabetic rat model as per the reported literature^{24,25}.

Acute toxicity assay

The acute toxicity assay was carried out as per the OECD Test Guidelines 425 (Up and Down Procedure). The fractions (CAFF, CATE, and CAAF) were selected based on the *in-vitro* alpha-amylase assay results. In this study, non-pregnant female albino mice with an age group of 8-10 weeks and 28 ± 4 g weight were selected. The mice fasted for 3–4 h before dosing but had access to water *ad libitum* and a single dose of 2000 mg/kg; p.o of the fractions (CAFF, CATE, and CAAF) were administered according to the bodyweight of the single mice from each test group. The animals were closely monitored initially for 30 min, then for four h for any sign of toxicity. The food was restored after 1–2 h of dosing. After the drug-treated mouse's survival, all the remaining four mice in each group were administered with the same dose. A similar protocol was carried out for all the vehicle control group mice by administering 1% CMC in the same volume as the treated group. After the single-dose administration of all the fractions, the groups were closely monitored for any toxic effect, and behavioral parameters were also recorded for the first 30 min, 4 hrs, and 24 hrs and after that at regular intervals

14 days. The bodyweight of mice was measured at regular intervals. At the end of the protocol, the mice excised by cervical dislocation under general anesthesia and organ weight of heart, liver, and kidney were measured. Blood samples were withdrawn by cardiac puncture and sent to the pathology laboratory to estimate biochemical and hematological parameters. The isolated organs, i.e., heart, liver, and kidney, were preserved in 10% formalin solution for histopathology evaluation^{22, 23}.

Biochemical analysis

All the samples were sent to the pathology lab (National Laboratories, Phagwara, Punjab) to analyze blood glucose, triglyceride, cholesterol, HDL, LDL, VLDL, and creatinine urea, bilirubin, AST, ALT, alkaline phosphate, total protein, globulins, and albumin.

Hematological analysis

The blood samples were analyzed by pathology lab (National Laboratories, Phagwara, Punjab) in tubes containing EDTA for hematological study. CBC parameters, total RBC, hemoglobin, MCH, MCV, MCHC, WBC count, platelet count, lymphocytes, neutrophils, eosinophils, basophils, and monocytes were estimated.

Histopathological study

The isolated vital organs (heart, liver, and kidney) of mice were fixed in 10% formalin after sacrificing, after processing fixed in paraffin wax. Paraffin sections (5mm) were stained with eosin and hematoxylin. The slides were kept beneath the light microscope, and magnified tissue structure images were captured for analysis.

In-vivo antidiabetic activity

Diabetes induction and *in vivo* experimental design

To evaluate and identify the potential novel antidiabetic agents from medicinal plants for the cure of type 2 diabetes mellitus, various fractions (CAFF, CATE, and CAAF) of *C. album* were selected using Sprague-Dawley rats (Both Male and Female) for the development of high-fat diet (HFD) feeding and administering a low dose (35 mg/kg) of Streptozotocin (STZ)^{22,23}. The rats were categorized into two different dietary regimens, i.e., NPD or HFD composition²⁶ (58% fat, 25% protein, and 17% carbohydrate, as a percentage of total kcal) *ad libitum*, respectively, for the initial period of 2 weeks. The NPD and HFD rats were further divided into NPD, HFD + STZ, HFD + STZ + Test compound CAFF, CATE, and CAAF having six animals in each group. The ingredients of HFD are according to²⁷. After two weeks of dietary manipulation, all the rats from the HFD-fed group were injected with STZ low dose (35 mg/kg; i.p.). After that, body weight and biochemical estimations were conducted on the 7th day. The test compounds CAFF, CATE, and CAAF, were fed orally at two different concentrations, i.e., 250mg/kg and 500 mg/kg continuous for seven days. Therefore, the higher dose (500 mg/kg) was selected as per the previously reported literature of the plant¹⁵ and rationalized the study; the lower dose was selected, i.e., 250 mg/kg. The blood samples were collected from rat's retro-orbital plexus under light anesthesia using capillary tubes to analyze the plasma glucose level, total cholesterol level, and triglycerides level. Histopathology of the pancreas was conducted after sacrificing 50% of the animals at the end of the protocol. The non-fasting rats with a plasma glucose level of ≥ 300 mg dl⁻¹ were considered diabetic and chosen for further experimental studies. Animal feed water intake was also measured. The rats were allowed to continue with the feed as per the protocol.

Histopathological study

The rats' isolated pancreas was fixed in 10% formalin after sacrificing and processing, fixed in paraffin wax. 5mm paraffin sections stained with eosin and hematoxylin. The slides were kept beneath the light

microscope, and magnified tissue structure images were captured for analysis.

Statistical analysis

The results were expressed as Mean \pm SD, and the statistical significance among the groups was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. $P \leq 0.05$ was considered statistically significant.

Liquid chromatography-mass spectrometry (LC-MS) analysis of CAFF

The bioactive CAFF fraction obtained from the plant's aerial parts was characterized through LC-MS Spectra for the bioactive molecules responsible for the activity. The LC-MS data components present in the CAFF fraction correlated the mass fragments with reported literature data^{28,29}.

Molecular docking

The molecular modeling software, Autodock-vina^{30,31}, was used to study molecular docking. PDB or proteins 3WY2, 4GQR, and 3SZ1 for alpha-glucosidase, alpha-amylase, and PPAR-gamma proteins were selected and extracted from the protein data bank. The structures of these molecules were drawn by ChemDraw and changed to 3D. Further minimizations of energy were carried out using the MM2 Interface program on ChemBio3D Ultra 12.0, and molecules were saved in pdb format (Cambridge Soft). For the identification of the most active molecule, initially, the removal of internal ligand was done and docking was carried out in the same manner as an actual ligand with set RMSD of 1.5A°.

RESULTS

Fractionation

There were various fractions were obtained from the plant *Chenopodium album*. The yield of CAFF, CATE, CAAF, and CASF was 2.67, 2.74, 1.70, and 2.90 % (w/w).

In-vitro Alpha-amylase inhibition assay

All the fractions of the plant were evaluated by alpha-amylases assay. The CAFF showed potent alpha-amylase inhibitory activity when compared with standard Acarbose having IC₅₀ values 122.18 \pm 1.15 and 812.83 \pm 1.07 respectively, whereas the CATE, CAAF found to be less active with that of the standard in terms of % inhibition., The CASF was found to be least active in comparison to the other fractions. The results are mentioned in Table 1.

Acute toxicity study

The acute toxicity was conducted as per OECD Guidelines. CAFF, CATE, and CAAF at a concentration of 2000 mg/kg were evaluated. No mortality was observed in any of the treated and vehicle control groups. All the animals were observed at a regular interval, and observations were noted during the study phase, i.e., 14 days.

Behavioral pattern and body weight

The itching was observed in all the treated groups in the first 30 min, and after that, sleepy and drowsing effects were observed in CAFF and CATE fraction during the first 4 hrs. The itching might be due to some mild toxicity caused by the fractions. During the acute toxicity study, it was observed that there is a slight increase in body weight in the treated and vehicle control group reported in Figure 1.

Organ to body weight index

There was no significant difference observed in organ to body weight index for both treated and vehicle control groups. There were no lesions

Table 1: Represents the % inhibition and IC₅₀ of fractions from *Chenopodium album*.

S. No	Conc. (µg/ml)	Log Conc.	% Inhibition Acarbose	% Inhibition CAFF	% Inhibition CATF	% Inhibition CAAF	% Inhibition CASF
1	50	1.70	8.75 ± 0.67	27.63 ± 0.93	7.89 ± 0.74	3.29 ± 0.87	2.63 ± 0.88
2	100	2.00	17.86 ± 0.87	40.13 ± 0.79	11.84 ± 0.79	5.26 ± 0.78	3.29 ± 0.72
3	150	2.18	21.32 ± 0.53	51.97 ± 1.02	16.45 ± 0.98	7.24 ± 0.99	4.61 ± 0.68
4	200	2.30	28.12 ± 0.78	63.82 ± 0.87	21.05 ± 0.86	9.21 ± 1.07	5.92 ± 0.57
5	250	2.40	34.67 ± 0.97	75.66 ± 0.68	26.97 ± 0.91	10.53 ± 1.02	6.58 ± 0.71
	IC ₅₀ (µg/ml)		812.83 ± 1.07	122.18 ± 1.15	----	----	---

Values are expressed as Mean ± SD; CAFF- *Chenopodium album* flavonoid fraction; CATF- *Chenopodium album* tannin fraction; CAAF- *Chenopodium album* alkaloid fraction; CASF- *Chenopodium album* saponin fraction.

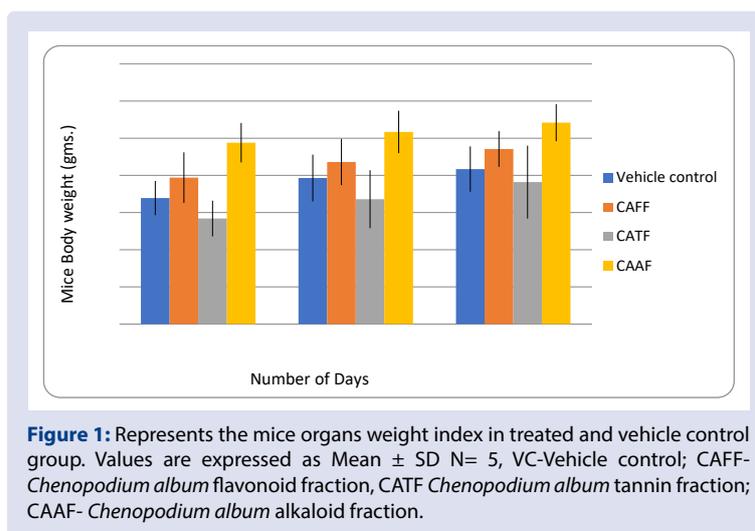


Figure 1: Represents the mice organs weight index in treated and vehicle control group. Values are expressed as Mean ± SD N= 5, VC-Vehicle control; CAFF- *Chenopodium album* flavonoid fraction, CATF *Chenopodium album* tannin fraction; CAAF- *Chenopodium album* alkaloid fraction.

observed at the organ level (heart, liver, and kidney) in any of the groups at a 2000 mg/kg concentration, p.o. The organ to body weight index was recorded and reported in Figure 2.

Biochemical analysis

In CAFF fraction, there is an increase in the total cholesterol, HDL, LDL, VLDL, triglycerides, urea, creatinine, protein, SGOT, SGPT levels when compared with vehicle control. Furthermore, in the CATF fraction, the levels of the total cholesterol, LDL, Triglycerides, urea, protein, SGOT, ALP were also increased, whereas in the CAAF fraction, there is an increase in total cholesterol, LDL, urea, SGOT, and ALP levels were recorded. The biochemical results indicate that all the fractions at 2000 mg/kg produce mild toxicity symptoms in albino mice without producing any severe toxicity at the organ level. The data are represented in Table 2.

Hematological analysis

Both fractions CAFF and CATF causes an increase in the levels of HGB, RBC, HCT, MCV, MCH, MCHC, PLT, PDW, MPV, and PCT, whereas the CAAF fraction significantly increases the levels of RBC, HCT, MCV, MCH, MCHC, WBC, PLT, PDW, MPV, and P-LCR. The research finding of a hematologic evaluation represents that all the fractions produce only mild toxicity symptoms in albino mice. The hematology data are represented in Table 3.

Histopathology analysis

All the fractions CAFF, CATF, and CAAF of the plant did not show any severe sign of toxicity at the organ level, suggesting that all the fractions are safe at a concentration of 2000 mg/kg, p.o. The histopathological research findings are summarized for the heart (Figure 3), Kidney (Figure 4), and liver (Figure 5). The results indicate that all the plant

fractions produce only mild toxicity at the heart, liver, and kidney, but the CAAF fraction produces mild to moderate liver toxicity. These results are also supported by the organ to body weight index, biochemical and hematology findings.

In-vivo Antidiabetic activity

Biochemical analysis

The plasma glucose, total cholesterol, and total triglycerides were compared on the 22nd and 29th day reported in Table 4. After one week of STZ injection, i.e., on the 22nd day, there is a highly significant increase in glucose, cholesterol, and triglyceride level in all the HFD+STZ treated groups that confirm the type 2 diabetic condition. After one week of treatment, i.e., 29th day, there is a highly significant decrease in the glucose, cholesterol, and triglyceride level in a CAFF fraction at a dose of 500 mg/kg, p.o. when compared with the experimental group and found to be more potent than the standard Acarbose. Furthermore, the CATF fraction also significantly reduces all the biochemical levels when compared with the experimental group. There is no significant difference observed with the CAAF fraction treated group than the experimental group (Figure 6).

Histopathology analysis

After a single dose of STZ administration, there is the development of type-2 diabetes and leads to necrosis of β -cell, which can be observed in the experimental group. After a week of administration of the CAFF fraction, the organs were isolated on the 29th day. The findings represented that the islet of pancreatic cells retains their standard structure, and mild necrosis was observed that supports the CAFF fraction's potential antidiabetic effect, whereas, in the CATF fraction, there is less recovery of islet cell. However, no recovery was observed in the CAAF treated group than the experimental control group (Figure 7).

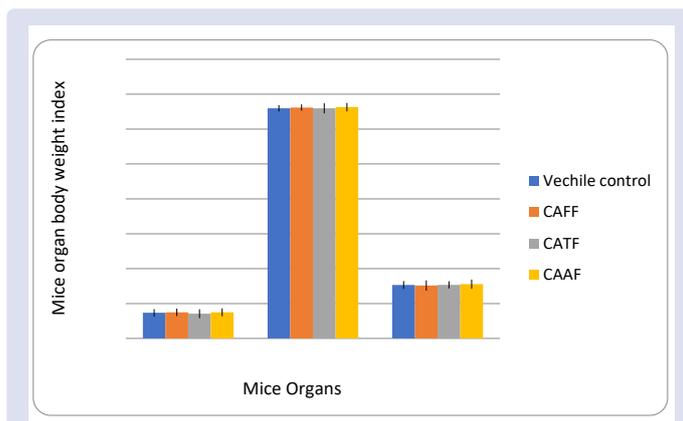


Figure 2: Effects of the *Chenopodium album* fractions on the body weight of mice. Values are presented as Mean \pm SD, N= 5; Organ-to-body weight index= (organ weight \times 100)/body weight VC-Vehicle control; CAFF-*Chenopodium album* flavonoid fraction, CATF *Chenopodium album* tannin fraction; CAAF- *Chenopodium album* alkaloid fraction.

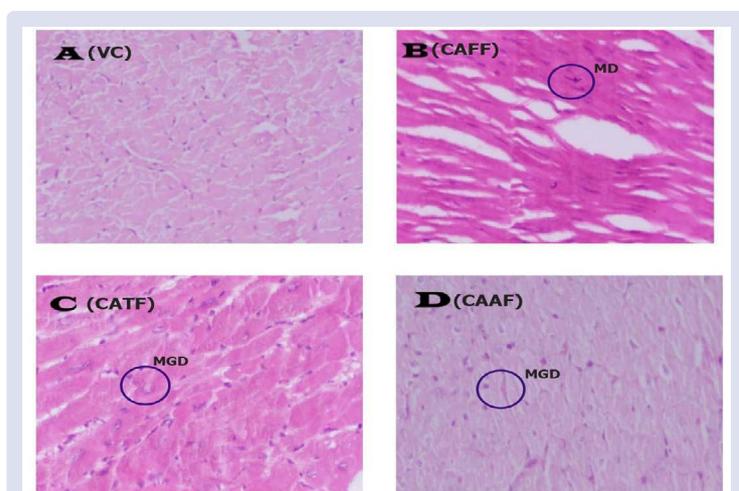


Figure 3: Represents the Histopathological observations of Heart, A (VC): Nothing abnormal detected; B(CAFF): Mild degenerative changes in the myocardial fibers; C (CATF): Mild granular degeneration in the myocardial fibers; D (CAAF): Mild granular degeneration in the myocardial fibers.

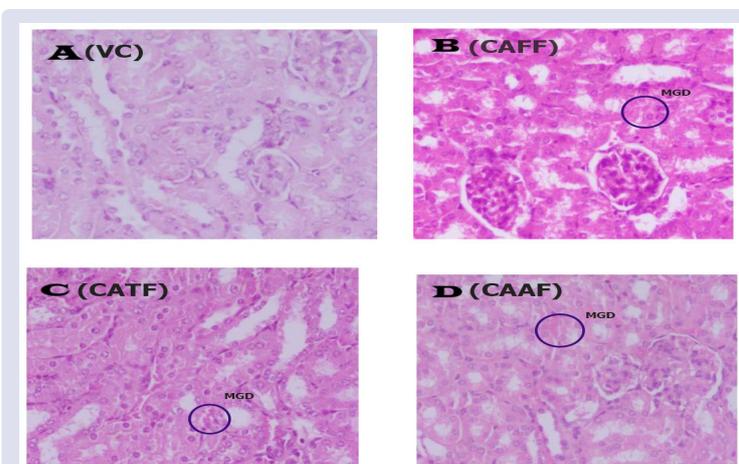


Figure 4: Represents the Histopathological observations of Kidney, A (VC): Nothing abnormal detected; B(CAFF): Mild granular degeneration and necrosis of the tubular epithelial cells; C (CATF): Mild granular degeneration and necrosis of the tubular epithelial cells; D (CAAF): Mild granular degeneration and necrosis of the tubular epithelial cells.

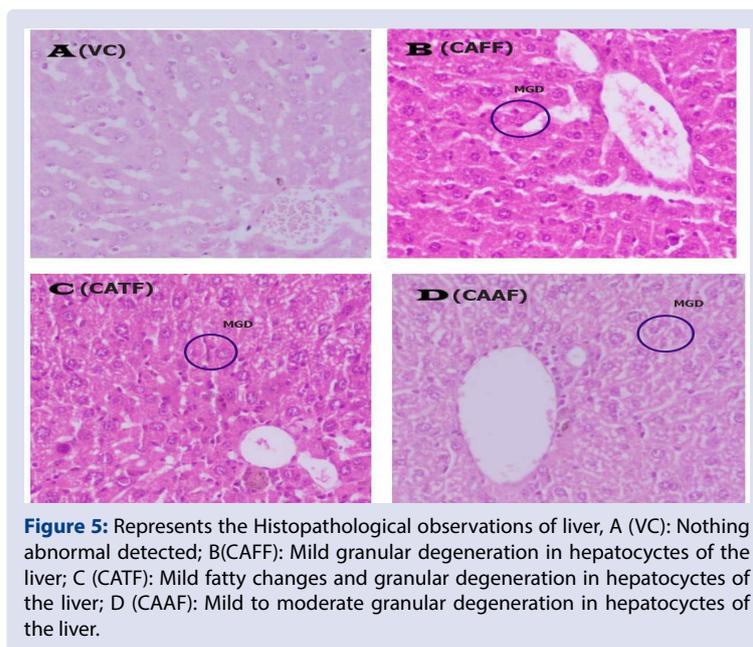


Table 2: Represents the Biochemical analysis of various fractions of *Chenopodium album*.

S. No	PARAMETERS	Units	VC	CAFF	CATF	CAAF
1	GLUCOSE	mg/dl	94.67 ± 1.96	91.37 ± 3.31	92.44 ± 2.88	95.54 ± 3.73
LIPID PROFILE						
2	TOTALCHOLESTEROL	mg/dl	98.67 ± 3.06	187.67 ± 2.52 #	108.00 ± 0.61 #	117.00 ± 2.00 #
3	HDL CHOLESTEROL	mg/dl	30.33 ± 1.53	44.80 ± 0.26 #	31.70 ± 0.61	32.23 ± 1.59
4	LDL CHOLESTEROL	mg/dl	45.93 ± 1.51	117.13 ± 1.42 #	55.35 ± 1.71#	62.43 ± 2.33 #
5	VLDL CHOLESTEROL	mg/dl	22.40 ± 0.60	25.73 ± 1.10 #	21.25 ± 1.26	22.33 ± 0.70
6	TRIGLYCERIDES	mg/dl	112± 3.00	128.67 ± 5.51 #	106.27 ± 6.29 #	111.67 ± 3.51
7	CHOL/HDL RATIO		3.26 ± 0.11	4.19 ± 0.03 #	3.42 ± 0.06	3.64 ± 0.21 #
8	LDL/HDL RATIO		1.52 ± 0.08	2.61 ± 0.02 #	1.75 ± 0.06 #	1.94 ± 0.15 #
KIDNEY FUNCTION TESTS						
9	UREA	mg/dl	37.33 ± 2.08	54.00 ± 2.00 #	56.00 ± 2.65 #	60.00 ± 2.00 #
10	CREATININE	mg/dl	0.53 ± 0.02	0.60 ± 0.02 #	0.57 ± 0.03	0.57 ± 0.02
LIVER FUNCTION TESTS						
11	BIT	mg/dl	0.67 ± 0.06	0.90 ± 0.05 #	0.61 ± 0.07	0.78 ± 0.09
12	BID	mg/dl	0.25 ± 0.05	0.30 ± 0.05	0.26 ± 0.06	0.35 ± 0.05 #
13	BII	mg/dl	0.42 ± 0.02	0.60 ± 0.10 #	0.34 ± 0.07	0.44 ± 0.04
14	PROTEIN	mg/dl	6.27 ± 0.21	7.03 ± 0.11 #	6.46 ± 0.06 #	6.23 ± 0.12
15	ALBUMIN	mg/dl	2.27 ± 0.06	2.95 ± 0.05 #	2.57 ± 0.06 #	2.62 ± 0.06 #
16	GLOBULIN	mg/dl	4.00 ± 0.17	4.08 ± 0.13	3.90 ± 0.04	3.61 ± 0.10 #
17	A:G/RATIO		0.57 ± 0.02	0.72 ± 0.03 #	0.66 ± 0.02 #	0.72 ± 0.02 #
18	SGOT/AST	IU/L	95.93 ± 1.79	151.00 ± 8.19 #	137.00 ± 2.65 #	125.10 ± 4.01#
19	SGPT/ALT	IU/L	65.97± 5.31	68.45 ± 4.30	65.37 ± 4.30	64.96 ± 4.38
20	ALP	IU/L	94.97 ± 6.05	128.33 ± 6.03 #	124.67 ± 3.51 #	129.67 ± 4.16 #

Values are presented as Mean ± SD, N= 5, Statistical analysis was performed using one way ANOVA followed by Turkey's multiple comparison test, # represents P<0.05 vs. Vehicle Control; VC-Vehicle control; CAFF- *Chenopodium album* flavonoid fraction, CATF *Chenopodium album* tannin fraction; CAAF- *Chenopodium album* alkaloid fraction.

Characterization by LC-MS

The *in-vitro* and *in-vivo* results showed that the CAFF possesses maximum activity among all the plant fractions. The CAFF fraction was analyzed by LC-MS Spectra and predicted two possible structures at retention time 7.13 min are Quercetin 3-O-(2",6"-di-O-rhamnosyl) glucoside (QRG) or Quercetin 3-O-(2",6"-di-Orhamnosyl) galactoside (QRGa) and 7.62 min. for Quercetin 3-O-rutinoside (rutin) (QR), as the major constituents that can be responsible for its activity Table 5.

Molecular docking

Based on LC-MS's prediction, major constituents were studied *in-silico* for their antidiabetic activity choosing α-glucosidase, α-amylase, and PPAR gamma receptors. Among the phytochemicals, QRGa is supposed to be most active with -6.5 kcal/mol, -9.3 kcal/mol, and -7.0 kcal/mol α-glucosidase, α-amylase, and PPAR gamma, respectively in comparison to standard Acarbose as mentioned in Table 6. A detailed interaction for QRGa was studied for α-glucosidase, α-amylase, and

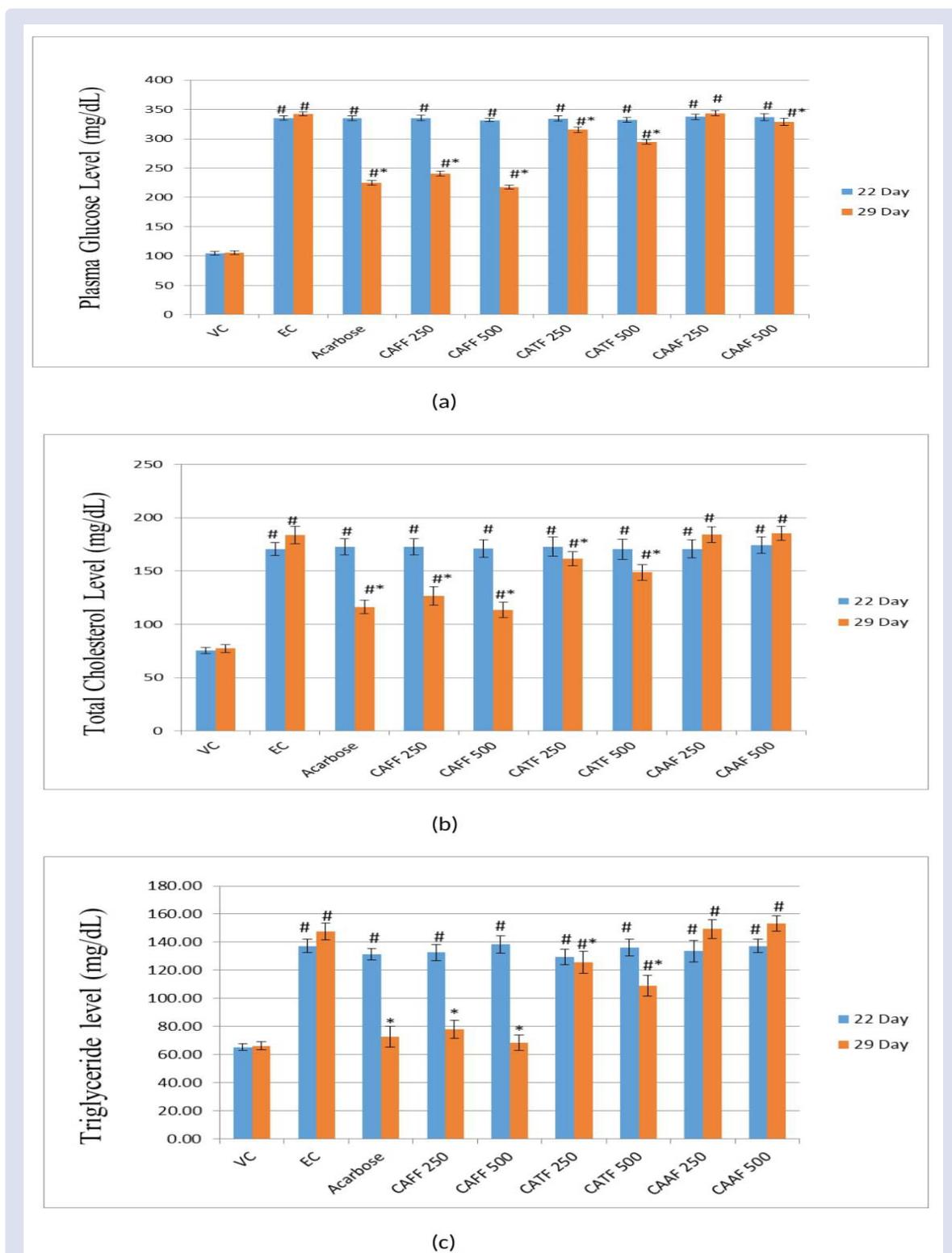


Figure 6: Effect of different groups on plasma glucose level (a), total cholesterol (b) and triglycerides (c). Values are presented as Mean± SD, n=6. Statistical analysis was performed using one way ANOVA followed by Turkey's multiple comparison test, # represents p< 0.05 vs. Vehicle Control, * represents p< 0.05 vs. Experimental Control; NPD: Normal pallet diet; CMC: Carboxymethyl cellulose; HFD: High fat Diet; CAFF- *Chenopodium album* flavonoid fraction; CATF *Chenopodium album* tannin fraction; CAAF- *Chenopodium album* alkaloid fraction.

Table 3: Represents the Hematological analysis of various fractions of *Chenopodium album*.

S. No	Parameters	Unit	VC	CAFF	CATF	CAAF
1	HGB	g/dl	13.35 ± 0.05	15.48 ± 0.08 #	15.32 ± 0.08 #	13.40 ± 0.05
2	RBCs	10 ⁶ /ul	8.48 ± 0.08	9.68 ± 0.04 #	10.05 ± 0.04 #	8.70 ± 0.03 #
3	HCT	%	44.68 ± 0.16	53.30 ± 0.11 #	52.69 ± 0.10 #	47.42 ± 0.08 #
4	MCV	fL	52.67 ± 0.52	55.08 ± 0.09 #	52.41 ± 0.09	54.53 ± 0.08 #
5	MCH	Pg	15.74 ± 0.19	16.00 ± 0.02 #	15.24 ± 0.02 #	15.41 ± 0.01 #
6	MCHC	g/dl	29.88 ± 0.07	29.05 ± 0.09 #	29.07 ± 0.09 #	28.26 ± 0.06 #
7	RDW-SD	fL	19.69 ± 0.11	21.80 ± 0.05 #	22.76 ± 0.06 #	22.27 ± 0.07 #
8	RDW-CV	%	19.06 ± 0.05	21.87 ± 0.08 #	20.66 ± 0.06 #	20.80 ± 0.09 #
9	WBCs	10 ³ /ul	4.94 ± 0.03	4.95 ± 0.04	4.89 ± 0.03	4.81 ± 0.04 #
10	NEUT%	%	20.45 ± 0.41	20.61 ± 0.92	21.45 ± 0.79	21.00 ± 0.26
11	LYMPH%	%	76.37 ± 0.33	75.52 ± 0.91	76.40 ± 0.77	74.96 ± 0.34 #
12	MONO%	%	1.00 ± 0.12	1.37 ± 0.05 #	1.31 ± 0.09 #	1.32 ± 0.07 #
13	EO%	%	2.18 ± 0.12	2.50 ± 0.05 #	2.64 ± 0.03 #	2.72 ± 0.04 #
14	BASO%	%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
15	IG%	%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16	NEUT#	10 ³ /ul	1.01 ± 0.02	1.02 ± 0.04	1.05 ± 0.04	1.01 ± 0.01
17	LYMPH#	10 ³ /ul	3.78 ± 0.03	3.74 ± 0.07	3.65 ± 0.04 #	3.61 ± 0.03 #
18	MONO#	10 ³ /ul	0.05 ± 0.01	0.07 ± 0.00 #	0.06 ± 0.00	0.06 ± 0.00
19	EO#	10 ³ /ul	0.11 ± 0.01	0.12 ± 0.00	0.13 ± 0.00	0.13 ± 0.00
20	BASO#	10 ³ /ul	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
21	IG#	10 ³ /ul	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22	PLT	10 ³ /ul	692.33 ± 2.52	821.67 ± 6.51 #	779.00 ± 4.58 #	705.00 ± 3.00 #
23	PDW	fL	6.75 ± 0.05	7.34 ± 0.04 #	6.92 ± 0.03 #	6.83 ± 0.04 #
24	MPV	fL	6.25 ± 0.05	6.50 ± 0.03 #	6.56 ± 0.06 #	6.45 ± 0.05 #
25	P-LCR	%	3.04 ± 0.01	3.82 ± 0.02 #	3.41 ± 0.04 #	3.15 ± 0.05 #
26	PCT	%	0.51 ± 0.02	0.75 ± 0.03 #	0.56 ± 0.01 #	0.49 ± 0.02

Values are presented as Mean ± SD, N= 5, Statistical analysis was performed using one way ANOVA followed by Turkey's multiple comparison test, # represents P<0.050 vs. Vehicle Control; VC-Vehicle control; CAFF- *Chenopodium album* flavonoid fraction; CATF *Chenopodium album* tannin fraction; CAAF- *Chenopodium album* alkaloid fraction.

Table 4: Represents the plasma glucose level, total cholesterol and triglyceride level in SD Rats.

Group Number	Plasma glucose level (mg/dL)		Total Cholesterol level (mg/dL)		Triglyceride level (mg/dL)	
	22 Day	29 Day	22 Day	29 Day	22 Day	29 Day
I	104.33 ± 3.06	105.33 ± 3.06	75.33 ± 3.06	77.33 ± 3.79	65.33 ± 2.52	66.33 ± 3.06
II	335.33 ± 3.51 #	342.33 ± 2.52 #	170.33 ± 6.03 #	183.67 ± 8.08 #	137.33 ± 4.73 #	147.67 ± 6.11 #
III	334.67 ± 4.16 #	224.33 ± 3.51 * #	172.67 ± 7.51 #	116.33 ± 6.11 * #	131.33 ± 4.16 #	72.67 ± 7.37 *
IV	335.33 ± 4.51 #	240.33 ± 4.51 * #	172.67 ± 7.64 #	126.67 ± 8.50 * #	130.67 ± 3.79 #	78.00 ± 6.56 *
V	331.67 ± 3.06 #	217.33 ± 3.51 * #	171.00 ± 8.19 #	113.33 ± 7.02 * #	134.33 ± 5.69 #	68.33 ± 5.51 *
VI	334.33 ± 4.73 #	315.33 ± 3.06 * #	172.67 ± 9.02 #	161.33 ± 6.51 * #	129.67 ± 5.51 #	125.67 ± 8.02 *#
VII	332.33 ± 4.16 #	294.33 ± 5.69 * #	170.33 ± 9.61 #	148.67 ± 7.37 *#	136.33 ± 6.03 #	109.00 ± 7.55 *#
VIII	337.33 ± 4.73 #	343.33 ± 4.51#	170.67 ± 8.50 #	184.00 ± 7.55 #	133.67 ± 7.51 #	149.33 ± 6.51 #
IX	336.67 ± 5.86 #	328.33 ± 3.51 *#	174.33 ± 7.64 #	185.33 ± 6.66 #	137.33 ± 4.73 #	153.33 ± 5.51 #

Values are presented as Mean± SD, n=6. Statistical analysis was performed using one way ANOVA followed by Turkey's multiple comparison test, # represents p< 0.05 vs. Vehicle Control, * represents p< 0.05 vs. Experimental Control.

PPAR gamma, respectively. QRGa showed four hydrogen bonding to ASN301 at the binding site of alpha-glucosidase (Figure 8a), while electrostatic interactions with alpha-amylase (Figure 8b), QRGa showed one hydrogen bond with PPAR-gamma protein (Figure 8c). The RMSD set for molecular docking was 1.5A°.

DISCUSSION

Type 2 Diabetes mellitus is the most common type of diabetes that arises from insulin production defects or reduced peripheral tissue response towards insulin^{32,33}. The complications associated with Type 2 diabetes

are nephropathy, retinopathy, neuropathy, and cardiovascular disease³⁴. The phytochemical treatment in type 2 diabetes has fewer adverse effects and might be considered a better replacement of the existing oral therapy^{35,36}. The inclusion of phytoconstituents in combination therapy provides more efficient treatment with significantly decreased side effects in diabetes management^{37,38}. The plant's available literature represents that the methanol extract was influential in the treatment of diabetes; however, the study was conducted at pilot scale only, and no reports were available for the responsible specialized metabolites or compounds responsible for its therapeutic activity. Additionally,

Table 5: Identification of major components in the CAFF fraction of *Chenopodium album*.

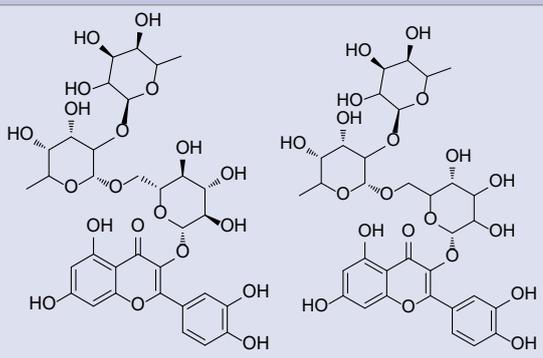
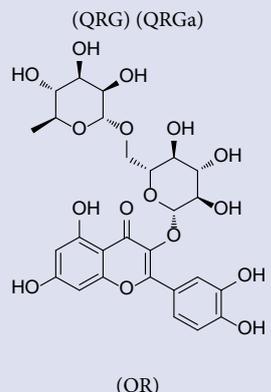
Retention time (min.)	% AUC	Mass (m/z)	Mass Fragments (m/z)	Identified component
7.13	75.86	756.21	779, 757, 611, 465, 303 (two possible structures, i.e. QRG or QRGa)	 <p>(QRG) (QRGa)</p>
7.62	23.14	610.15	633, 611, 465, 449, 303	 <p>(QR)</p>

Table 6: Molecular docking results of the bioactive CAFF Fraction.

S. No.	Molecule	Binding affinity (kcal/mol), RMSD Value 1.5Å ^o		
		Alpha -glucosidase (3WY2)	Alpha-amylase (4GQR)	PPAR gamma (3SZ1)
1	QRG	-3.9	-9.3	-6.0
2	QRGa	-6.5	-9.3	-7.0
3	QR	-5.3	-8.9	-6.7
4	Acarbose	-4.4	-6.9	NA

toxicity studies were also not conducted to establish the safety profile of the plant¹⁵. Therefore, our research mainly focuses on exploring the safety profile, *in-vitro* and *in-vivo* antidiabetic potential of the plant at the fraction level, identifies the responsible phytoconstituents, and predicts its plausible mechanism of action.

The plant's aerial parts were fractionated into different fractions and screened for *in-vitro* alpha-amylase assay. The CAFF fraction showed potent alpha-amylase inhibitor activity compared to standard Acarbose with IC₅₀ values 122.18 ± 1.15 and 812.83 ± 1.07 µg/mL, respectively. Other fractions (CATF and CAAF) showed less potent activity. However, the CASF fraction did not produce any significant *in-vitro* alpha-amylase inhibition activity. Therefore, it is predicated from an alpha-amylase assay that the CAFF fraction plays a vital role in starch and glycogen metabolism, i.e., by decreasing the blood glucose level, thereby reducing the conversion rate of starch to monosaccharides. The fractions (CAFF, CATF, and CAAF) were analyzed for their safety profile based on the alpha-amylase activity. These fractions were screened for acute toxicity at a concentration of 2000 mg/kg, p.o. in albino mice as per OECD guideline 425. The results represent itching for the first 30 min in all the fractions, and afterward, sleepy and drowsing effects were observed in CAFF and CATF fraction. No significant difference was observed in organ body weight index; lesions were not observed in any

isolated organs. However, the initial behavioural changes were observed during the first 4 hrs., probably indicating mild toxicity symptoms.

Furthermore, the biochemical parameters represent a significant increase in the lipid profile, i.e., total cholesterol, LDL, VLDL, triglycerides levels; urea in kidney function test; protein, albumin, SGOT/AST levels in liver function test. The increased levels of biochemical parameters in all the fractions indicate the mild toxicity symptoms in CAFF and CATF fractions whereas mild to moderate toxicity symptoms was observed in CAAF fraction.. The hematology parameters also represent the significant increase in the platelets and various blood cells. A significant difference was observed in WBC only in the CAAF fraction in comparison to the control group. These findings indicate the mild toxicity of the fractions. The biochemical results were supported by histopathology results, representing that the fractions (CAFF and CATF) produce mild granular degeneration in the heart, liver, and kidney, whereas the CAAF exhibits mild to moderate granular degeneration in all the organs. Therefore the acute toxicity study indicates that all the fractions have an LD₅₀ more than 2000 mg/kg. Despite this, it is recommended to explore the mechanistic insights for the mild toxicity by performing sub-acute and chronic toxicity of the bioactive fractions with detailed mechanistic studies.

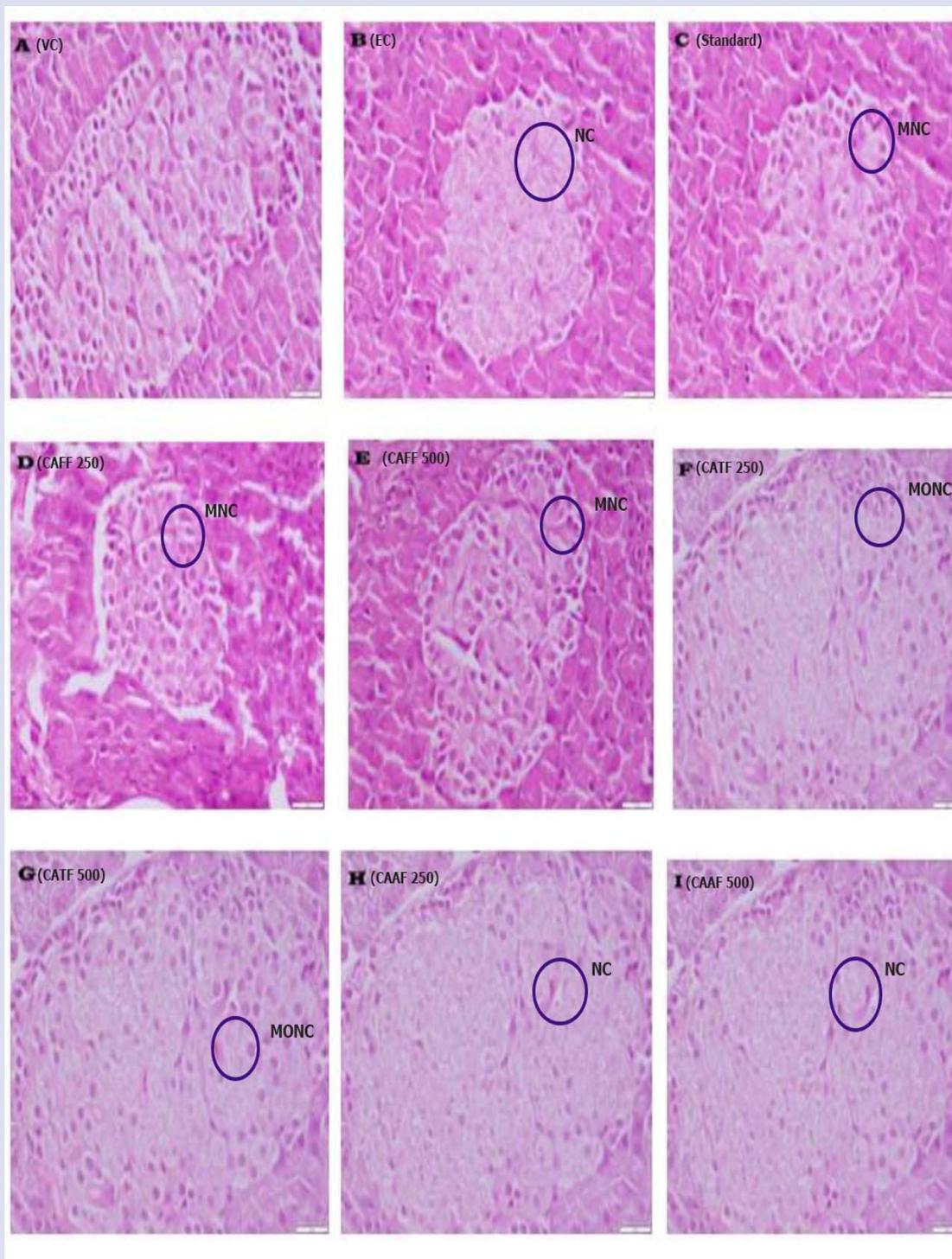


Figure 7: Represents the Histopathological observations of Pancreas, A (VC): Nothing abnormal detected; B(Experimental Control): Necrosis of Islets of Langerhans; C (Standard): Mild necrosis of Islets of Langerhans; D (CAFF 250 mg/kg): Mild necrosis of Islets of Langerhans; E (CAFF 500 mg/kg): Mild necrosis of Islets of Langerhans; F (CATF 250 mg/kg): Moderate degeneration and necrosis of Islets of Langerhans; G (CATF 500 mg/kg): Moderate degeneration and necrosis of Islets of Langerhans; H (CAAF 250 mg/kg): Necrosis of Islets of Langerhans; I (CAAF 500 mg/kg): Necrosis of Islets of Langerhans.



The fractions (CAFF, CATF, and CAAF) were evaluated for their *in-vivo* antidiabetic potential in HFD+ STZ induced Type 2 diabetes. The result represents that after one week of CAFF administration at a 500 mg/kg dose, p.o. There is a highly significant decrease in the glucose, cholesterol, and triglyceride level compared with the experimental group, which supports the potential role of CAFF as it was found to be more potent than the standard Acarbose that acts as a positive control. The histopathology findings of the CAFF fraction indicate that the islet of pancreatic cells retains their standard structure, and mild necrosis was observed that supports the potential anti-diabetic effect of the CAFF fraction, whereas, in the CATF fraction, moderate necrosis was observed. However, no recovery is observed in CAAF treated group when compared to the experimental control group. The *in-vivo* and *in-vitro* antidiabetic study represent that the CAFF fraction exhibit potential antidiabetic activity among all the fractions.

The highly active fraction CAFF fraction was further explored by LCMS, and three principal components were identified, i.e., QR, QRG, and QRGa, which are probably responsible for the therapeutic activity of the fraction, reported in Table 5. In literature, these flavonoids QR, QRG, and QRGa are reported for antidiabetic and antioxidant activity³⁹⁻⁴⁴ and mainly act by interfering in the carbohydrate absorption in the form of glucose and also by assimilation of glucose via insulin release. This represents that due to these flavonoids' presence, the CAFF fraction showed an antidiabetic effect³⁹⁻⁴⁴.

Thus, to predict the CAFF fraction mechanism, the molecular docking study was carried out against various targets. A detailed interaction for QRGa was studied for alpha-glucosidase, alpha-amylase, and PPAR gamma, respectively. The molecular docking results represent that the QRGa was most active with -6.5 kcal/mol, -9.3 kcal/mol, and -7.0 kcal/mol alpha-glucosidase alpha-amylase, and PPAR gamma, respectively, and it is predicated that CAFF fraction mainly acts as an α -amylase inhibitor.

SUMMARY AND CONCLUSION

The current study results represent that CAFF fraction was found to possess potent antidiabetic activity dose-dependently in both *in vitro* and *in vivo* diabetic models. In contrast, the CATF was less active, and the CAAF fraction did not produce any therapeutic activity. Furthermore, all the fractions did not produce any sign of severe toxicity. Only mild toxicity was observed at a dose of 2000 mg/kg, p. o. In conclusion, the CAFF fraction enhanced the overall diabetic situation by acting as an alpha-amylase inhibitor and further exploring the formulations.

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ABBREVIATIONS

ALP, Alkaline phosphates; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BASO, Basophils; BID, Bilirubin direct; BIL, Bilirubin indirect; BIT, Bilirubin total; CMC, Carboxymethyl cellulose; EO, Eosinophils; HCT, Hematocrit; HDL, High-density lipoprotein; HFD, High fat diet; HGB, Hemoglobin; IG, Immature granulocyte count; LDL, Low-density lipoprotein; LYMP, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, Monocytes; MPV, mean platelet volume; NEUT, Neutrophils; P-LCR, Platelet large cell ratio; PCT, Plateletcrit; PDW, Platelet distribution width; PLT, Platelet; RBC, Red blood cells; RDW-CV, Red blood cell distribution width-coefficient of variance; RDW-SD, Red blood cell distribution width- standard deviation; SGOT, Serum glutamic oxaloacetic transaminase; SGPT, Serum glutamic pyruvic transaminase; STZ, Streptozotocin; VLDL, Very low-density lipoprotein; WBC, White blood cells.

CONFLICTS OF INTEREST

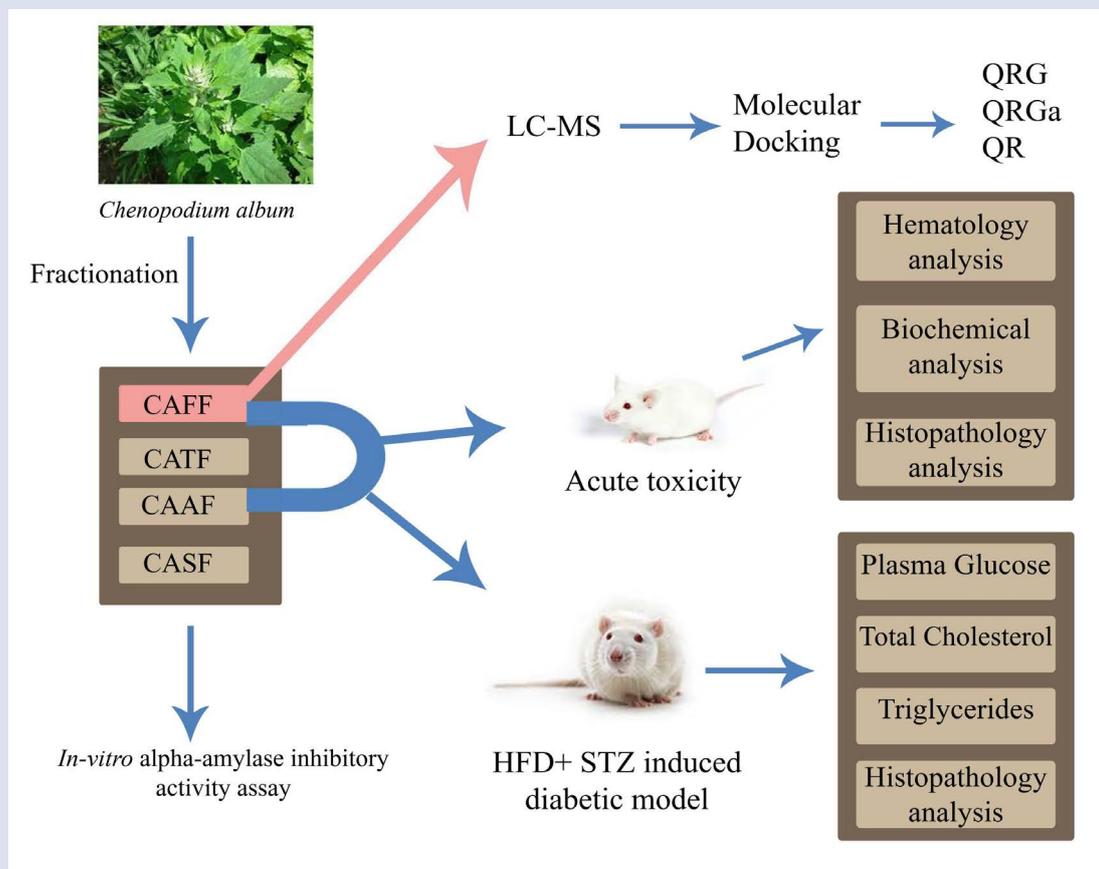
The authors declare no conflicts of interest.

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GRAPHICAL ABSTRACT



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