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Current Drug Discovery Technologies, 2024, *21*, e201023222477

RESEARCH ARTICLE

Toxicological Assessment and Anti-diabetic Effects of Combined Extract of Chirata, Fenugreek and Sesame on Regulating TNF-α, TGF-β and Oxidative Stress in Streptozotocin-Induced Diabetic Rats

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Abstract: ***Background*:** *Swertia chirayita, Trigonella foenum-gracum* and *Sesamum indicum* are used as traditional medicines to treat diabetes mellitus. A collection of metabolic illnesses known as diabetes mellitus (DM) involves chronic hyperglycemia caused by flaws in insulin secretion, function, or both. Innate immunity and inflammation both play important roles in the etiology of diabetes-related microvascular problems.

***Objective*:** This study aims to examine the anti-diabetic effects and the acute toxicity of combined extract (1:1:1) of *Swertia chirayita, Trigonella foenum-gracum* and *Sesamum indicum.* To address the demand for higher effectiveness and safety, the current effort aims to construct anti-diabetic preparations containing methanolic extract from herbal medications.

***Methods*:** The OECD 423 method was used to investigate acute toxicity in rats. Rats were used as test subjects, and rats were given a 35 mg/kg BW injection of streptozotocin to develop diabetes. The diabetic control group was given Glibenclamide 0.25 mg/kg BW, while the experimental group's diabetic rats received 125 mg/kg BW and 250 mg/kg BW of a combined methanolic extract of all plants. Among the measurements looked at were acute oral toxicity, behavioral changes, body weight, serum glucose levels, lipid profiles, oxidative stress, renal function tests, and inflammatory mediators. All the rat groups' histopathologies of the kidney, liver, and stomach were compared. The data were evaluated using analysis of variance, and a post hoc test was then carried out.

***Results*:** The combined extracts' medium lethal doses (LD_{50}) were higher than 2000 mg/kg, indicating that they are not poisonous under the conditions that can be observed. Streptozotocin-induced diabetic rats' elevated blood glucose was found to be considerably lower (p 0.01) in the treated group of rats. In the treated group of rats, it was discovered that the damage and disarray in the cells typical of Streptozotocin-induced DM had been repaired. The treated group of rats returned to normal levels of the lipid profile, hyperglycemia, decreased serum protein and liver glycogen, increased liver function, and kidney function markers seen in the rats of the DM control group.

***Conclusion*:** The evaluated combined methanolic extract can be considered safe for use in rats. Combining methanolic extract from all selected medicinal plants (*Swertia chirayita, Trigonella foenum-gracum* and *Sesamum indicum)* has a potential anti-diabetic effect and can be safely developed as an alternative medicine.

Keywords: Diabetes mellitus, streptozotocin, anti-oxidants, acute oral toxicity, anti-diabetic, innate immunity.

ARTICLE HISTORY

Received: March 06, 2023
Revised: July 12, 2023
Accepted: August 13, 2023

DOI:
10.2174/0115701638252203230919092315

1. INTRODUCTION

Diabetes mellitus (DM), a chronic disease, is characterized by a persistent rise in blood sugar levels. Moreover, it exhibits polydipsia, polyurea, ketonemia, and ketonuria. The prevalence of type 2 diabetes mellitus (T2DM), commonly known as non-insulin-dependent diabetes, is increasing by 90-95% annually. Due to their increasing frequency, chronic diseases are becoming a serious threat to people's health, quality of life, and life expectancy. Diabetes mellitus (DM), one of the most common chronic diseases, is a complex metabolic condition characterized by elevated blood sugar levels, oxidative stress, and abnormal lipid and protein metabolism [1, 2]. Insulin sensitivity and insufficient pancreatic insulin production are two of the primary pathogenic processes of DM [3]. A range of potentially catastrophic health disorders, such as coronary artery disease, cardiovascular

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1570-1638/XX $65.00+.00

diseases, renal failure, blindness, limb amputation, neurological problems, and early death, can result from prolonged hyperglycemia in diabetic people. Currently, dietary adjustments combined with insulin injections or the use of oral hypoglycaemic medicines are the primary methods utilized to treat hyperglycemia and other diabetes-related issues. Yet, maintaining diabetes under control while taking these drugs can frequently be unpleasant because practically all of them have side effects. Researchers have attempted to discover novel drugs with fewer side effects by looking into natural substances derived from medicinal plants, which include secondary metabolites like polyphenols and flavonoids, which have antioxidant and anti-diabetic properties [4].

Swertia chirayita (Gentianaceae), a popular medicinal herb is used in traditional medicine to treat numerous ailments such as liver disorders, malaria, and diabetes and is reported to have a wide spectrum of pharmacological properties [5]. Sesame (*Sesamum indicum* L.) has been used as a traditional plant-based therapy worldwide, most commonly in Asian regions. Sesame seeds are good sources of lignan compounds (sesamin, sesamolin, sesamol, and epi sesamin). These lignans are responsible for most medicinal actions of sesame, including antioxidant activity, anti-inflammatory actions, and hypoglycemic effects [6]. *Trigonella foenum-graecum* (Methi), a green leafy vegetable belongs to the Leguminosae family. Its seed possesses anti-diabetic, anti-hyperlipidemic, and antioxidant properties [7].

2. MATERIALS AND METHODS

2.1. Chemicals

Streptozotocin was available from the chemical store of the IFTM University School of Pharmaceutical Sciences. Glibenclamide was a gift from Hyderabad's Aurobindo Pharma Ltd. We bought biochemical assay kits from Coral Diagnostics Ltd. in Bombay for measuring glucose, total cholesterol, total protein, triglycerides, high-density lipoprotein (HDL) cholesterol, serum glutamic pyruvate transaminase (SGPT), and serum glutamic oxaloacetic transaminase (SGOT). Other ingredients were purchased from SD Fine Chemicals Limited in India and were of analytical grade.

2.2. Collection and Authentication of Drug

Based on the literature, plants that have been reported to possess antidiabetic, anti-inflammatory, and antioxidant activities are selected. The locally available plants were freshly collected and the remaining herbs were purchased from an Ayurvedic shop in Kundarki, Moradabad District of Uttar Pradesh, India. The plant was authenticated by the Raw Material and Herbarium Museum, Delhi (RHMD), India. The reference numbers of these plants are NISCAIR/RHMD/Consult/2021/4141-42-1 for *Swertia chirayita*, NISCAIR/RHMD/Consult/2021/4141-42-2 for Fenugreek, and NISCAIR/RHMD/Consult/2021/4141-42-3 for *Sesamum indicum.*

2.3. Extraction of Material

Swertia chirayita, Trigonella foenum-graecum, and *sesamum indicum* dried powdered plant material (500 g) were extracted using customary extraction methods in a Soxhlet apparatus on heating mantle, first with petroleum ether to remove lipids and then with methanol (3 litres each) at a temperature not exceeding 45°C separately until the extraction was finished. Under lowered pressure, the methanolic extract of several plants was concentrated on Rota vapors and kept in a tight container for further use [8].

2.4. Animals

The Wistar albino rats (180 ± 20 g BW) were acquired from the animal house at the IFTM University's School of Pharmaceutical Sciences in Moradabad, India. Large, roomy polyacrylic cages with a 12-hour light/12-hour dark cycle were employed to house the animals. Rats have unrestricted access to rat pellets and water. The Institutional Animal Ethics Committee of the School of Pharmaceutical Sciences at IFTM University in Moradabad, India gave its approval to the project. The Committee for the Purpose of Regulation and Supervision of Experiments on Animals' rules were followed for all animal experiments.

2.5. Acute Oral Toxicity Studies

Acute oral toxicity tests were performed on all plant materials using methanolic extracts following the revised Draught Guidelines 423 of the Organization for Economic Cooperation and Development. The goal is to acquire enough information on the test drug's acute toxicity to enable its classification utilizing a step-by-step method that involves the fewest number of animals possible at each stage. Three healthy Wistar rats (any sex) were used in the experiment, one dose per animal. Rats that had fasted overnight were given oral dosages of the combination methanolic extract (CME) at levels of 5, 50, 300, and 2000 mg/kg BW, respectively. The rats were closely monitored for changes in their behavioral, neurological, and autonomic features for 24 hours after injection. Following 24 hours, animals were observed for 14 days (at least twice per day) to evaluate any changes in their behavioral, neurological, autonomic, and mortality profiles [9, 10].

2.6. Anti-diabetic Effect of Combined Methanolic Extracts

Healthy, mature male Wistar albino rats weighing 180–200 g were used for the experiment. Each group contained six animals. To induce diabetic mellitus in overnight-starved rats, one intraperitoneal injection of newly made 50 mg/kg BW STZ was administered, followed by 120 mg/kg of nicotinamide (NIC) in 0.1 M citrate buffer (pH 4.5). Rats were given 5% w/v glucose solution (2 ml/kg BW) after 24 hours of diabetes mellitus induction to lower hypoglycemia mortality. Diabetes mellitus was confirmed by measuring the fasting blood glucose level using a tail vein blood sample after 48 hours of induction. In the tests that followed, diabetic rats defined as those with fasting blood glucose levels higher than 200 mg/dl were used. Diabetes-affected animals were randomly assigned to one of five groups (Groups II–V).

Group I: Normal control.

Group II: Diabetic control.

Group III: Diabetic animals treated with glibenclamide (0.25 mg/kg).

Group IV: Diabetic animals treated with CME (125 mg/kg).

Group V: Diabetic animals treated with CME (250 mg/kg).

Animals in groups I and II received 0.5% w/v carboxymethyl cellulose (normal control and diabetic control, respectively) (CMC). Animals in groups IV-V received CME at dosages of 125 and 250 mg/kg BW, whereas those in group III received glibenclamide at a dose of 0.25 mg/kg BW. Based on the toxicological study, the CME dosages were selected. For a total of 28 days, the standard and test drugs were administered orally after being dissolved in 0.5% w/v CMC. A few drops of venous blood were drawn on the seventh and fourteenth days of the experiment, and they were immediately used to estimate blood glucose (in whole blood) using a glucometer [11]. Throughout the investigation, the variations in BW in the test animals were periodically monitored. Blood samples were obtained from each experimental animal *via* a retro-orbital plexus puncture at the end of the trial (*i.e*., after 28 days), and the serum was separated and used for biochemical analysis.

2.7. Fasting Blood Glucose

After the 28-day experiment, the rats in all groups were kept on a strict fast, and blood was drawn from the tail area to measure the rats' blood glucose levels. After the trial, body weight, food, and water consumption were also noted and examined [12].

2.8. Biochemical Parameters

On days 0, 7, 14, 21, and 28 of the trial, blood samples were taken by puncturing the retro-orbital plexus while being given lite anaesthesia. Animals who starved for 18 hours were slaughtered on day 21 *via* cervical dislocation. Blood was drawn into a dry test tube, where it was left to clot for 30 minutes at room temperature. For the estimation of glucose and total cholesterol, serum was centrifuged at 3000 rpm for 10 minutes [13], and levels of total triglycerides, LDL, VLDL, and HDL (low-density lipoprotein, very low-density lipoprotein, and high-density lipoprotein) [14]. To homogenize the liver, tris, and phosphate buffers were used to separate the liver from the killed animals, weigh it, wash it with saline, blot it dry, and do other steps. The homogenate's total glycogen content was determined [15, 16].

2.9. Estimation of Renal Function Parameters in Serum and Urine

Serum creatinine levels were calculated using the Erba Chem-7 commercially available kit. For 24 hours, each rat in each group was maintained in a metabolic cage alone. The urine samples were taken and measured with a measuring cylinder, and the urine volume (ml/24 h) was recorded [17]. Using commercially available Erba Chem-7 kits, the obtained urine samples were used to estimate urinary proteins (biuret technique), urea (urease L-glutamate dehydrogenase method), and creatinine (Jaffe method). By using the test procedure provided by Abcam's enzyme-linked immunosorbent assay (ELISA) kits, type IV collagen levels in urine samples were assessed (ab 6586). Using a previously published formula, the 24-hour urine albumin excretion rate (UAER) was calculated [18, 19].

2.10. Estimation of Inflammatory Cytokines

Inflammatory cytokines in serum: interleukin-6 (IL-6), transforming growth factor (TGF)-β1, and tumor necrosis factor-alpha (TNF-α) levels were estimated according to the procedure by ELISA kits [20, 21].

2.11. Histopathological Study

The retrieved kidneys and liver were fixed for 48 hours by submersion in 10% buffered formaldehyde (BNF). Then, using a tissue processor with a timer, the fixed tissue was cut into pieces that were between 0.5 and 1 cm broad, placed into an embedding cassette, and treated. The embedding fluid comprised 90% absolute ethanol, 5% methanol, and 5% isopropanol for around two hours [22]. The tissue was embedded, then put into a mould and covered with paraffin. After the paraffin had a chance to solidify, it was taken out of the mould, thinly cut using a microtome (4-5 ck), and stained with hematoxylin and eosin. Comparing the histology tissue from the treatment and control groups allowed for the examination of the kidney and liver. Vacuolization, organ fattening degeneration, and necrotic and hydrophilic degeneration are the traits that have been seen. The microscopic observation was scored, and a descriptive analysis was done [23].

2.12. Statistical Analysis

All the results are expressed as mean ± SEM, analyzed by two-way ANOVA followed by Tukey's multiple comparison analysis as a post-hoc test. The $P<0.05$ was considered to be statistically significant. GraphPad Prism (version 5.0) software, Inc. La Jolla, California, (USA) was used for all statistical analysis.

3. RESULTS

3.1. Acute Toxicity Study

The combined methanolic extract was found to be practically nontoxic when administered orally to rats and its LD_{50} value was found to be higher than 2000 mg/kg body weight.

3.2. Serum Glucose Level Estimation

The results were recorded as shown in Table **1**. When the tests were performed on day 28 of the experimentation the treated groups IV and V showed a reduction of blood glucose which was recorded as 125.10 and 113.25 mg/dl. The results are substantial when compared with the results of Group II (Diabetic control) from week 12.

3.3. Body Weight, Food Intake (FI), Water Intake (WI), and Urine Out (UO)

From week two to week twelve, both groups IV and V of rats put on weight. Since diabetes is a condition where weight growth is the reverse of weight reduction, treatment

Table 1. Effect of CME on fasting blood sugar level during the course of anti-diabetic study model (Post induction of diabetes).

Groups	Fasting Blood Glucose Level in mg/dl			
Treatment	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Group I	98.6 ± 1.89	96.56 ± 0.56	97.23 ± 1.43	95.56 ± 1.72
Group II	163.54 ± 1.36a	245.26 ± 2.45a	289.12 ± 0.28a	315.31 ± 1.45a
Group III	135.56 ± 1.31***	155.58 ± 1.72***	132.12 ± 1.56***	109.67 ± 2.31***
Group IV	143.21 ± 1.32***	165.60 ± 1.76***	145.12 ± 1.22***	125.10 ± 3.45***
Group V	146.14 ± 1.43***	126.25 ± 2.43***	122.51 ± 1.43***	113.25 ± 1.55***

Note: Values are expressed as Mean ± SEM; n =6. One-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a $P< 0.001$ in comparison with normal control and ***$P < 0.001$ in comparison with the diabetic control.

Table 2. The effect of CME on feed intake, water intake, urine output and body weight of the STZ induced diabetic rats.

Groups	Body Weight (g)	FI (g/day)	WI (mL/day)	UO (mL/day)
Group I	165 ± 5.12	25.75 ± 5.25	38.33 ± 6.62	6.42 ± 2.43
Group II	158.30 ± 4.45a	42.67 ± 3.56a	85.55 ± 3.51a	32.37 ± 4.60a
Group III	171.20 ± 5.12a***	42.38 ± 4.35***	67.37 ± 4.40***	12.34 ± 3.52***
Group IV	186.30 ± 6.3***	32.45 ± 5.32***	51.54 ± 6.22***	14.55 ± 2.05***
Group V	187.31 ± 4.32a***	28.54 ± 3.56***	55.32 ± 6.35***	13.32 ± 3.78***

Note: Values are expressed as Mean ± SEM; n =6. One-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a $P< 0.001$ in comparison with normal control and ***$P < 0.001$ in comparison with the diabetic control

groups with average weights of 186.30 ± 6.3 and 187.31 ± 4.32 g and diabetic groups with average weights of 158.30 ± 4.45 g both showed gains in health. As compared to Group II (Diabetic control) data from week 12, the results are substantial. The results are displayed in Table **2**.

3.4. Biochemical Parameters

On the 28th day of the study, diabetic animals showed a rise in triglyceride total cholesterol and LDL levels, whereas HDL levels decreased, compared to normal control animals. The animals treated with Combined Methanolic Extract (250 mg/kg), Glibenclamide (0.20 mg/kg) have shown significant ($P < 0.05$) improvement in these altered levels shown in Table **3**.

3.5. Effect of CME on Oxidant–Antioxidant Status in Liver and Kidney

To determine the degree of oxidative stress experienced by diabetic rats, lipid peroxidation expressed as malondialdehyde (MDA) and enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH) were examined. MDA significantly increased ($p<0.05$) in the parenchymal cells of the liver and kidney, while the measured antioxidant levels significantly decreased. Nevertheless, oral administration of CME effectively reversed these changes in a dose-dependent manner, even returning the levels to normal at the highest dose used (250 mg/kg) (Tables **4** and **5**).

3.6. Effect of CME on Renal Function Tests

STZ-DN rats displayed a significant decrease in 24-h urine volume, urinary urea, and urine creatinine ($P < 0.01$) and an increase in serum creatinine, protein in the urine, urinary albumin excretion in rats (UAER), AGES, and type IV collagen excretion ($P < 0.01$). Combined methanolic extract 125 and 250 mg/kg body weight (bw) doses demonstrated a significant increase in 24-h urine volume, urine creatinine ($P < 0.01$), and urinary urea ($P < 0.05$ and $P < 0.01$) and a significant decrease in protein in the urine, UAER, AGES, and type IV collagen excretion ($P < 0.01$) in correlation with DN rats (Tables **6** and **7**).

Values are expressed as Mean ± SEM; n=6. One-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a $P< 0.001$ in comparison with normal control and ***$P<0.001$ in comparison with the diabetic control.

3.7. Effect of CME on Inflammatory Mediators

STZ-DN rats displayed a considerable rise in IL-6, TGF-β1, and TNF-α ($P < 0.01$). DN rats with PHF at 250 and 500 mg/kg bw doses demonstrated a significant decrease in IL-6, TGF-β, and TNF-α ($P < 0.01$) in correlation with DN rats (Table **8**).

Table 3. Effect of CME on serum lipid profile.

Groups	Lipid Profile (mg/dl)			
Treatment	**Serum cholesterol**	**Serum triglycerides**	**Serum HDL**	**Serum LDL**
Group I	72.56 ± 2.34	74.92 ± 1.67	39.43 ± 1.32	32.34 ± 1.45
Group II	149.38 ± 1.72	139.45 ± 1.45*	28.53 ± 1.78	96.28 ± 1.48**
Group III	78.67 ± 0.35***	72.89 ± 0.45***	35.73 ± 1.49***	32.41 ± 0.36 ***
Group IV	75.48 ± 1.32***	65.65 ± 1.65***	36.83 ± 1.69***	36.32 ± 0.45 ***
Group V	71.75 ± 1.46***	66.15 ± 1.45***	33.45 ± 1.59***	34.72 ± 2.43***

Note: Values are expressed as Mean ± SEM; n =6. One-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a *P*< 0.001 in comparison with normal control and ****P* < 0.001 in comparison with the diabetic control

Table 4. Effect of CME on hepatic oxidant-antioxidant parameters.

Group	SOD (Unit/mg Protein)	CAT (µmol/min/mg Protein)	GPx (µmol/min/mg Protein)	GSH (mM/100 mg Tissue)	MDA (µmol/100 mg Tissue)
Group I	8.36 ± 1.12	94.8 ± 3.45	12.2 ± 1.09	55.6 ± 2.50	1.79 ± 0.54
Group II	4.50 ± 0.86 a	38.4 ± 2.32 a	5.32 ± 0.80 a	27.6 ± 1.45	2.35 ± 0.60
Group III	7.49 ± 1.45 **	58.1 ± 4.23 **	8.38 ± 2.21 **	39.3 ± 2.50 **	1.80 ± 0.32**
Group IV	7.50 ± 0.43 **	35.6 ± 2.45 **	7.75 ± 1.54 **	33.3 ± 2.34**	1.25 ± 0.68 **
Group V	8.45 ± 0.38***	65.7 ± 3.78 ***	9.54 ± 1.35 ***	35.4 ± 2.44 ***	1.58 ± 0.51 **

Note: Values are expressed as Mean ± SEM; n =6. One-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a *P*< 0.001 in comparison with normal control and ****P* < 0.001 in comparison with the diabetic control.

Table 5. Effect of CME on renal oxidant-antioxidant parameters.

Group	SOD (unit/mg Protein)	CAT (µmol/min/mg Protein)	GPx (µmol/min/mg Protein)	GSH (mM/100 mg Tissue)	MDA (µmol/100 mg Tissue)
Group I	7.56 ± 1.12	92.8 ± 3.42	13.2 ± 1.20	54.6 ± 2.60	1.45 ± 0.94
Group II	4.45 ± 0.86 a	35.4 ± 2.32 a	7.52 ± 0.80 a	23.6 ± 1.55	2.56 ± 0.80
Group III	8.55 ± 1.45 **	52.1 ± 4.25 **	9.38 ± 2.25 **	36.2 ± 2.60 **	1.67 ± 0.62**
Group IV	6.54 ± 0.42 **	37.2 ± 2.42 **	9.75 ± 1.44 **	35.4 ± 2.24**	1.25 ± 0.48 **
Group V	8.56 ± 0.32***	62.5 ± 3.72 ***	10.15 ± 1.85 ***	38.5 ± 2.54 ***	1.58 ± 0.41 **

Note: Values are expressed as Mean ± SEM; n =6. One-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a *P*< 0.001 in comparison with normal control and ****P* < 0.001 in comparison with the diabetic control.

Table 6. Effect of CME on renal function tests.

Group	Urine Volume (ml/day)	Urinary Urea (mg/dl)	Serum Creatinine (mg/dl)	Urine Creatinine (mg/dl)
Group I	14.5 ± 0.90	7.35 ± 0.24	0.98 ± 0.05	25.6 ± 1.24
Group II	4.8 ± 0.28**	1.49 ± 0.01**a	2.80 ± 0.52**a	5.8 ± 0.99**a
Group III	13.8 ± 1.75***	2.55 ± 1.90***	1.55 ± 0.02***	22.1 ± 1.18***
Group IV	13.4 ± 1.45***	4.28 ± 0.53***	0.78 ± 0.05***	23.6 ± 0.50***
Group V	14.2 ± 1.55***	5.45 ± 0.65***	0.95 ± 0.03***	24.6 ± 0.50***

Note: Values are expressed as Mean ± SEM; n =6. One-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a *P*< 0.001 in comparison with normal control and ****P* < 0.001 in comparison with the diabetic control.

Table 7. Effect of CME on renal function tests.

Group	Protein in Urine (mg/day)	UAER (µg/day)	AGES (AU)	Type IV Collagen Excretion (µg/day)
Group I	0.69 ± 0.072	1.5 ± 0.33	192 ± 12.3	16.3 ± 3.55
Group II	18.50 ± 0.32a**	15.8 ± 1.50a**	345 ± 25.7a**	56.5 ± 6.5a**
Group III	0.85 ± 0.054***	4.2 ± 0.45***	195 ± 15.2***	88.0 ± 5.23***
Group IV	0.65 ± 0.010***	3.35 ± 0.35***	198 ± 22.0***	32.5 ± 5.43***
Group V	0.63 ± 0.040***	3.65 ± 0.24***	192 ± 20.4***	33.5 ± 5.56***

Note: Values are expressed as Mean ± SEM; n=6. One-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a $P< 0.001$ in comparison with normal control and ***$P<0.001$ in comparison with the diabetic control.

Table 8. Effect of CME on inflammatory mediators.

Group	IL-6 (Pg/ml)	TGF-β1 (%)	TNF-α (Pg/ml)
Group I	172 ± 3.60	14.6 ± 2.55	36.2 ± 4.52
Group II	368 ± 8.6a**	26.4 ± 2.55a**	285 ± 35.5a**
Group III	268 ± 4.52***	18.2 ± 1.75***	36.4 ± 10.6***
Group IV	254 ± 7.65***	16.2 ± 1.32***	48.0 ± 7.65***
Group V	255 ± 8.62***	15.2 ± 1.52***	38.5 ± 4.55***

Note: Values are expressed as Mean ± SEM; n =6. One-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a $P<0.001$ in comparison with normal control and ***$P < 0.001$ in comparison with the diabetic control.

3.8. Histopathology Analysis

Under a microscope, the organs of the animals administered the extract did not differ in colour from controls in any way. After the experiment period, autopsies from the control and treated rats' testicles and stomachs revealed no evident abnormalities in the histopathology report. The microscopic representation of the organs in Fig. (**1**) shows insignificant differences between the control and test groups. Rat stomachs, livers, and kidneys were investigated histopathologically, and the alterations seen in Figs. (**2, 3** and **4**) were noted under a microscope.

4. DISCUSSION

The combined methanolic extracts contained *Trigonella foenum-graecum* seeds, *Swertia chirayita* leaves, and *Sesamum indicum* seeds. Preliminary investigations have shown that the combination of the ingredients mentioned above has anti-diabetic benefits in test animals [24, 25]. Information about the toxicity, pharmacological, pharmacokinetic, and other impacts of pharmaceuticals on humans can be found in the results of preclinical tests. The preclinical test in this study assessed the acute and subacute toxicity of CME as an anti-diabetic following the Organization for Economic Cooperation and Development's (OECD) standards 423 and 407 [26]. There were no signs of tremor, salivation, convulsions, diarrhea, or coma based on the observations of the animal's behavior at each dose level [27]. Moreover, rats who were given doses of 175 and 550 mg/kg did not exhibit any negative side effects. Fourteen days following the therapy, the test animals' eyes, skin, mucous membranes, and right reflex were still normal, but their motor activity had changed [28]. The combined methanolic extract's LD_{50} value was over 2000 mg/kg based on the results. The medication was found to be safe because it had an LD_{50} value of 2000 mg/kg and higher, which was categorized as unclassified by the Organization for Economic Cooperation and Development (OECD) guidelines for acute oral toxicity. The acute toxicity effect of the mixed methanolic extract was examined in the current investigation up to a dose of 2000 mg/kg, and neither toxicity nor death was reported. To complement the findings of the acute toxicity test, rats were given CME once daily for 28 days to perform oral sub-acute toxicity testing [29]. From day 0 to day 28, there were fluctuations in the mean body weight of the rats, both male and female. For each group, the body weight didn't significantly change, though. This demonstrated that there were no signs of toxicity or illnesses that cause appetite loss, as a large drop in body weight is a sign of toxicity from the test preparation used [30]. When weight fluctuations are fluctuating, fat storage and the body's adaptive response to the herbal treatment may be to blame rather than toxicity [31]. There were no discernible differences between the normal and dose groups in the aspartate aminotransferase, alanine aminotransferase, and albumin liver function tests ($p> 0.05$). ASR and ALT levels that are higher than in the control imply that the hepatocyte function is being disrupted, resulting in the release of both AST and ALT into the bloodstream [32]. Reduced albumin levels are associated with disruption of hepatocyte synthesis activity, especially in chronic hepatocyte lesions. Moreover, there were no appreciable differences in the blood urea nitrogen (BUN) and creatinine

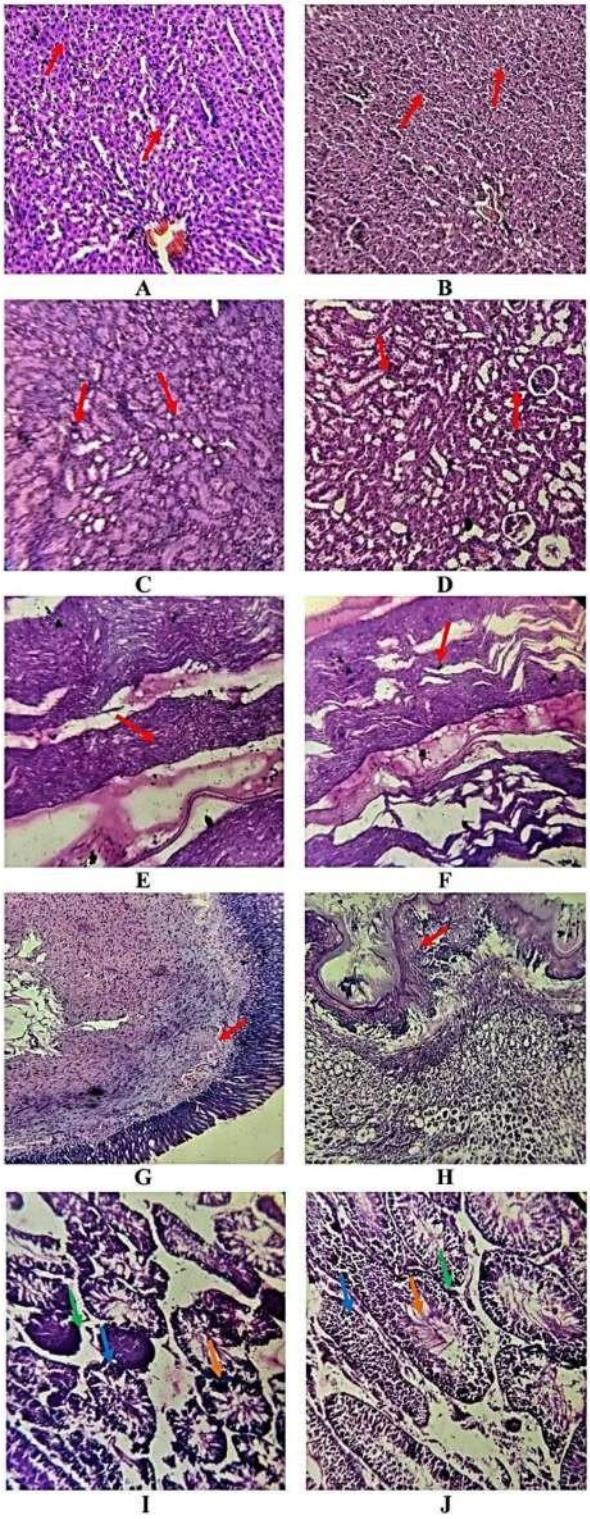

Fig. (1). Histopathology of rat's organs for acute oral toxicity studies. (**A**) liver showing normal hepatocytes (arrow); (**B**) liver showing normal hepatocytes (arrow); (**C**) The renal tissue shows fairly well-preserved renal parenchyma. The renal tubules around renal corpuscles appeared apparently normal. Inflammatory changes in the renal parenchyma are not evident; (**D**) The renal tissue shows well preserved renal parenchyma. The renal tubules around renal corpuscles appeared apparently normal. Inflammatory changes in the renal parenchyma are not evident; (**E**) Showed the normal arrangement of fibers; (**F**) Showed the normal arrangement of fibers; (**G**) Normal histological structure of gastric layers, arrows show mucosa; (**H**) Normal histological structure of gastric layers, arrows show mucosa; (**I**) Normal testicular histology. The active seminiferous tubules (orange arrow) and interstitial spaces (green arrow). The seminiferous epithelium (blue arrow) showing active spermatogenesis; (**J**) Normal testicular histology. The active seminiferous tubules (orange arrow) and interstitial spaces (green arrow). The seminiferous epithelium (blue arrow) showing active spermatogenesis. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

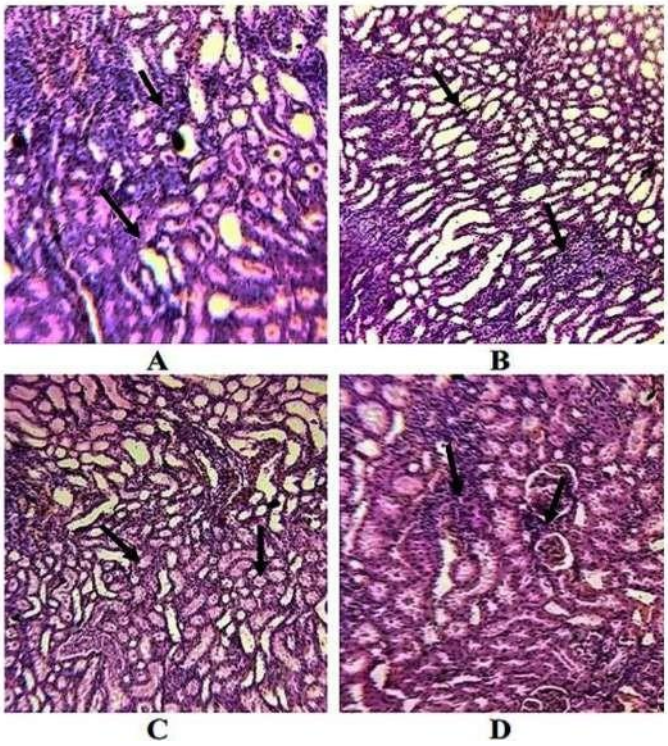

Fig. (2). Histopathology of kidney of rats. (**A**) Diabetic control-arrow indicates the improper arrangement of the nephron cell with the presence of high endocytic vacuoles, (**B**) CME 125 mg/kg treated group-arrow indicate partly recovered nephron cells, (**C**) CME 250 mg/kg treated group animals-arrow indicate recovered normal arrangement of the nephron cells with normal glomeruli, (**D**) Glibenclamide treated Standard group-arrow indicates recovered normal arrangement of the nephron cells with normal glomeruli and low endocytic vacuoles. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

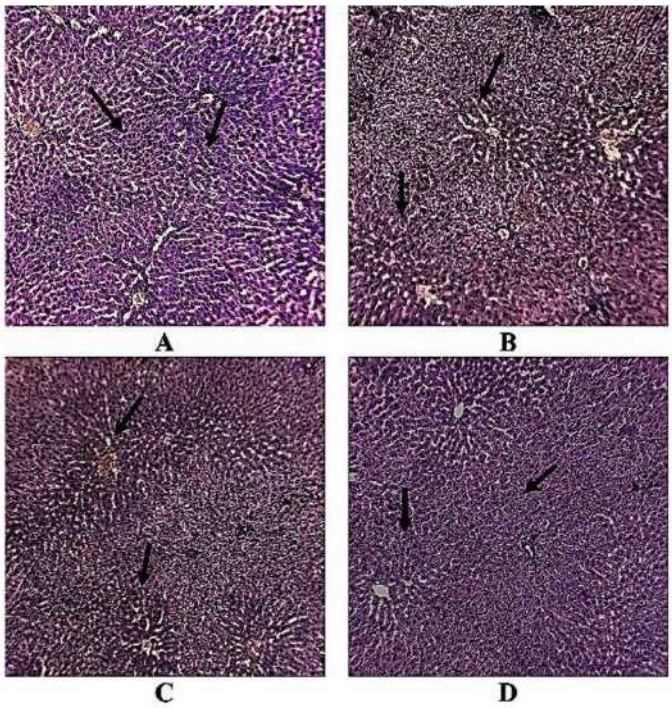

Fig. (3). Histopathology of liver of rats. (**A**) Diabetic control rat liver showing increase vacuolation in the cytoplasm of hepatocytes appeared as indistinct clear vacuoles (arrows) indicate glycogen infiltration in diabetes, (**B**) Liver of diabetic rat treated with CME 125 mg/kg showing normal liver architecture with hepatocytes arranged in normal sheets or cord around the central vein, (**C**) Liver of diabetic rat treated with CME 250 mg/kg showing better liver architecture with normal hepatocytes arranged in normal sheets or cord around the central vein, (**D**) Standard group diabetic rat treated with metformin is showing normal hepatocytes and normal hepatic architecture with mild vacuolation of hepatocytes (arrow). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

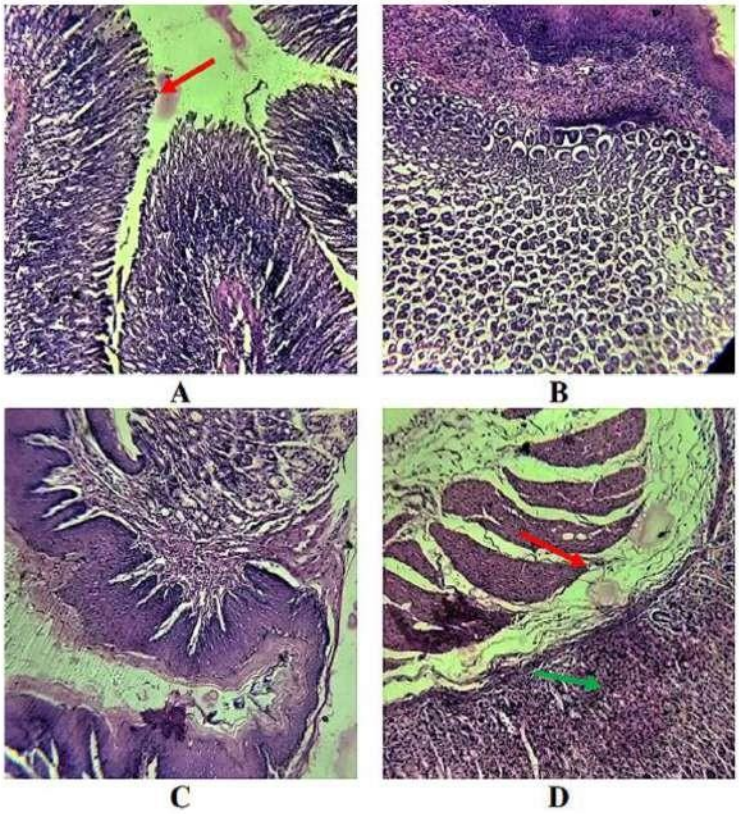

Fig. (4). Histopathology of stomach of rats. (**A**) diabetic ontrol group rats, showing multiple focal necrosis of gastric mucosa (arrow), (**B**) CME 125 mg/kg treated rat shows a normal histological structure of gastric layers, (**C**) CME 250 mg/kg treated rat shows normal epithelial tissue, (**D**) Glibenclamide treated standard group showing normal gastric mucosa (Red arrow) and submucosa (Green arrow). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

values between the normal and dose groups ($p > 0.05$) [33]. Under normal kidney function, blood creatinine levels should remain constant; any increase is a sign of impaired kidney health. BUN readings could be used to monitor the effectiveness of hemodialysis, the progression of renal disease, and kidney function [34]. Blood BUN levels are associated with glomerulus injury, although they can also be triggered by hepatotoxins or starvation. The polyherbal formula had no impact on the liver and renal function in male and female rats, as shown by the fact that 10% extract of *S. sonchifolius* at doses of 14 g/kg and 0.32 g/kg did not cause mouse mortality. After 90 days of daily dosage at doses of 0.07, 0.14, and 0.28 g/kg, the haematology, biochemical, and histopathological markers did not significantly change. It was also reported that there was very little subacute toxicity and changes in body weight, haematological, serum, urine, and histological parameters after 28 days of extract therapy with doses of 625, 1250, and 2500 mg/kg to rats. When dosages of 500, 1000, and 2000 mg/kg per day for 90 days were utilised in a study on the toxicity of S. rebaudiana leaf extract, similar results were observed [34]. Rats given *S. polyanthum* leaf extract at doses of 100, 400, and 1000 mg/kg per day for 90 days saw comparable results, although the female rats' livers were fatty and necrotic [35]. These arguments suggest that the effect of a single plant is insufficient to provide diabetic patients with a therapeutic outcome. These plants could be combined in a certain ratio in a polyherbal composition to get a better outcome with less toxicity [36].

CONCLUSION

In rats given streptozotocin-induced, simultaneous treatment with methanolic extract in a 1: 1: 1 ratio for twenty-eight days may lower fasting blood glucose levels and somewhat enhance pancreatic beta-cell function. The lipid profile was also within acceptable bounds. After oral administration of a single dose, combined methanolic extract combinations up to a dose of 2000 mg/kg BW were considered safe. The combination of the methanolic extract can be created as a secure anti-diabetic medication because body weight, relative organ weight (liver and kidneys), and hematological, and biochemical indicators were not substantially different from the control group. The anti-diabetic mechanism of action of this combination and the variations in the dose ratio of the combined methanolic extracts hence require additional research. Also, further sub-chronic and chronic toxicology studies to confirm the safe use of combined methanolic extracts are needed.

LIST OF ABBREVIATIONS

BUN	=	Blood Urea Nitrogen
bw	=	Body Weight
CAT	=	Catalase
CME	=	Combination Methanolic Extract
DM	=	Diabetes Mellitus

ELISA	=	Enzyme-linked Immunosorbent Assay
GPx	=	Glutathione Peroxidase
HDL	=	High-density Lipoprotein
IL-6	=	Interleukin-6
MDA	=	Malondialdehyde
NIC	=	Nicotinamide
OECD	=	Organization for Economic Cooperation and Development's
SGOT	=	Serum Glutamic Oxaloacetic Transaminase
SGPT	=	Serum Glutamic Pyruvate Transaminase
SOD	=	Superoxide Dismutase
T2DM	=	Type 2 Diabetes Mellitus
TGF-β1	=	Transforming Growth Factor
TNF-α	=	Tumor Necrosis Factor-alpha
UAER	=	Urine Albumin Excretion Rate

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained to conduct pharmacological activity on animals from CPCSEA/Institutional Animal Ethics Committee (IAEC) registration number 837/PO/Re BiBt/S/04/CPCSEA with reference number IFTM/IAEC/ 2022/02/03. These recommendations complied with the widely recognized standards for the usage and maintenance of laboratories.

HUMAN AND ANIMAL RIGHTS

All the animal experimentation was performed according to the Guide for the Care and Use of Laboratory Animals.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors are grateful to the Prof. Sushil Kumar (Director) School of Pharmaceutical Sciences, Prof. Navneet Verma (Dean Faculty of Pharmacy) and Prof. M. P. Pandey (Vice Chancellor) IFTM University for providing the facility for the research work.

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HOW TO CITE:

Shivam*, Gupta Kumar Asheesh, Toxicological Assessment and Anti-diabetic Effects of Combined Extract of Chirata, Fenugreek and Sesame on Regulating TNF-α, TGF-β and Oxidative Stress in Streptozotocin Induced Diabetic Rats, Current Drug Discovery Technologies 2024; 21 (1) : e201023222477 . https://dx.doi.org/10.2174/0115701638252203230919092315.