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Cardioprotective effect of bioassay-guided fractions of *Cleome viscosa* methanolic extract in streptozotocin-induced diabetic ratsSuresh Yarrappagaari¹, Lavanya Thopireddy², Srinivasulu Cheemanapalli³, Venkata Ramireddy Narala⁴, Rajeswara Reddy Saddala[✉]¹Division of Ethnopharmacology, Department of Biotechnology, School of Herbal Studies and Naturo Sciences, Dravidian University, Kuppam-517 426, Andhra Pradesh, India²Department of Zoology, KVR Government College for Women, Kurnool-518 004, Andhra Pradesh, India³Regional Ayurveda Research Institute, Itanagar-791111, Arunachal Pradesh, India⁴Department of Zoology, Yogi Vemana University, Kadapa- 516005, Andhra Pradesh, India

ABSTRACT

Objective: To evaluate the cardioprotective effect of bioassay-guided isolated fractions of *Cleome viscosa* methanolic extract in streptozotocin-induced diabetic rats.

Methods: Total phenolics, flavonoids, and *in vitro* antioxidant activities of the methanolic extract of *Cleome viscosa* were evaluated. The FD-40 fraction from this extract was further evaluated for antihyperglycemic efficacy (insulin and HbA1c), antioxidant activity, and cardioprotective effects (creatine kinase-MB, lactate dehydrogenase, and histopathology) in streptozotocin-induced diabetic rats. *In silico* studies were also conducted to assess the bioactivity of FD-40.

Results: *Cleome viscosa* methanolic extract exhibited the highest antioxidant activity in DPPH, ABTS, H₂O₂, and FRAP assays compared to other extracts. Treatment with FD-40 (40 mg/kg b.w.) isolated from this extract normalized blood glucose, insulin, HbA1c, creatine kinase-MB, and lactate dehydrogenase levels in diabetic rats. It also significantly reduced oxidative stress by increasing antioxidant enzymes, decreasing lipid peroxidation as well as restoring the levels of ascorbic acid and glutathione. Histological study demonstrated that FD-40 treatment improved cardiac structure in diabetic rats. Molecular docking analysis revealed that phytochemicals from FD-40 had strong binding affinities with cardiac markers and oxidative enzymes. Hexose (5TMS) demonstrated greater binding affinity with xanthine oxidase and lactate dehydrogenase.

Conclusions: FD-40 of *Cleome viscosa* methanolic extract exhibits significant cardioprotective effects in diabetic rats by regulating cardiac markers and reducing oxidative stress. The underlying mechanisms need to be elucidated in the future.

KEYWORDS: *Cleome viscosa* L.; Cardioprotection; Cardiac markers; Docking; CK-MB; GC-MS; Diabetes

1. Introduction

More than 537 million people worldwide have diabetes mellitus, and the number is expected to rise to over 643 million by 2030 and 783 million by 2045[1]. One-third of the people with diabetes worldwide have type 2 diabetes mellitus. The cost of treating

Significance

Cleome viscosa has traditionally been used for various ailments, but its specific cardioprotective effects in diabetes have not been extensively studied. This research demonstrates that the bioassay-guided isolated fractions of *Cleome viscosa* methanolic extract exhibit significant cardioprotective effects in streptozotocin-induced diabetic rats. The study highlights the potential of bioassay-guided phytochemicals in mitigating cardiac damage and underscores the need for further clinical validation.

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diabetes-related health issues and their coexisting conditions is steadily increasing, placing a heavy financial burden on diabetics and, particularly, in developing nations[2]. Type 2 diabetes mellitus is a significant global health issue, and among other factors, the likelihood of developing diseases increases with age, obesity, stress, nutrition, ethnicity, inactivity, and inflammation.

Protein glycation has been associated with chronic hyperglycemia, which can result in cardiovascular disease, renal damage, liver damage, fatigue, mental confusion, weight gain, and skin conditions[3]. Antioxidant therapy has the potential to alter the natural history of a number of diseases because oxidative stress is crucial in the etiology of many clinical disorders and aging. Reactive oxygen species (ROS) are produced as a result of hyperglycemia by a metabolic pathway involving diacylglycerol, protein kinase C, and NADPH-oxidase[4]. In individuals with diabetes and hyperglycemia, ROS are hypothesized to be generated by mitochondrial respiratory chain enzymes, xanthine oxidase (XOD), lipoxygenase, cyclooxygenase, nitric oxide synthase, and peroxidase[5].

With few adverse effects, natural compounds and molecules produced from them may be effective treatments for type 2 diabetes and associated consequences. Numerous potent medicinal plants and their naturally occurring bioactive components have already demonstrated their anti-diabetic capabilities. A variety of medicinal herbs have been used to treat and prevent diabetes and its complications since ancient times[6].

The most recent list of approved medicines demonstrates the continued significance of plant-derived compounds in the development of new medicines. Plant-based medicines have demonstrated some defense against the pathophysiology of diabetes mellitus by blocking the markers associated with cardiovascular disease. Animals have been used to evaluate and validate the hypoglycemic activity of several plant extracts[7]. According to recent research, plant-based formulations that serve as antioxidants, anti-inflammatory agents, and glucose-lowering agents can help manage diabetes[8].

Major Indian-Ayurvedic and Chinese traditional medicines, including *Cleome viscosa* (*C. viscosa*) L. (Cleomaceae), have been used to treat rheumatoid arthritis, hypertension, malaria, neurasthenia, and wound healing. *C. viscosa* is common throughout India and is also known as Asian spider blossom. It has been formulated, documented, and grouped into various medical systems, including Ayurveda, Siddha, Unani, and others[9]. Dropping whole plant juice into the ear can help relieve fever and earache. The paste of the leaf and the bark can be applied to wounds. The leaf drops are applied as nasal drops to relieve the headache. The seeds and roots are used to reduce blood pressure, diabetes, and cardiac disorders. The leaves are applied for earaches and boils. To reduce the fever, the plant juice can be taken orally after being diluted with water. According to Mali, tribal people can instill leaf juice in their ears to relieve toothache[10]. We have previously shown that *C. viscosa* crude extracts and separated fractions exhibit anti-inflammatory, anti-

diabetic, hepatoprotective, and anti-hyperglycemic properties[11]. The current study focused on evaluating the cardioprotective effects of an isolated fraction of *C. viscosa* methanolic crude extract (FD-40) using *in vitro* and *in vivo* models, and *in silico* analysis.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and standards used for antioxidants and antidiabetics were purchased from HiMedia and Sigma-Aldrich (Bangalore, India). Analytical-grade chemicals were used for reagents and buffer solutions.

2.2. Plant collection and preparation of *C. viscosa* crude extracts

The whole plant of *C. viscosa* was collected in the vicinity of Dravidian University in Andhra Pradesh, India, and identified and validated (No. NY/531) by Prof. N. Yasodamma, a taxonomist, Department of Botany, S.V. University, Tirupati, India. The plant was shade-dried and then ground into a fine powder using an electric grinder. Soxhlet apparatus was used to extract dried *C. viscosa* powder (500 g per extract) at 50–60 °C for 72 h, using 5 L of methanol, distilled water, ethyl acetate, and *n*-hexane. Extract was then concentrated in a rotary shaker, filtered under vacuum, and lyophilized to produce 93 g, 90 g, 10 g, and 3 g of solid crude extract from respective solvents.

2.3. Determination of phytochemical content in crude extracts

2.3.1. Total phenolic content

Total phenolic content was determined using the recommended method[12]. Briefly, 6.0 mL of distilled water, 2.5 mL of 20% sodium carbonate, 0.5 mL of Folin-Ciocalteu's reagent, and 100 mg/mL of *C. viscosa* plant crude extract were mixed. The final mixture was blended and incubated for 30 min at 40 °C in a water bath with shaking. The absorbance of the reaction mixture was measured at 760 nm. A calibration curve was created using gallic acid as the standard.

2.3.2. Total flavonoid content

The aluminium chloride colorimetric technique was used to determine the total concentration of flavonoids as previously described[13]. Quercetin (50–100 mg/mL) was used as a standard and 100 mg of the crude extracts of *C. viscosa* was diluted in 1 mL of distilled water. Additionally, 0.5 mL of this solution was mixed with 2.8 mL of distilled water, 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, and 0.1 mL of 1 M potassium acetate. Absorbance was measured at 415 nm after 30 min of incubation at room temperature.

2.4. *In vitro* antioxidant activity of crude extracts

2.4.1. ABTS^{•+} scavenging assay

ABTS radical scavenging activity was measured according to the previous method[14]. The stock solution included 2.4 mM potassium persulfate and 7 mM ABTS. Various quantities of crude extracts from the *C. viscosa* plant (20-100 µg/mL) and standard ascorbic acid were allowed to react for 7 min before determining the absorbance at 734 nm.

2.4.2. DPPH radical scavenging assay

The DPPH scavenging activity of the crude extracts was determined using a modified Brand-Williams method[15]. Briefly, various concentrations of each extract (20-100 µg/mL) and the standard antioxidant gallic acid were prepared in methanol. A 60 µM DPPH solution was freshly prepared in methanol, and 3 mL of this solution was added to 1 mL of each extract concentration. The mixtures were incubated for 30 min in the dark at room temperature. After incubation, the absorbance was measured at 517 nm against a methanol blank. The DPPH radical scavenging activity was calculated as a percentage of the control.

2.4.3. H₂O₂ scavenging assay

The H₂O₂ scavenging activity of *C. viscosa* crude extracts was assessed using the previous method[16]. A neutral phosphate buffer was used to prepare a 2 mmol/L H₂O₂ solution. Then 0.6 mL of H₂O₂ solution was added to the plant extracts (20-100 µg/mL). After incubation (10 min), the absorbance of H₂O₂ was measured at 230 nm against a blank, and ascorbic acid was used as a standard to compare the radical scavenging activity.

2.4.4. Ferric reducing antioxidant power (FRAP) assay

FRAP reagent was prepared by mixing solution A (300 mM acetate buffer, pH-3.6), solution B (10 mM TPTZ in 40 mM HCl), and solution C (20 mM FeCl₃) in 10:1:1 (*v/v/v*) ratio, then it was kept in the dark for 30 min. In the experiment, 0.1 mL of solvent extracts containing 20-100 µg/mL and ascorbic acid were mixed with 2.9 mL of FRAP reagent individually and then incubated for 30 min in the dark at a water bath (37 °C)[17]. The absorbance of the reaction mixture was measured at 593 nm using a UV-visible spectrophotometer. Ascorbic acid equivalents (AAE) in g of sample dry weight (DW) were used to determine FRAP activity.

2.5. Bioassay guided fractionation of methanolic crude extract

We used silica-packed column chromatography to fractionate the active components in the methanolic crude extracts. Before the sample was loaded, 30 g of silica (60×120 mesh size) were packed

on a 40 mm×330 mm column and preconditioned for 3 h with analytical grade *n*-hexane. Subsequently, 20 g of the methanolic crude extracts were loaded onto the activated silica and achieved the elution of different fractions using a gradient mobile phase of analytical grade solvents such as 80%:20%, 60%:40%, 40%:60%, and 20%:80% of methanol: *n*-hexane, methanol: ethyl acetate, and methanol: aqueous combinations. Moreover, 100% methanol, 100% aqueous, 100% ethyl acetate, and 100% hexane combinations were used. The fractions were chromatographed on silica plates for thin layer chromatography (TLC) using the model 60F254 (Merck). The mobile phase was a 1:2 mix of *n*-hexane and ethyl acetate (*v/v*). The plates were air-dried, 30% iodine-sprayed, and oven-dried for UV light detection (254-365 nm). The rotor-evaporated fractions were combined with similar retention factor (*R_f*) values to test their ability to lower blood glucose in streptozotocin (STZ)-induced rats. This procedure was guided by TLC monitoring and evaluation.

2.6. *In vivo* antihyperglycemic activity

2.6.1. Animals

Animals were purchased from Sri Venkateswara Enterprises, Subramanya Nagar, Malleswaram, Bangalore, Karnataka, India. Six healthy male Wistar albino rats were used in each group for this study. The rats were kept in polypropylene cages at 25-30 °C with a cycle of 12 hours of light and 12 hours of darkness. The rats were fed a commercial meal and given unrestricted access to water.

2.6.2. Induction of diabetes

A freshly prepared single dose of STZ (45 mg/kg/bw) was intraperitoneally injected into rats for induction of diabetes. A glucose (15%) solution was given to rats for the next 24 h after the 8-hour STZ administration to prevent hypoglycemia or coma. After 48 h, an Accu-Check (Roche Diagnostics Co., USA) glucometer was used to estimate the fasting blood glucose levels (FBGL) in STZ-induced rats through the punctured tail vein. In this examination, FBGL ≥ 270 mg/dL was considered diabetic. Following seven days, when observed, the balanced state of diabetes was marked as hyperglycemia (FBGL ≥ 250 mg/dL), which was chosen for this study. The study design and treatment for the acute antihyperglycemic activity of isolated pooled fractions are summarized in Supplementary Figure 1. Using this method, the percentage of glycemic change was calculated.

$$\% \text{ of glycemic change} = (G_x - G_i) / G_i \times 100$$

Where G_x=glycemia at 'x' time; G_i=glycemia at initial time.

2.6.3. Grouping of animals

The study consisted of five groups of rats, each containing 6 animals, and treatments were administered daily for a duration of 28 d as outlined below:

Group I (NC): Normal control rats were given standard feed and water without any additional treatment for 28 d;

Group II (DC): Diabetic control rats were induced with diabetes and received no treatment other than standard feed and water for 28 d;

Group III (D + GLB): Diabetic rats were treated with glibenclamide at a dose of 20 mg/kg body weight (b.w.) per day orally for 28 d;

Group IV (D + FD-40): Diabetic rats were treated with FD (40 mg/kg b.w./day) orally for 28 d;

Group V (N + FD-40): Normal rats were treated with FD (40 mg/kg b.w./day) orally for 28 d.

2.7. Measurement of FBGL and body weight

FBGL was measured by using a glucometer (Accu-check) from the collection of blood samples commencing with a 12-15 h fasted rat tail vein on 0, 7, 14, 21, and 28 days (Supplementary Figure 2). During the treatment period, animal body weights were measured on 0, 7, 14, 21, and 28 days with an electronic weighing balance (Supplementary Figure 2).

2.8. Blood collection and plasma preparation

The animals were fasted for 12 to 15 h on the 29th day after the experiment concluded. Blood was collected in EDTA-coated tubes *via* the retroorbital plexus. After centrifuging the entire blood for 15 min at 3 500 rpm at ambient temperature (20-25 °C), the plasma was collected in dry tubes and stored at -40 °C for later use.

2.9. Preparation of tissue homogenate

Rats were sacrificed and the heart tissue was removed immediately and stored in 10 mmol/L of ice-cold phosphate-buffered solution (pH 7.4). Then 25% tissue homogenate was prepared using an ice-cold phosphate-buffered solution. The cellular debris was washed away *via* 10-minute centrifugation at 4 °C, and the supernatant was collected and kept at -80 °C until biochemical estimation.

2.10. Assessment of antihyperglycemic activity of FD-40 from *C. viscosa* methanolic extracts

2.10.1. Plasma insulin resistance, insulin sensitivity, and β -cell function

The serum insulin levels of rats treated with FD-40 for 28 d were measured using an ELISA kit (Boehringer Mannheim Diagnostics, Germany). Insulin resistance was assessed using a homeostasis model. Insulin resistance (HOMA-IR), β -cell function (HOMA- β), and the quantitative insulin sensitivity check index (QUICKI) were calculated using the following equations:

$$\text{HOMA-IR} = \text{fasting plasma insulin levels (FPIL, } \mu\text{U/L)} \times \text{FBGL (mg/dL)} / 405;$$

$$\text{HOMA-}\beta = 360 \times \text{FPIL (}\mu\text{U/L)} / \text{FBGL (mg/dL)} - 63;$$

$$\text{QUICKI} = 1 / [\log (\text{FPIL, } \mu\text{U/L)} + \log (\text{FBGL, mg/dL})].$$

2.10.2. Determination of glycosylated hemoglobin (HbA1c) levels

HbA1c levels were measured according to a standard method[18]. Hemoglobin (Hb) absorption was measured at 10 mg/dL with normal saline. Then, 2 mL of hemolytic solution containing 10 mg/dL of Hb was withdrawn and mixed with 1.0 mL of 0.3 mol/L oxalic acid. The mixture was kept in a water bath for 1 h and thus solidified at room temperature. Then 1 mL trichloro acetic acid 40% was included. The material was mixed and centrifuged at 3 000 rpm for 10 min, and 2 mL of the supernatant was collected and mixed with 0.5 mL of 0.7% thiobarbituric acid. The total test solution was kept at 37 °C for 40 min and the absorbance was measured at 443 nm against the blank.

2.11. Assessment of cardioprotective activity of FD-40

2.11.1. Determination of serum creatine kinase-MB (CK-MB) activity

CK-MB activity was analyzed using a commercial kit with the Beckman Coulter AU Analyzer. The Beckman Coulter AU Analyzer was calibrated, and Reagent 1 (Buffer) was loaded into the reagent compartment. For each reaction, 180 μL of Reagent 1 was mixed with 45 μL of Reagent 2 and 10-15 μL of the sample was added to each reaction well. The analyzer automatically mixed the reagents and the sample, and CK-MB catalyzed reactions that generated NADPH. The NADPH production was measured by recording the absorbance at 340 nm, and CK-MB activity was calculated in U/L based on the rate of NADPH formation.

2.11.2. Determination of serum lactate dehydrogenase (LDH) activity

The test was conducted based on a kinetic reaction method[19]. In each well of a 96-well plate, 200 μL of the LDH assay buffer, 50 μL of the 6.6 mM NADH solution, 10-20 μL of the test sample, and 50 μL of the 30 mM sodium pyruvate were added to initiate the reaction. A blank reaction was prepared by adding distilled water instead of the test sample. Immediately after adding sodium pyruvate, the reaction was gently mixed by pipetting. Absorbance was measured at 340 nm every minute for 3-5 min at 37 °C. Finally, the rate of change in absorbance ($\Delta\text{A/min}$) during the linear phase of the reaction was determined to calculate LDH activity.

2.12. Estimation of FD-40 enzymatic and non-enzymatic antioxidant activity

2.12.1. Enzymatic antioxidants

2.12.1.1. Superoxide dismutase (SOD) activity

The Misra and Fridovich method[20] was used to determine the SOD activity. For this assay, 14 μL of MTT solution, 8 μL of NAD solution, 8 μL of phenazine methosulfate solution, and 170 μL of substrate buffer were mixed by adding each component at a time.

The change in absorbance (optical density) was measured at 480 nm for 3 min. The results were expressed as the change in optical density per minute per milligram of protein ($\Delta OD/\text{min}/\text{mg}$ protein).

2.12.1.2. Catalase activity

According to Sinha[21], catalase was calculated calorimetrically at 620 nm and expressed as μmol of H_2O_2 consumed/min/mg protein. Briefly, 50 μL of supernatant was added to the reaction mixture consisting of 2 mL of phosphate buffer (pH 7.4) and 1 mL of H_2O_2 solution (30 mM) in a cuvette. After incubation (30 min, 37°C), catalase activity was measured. The process was halted by mixing 5% potassium dichromate with glacial acetic acid in 2 mL of a 1:3 ratio dichromate-acetic acid reagent.

2.12.1.3. Glutathione-S-transferase (GST) activity

GST activity was determined using the method of Habig *et al.*[22]. A conical flask was filled with 0.1 mL of 1-chloro-2, 4-dinitrobenzene solution, 1 mL of phosphate buffer, and 1.7 mL of distilled water. After 5 min of incubation at 37°C , the reaction mixture was then mixed with 0.1 mL of tissue homogenate and 0.1 mL of glutathione-reduced solution. Absorbance was measured at 340 nm for 5 min at 60 s intervals.

2.12.2. Non-enzymatic antioxidants

2.12.2.1. Vitamin C (ascorbic acid)

The method of Jacques-Silva *et al.*[23], was used to measure the levels of vitamin C (ascorbic acid). In brief, 10% homogenate was prepared with 5% ice-cold TCA and centrifuged for 20 min at 3500 *g*. Subsequently, 1 mL of the supernatant was mixed with 0.2 mL of dinitrophenyl hydrazine reagent and incubated for 3 h at 37°C . Then 1.5 mL of ice-cold 65% sulfuric acid was added and mixed well, and the solutions were allowed to stand at room temperature for an additional 30 min. Absorbance was determined at 520 nm. The ascorbic acid (vitamin C) concentration was shown as $\mu\text{g}/\text{mg}$ of protein.

2.12.2.2. Glutathione (GSH)

The method of Carlberg and Mannervik[24] was used to measure GSH levels. The assay solution included bovine serum albumin (10%), phosphate buffer (50 mM, pH 7.6), NADPH (2 mM), and 20 mM oxidized glutathione. At 25°C , absorbance was measured at 340 nm.

2.13. Oxidative stress assessment

2.13.1. Estimation of lipid peroxidation

Lipid peroxidation was assessed in the heart tissue using the methods of Niehaus and Samuelsson[25] with some modifications. Sera were treated with trichloroacetic acid to precipitate proteins, vortexed for 30 s, and centrifuged at 3000 rpm for 10 min to obtain a clear supernatant. Then 0.1 mL supernatant was added with Tris-HCl Buffer (pH 7.5), and TBA-TCA-HCL (1:1:1 ratio) reagent,

placed in a water bath for 15 min, cooled, and centrifuged again at room temperature for 10 min. In order to determine the amount of malondialdehyde (MDA) generated, a clear supernatant solution was collected, and the absorbance was measured at 535 nm against the blank. The following equation was used to compute MDA levels.

$$\text{MDA} = A/E \times L$$

Where A = absorbance; E = extinction coefficient (1.56×10^5 mol/L/cm); L = length of the light path (1 cm).

2.13.2. XOD assay

The XOD activity was measured using the previously established method[26]. Heart tissue was homogenized in 0.01 M Tris-HCl (pH 8.0) with 1 mM EDTA. The homogenate was centrifuged and the supernatant was collected and then kept at -80°C . The enzyme assay mixture consisted of 2 mL of supernatant and potassium phosphate buffer (50 mM, pH 7.5) with 0.3 mM of EDTA. In addition, 50 M of xanthine was added as a substrate. The absorption velocity at 292 nm wavelength indicates that uric acid was formed at 10-minute intervals at varying temperatures. The percentage of XOD inhibitory activity was calculated using the following formula.

$$\text{XOD inhibition (\%)} = (\text{Abs control} - \text{Abs sample}) / \text{Abs control} \times 100$$

Where Abs control = Absorbance of the control reaction (without test sample); Abs sample = Absorbance of the test sample.

2.14. Histopathological study

On day 29 following the experiment, the rats underwent an overnight fast. Cervical dislocation was used under general anesthesia to euthanize the rats in each group. The heart was isolated, cleaned with saline, fixed in a 10% neutral formalin buffered solution for 48 h, embedded in paraffin, and then cut into 0.30 mm–0.50 mm slices for histopathological analysis using hematoxylin and eosin staining. Sections were examined under a light microscope at $10\times$ magnification.

2.15. Gas column-mass spectrophotometry (GC-MS) analysis of bioactive FD

In this experiment, a 5 mg sample was pulverized, transferred to a sterile, clean test tube, and added with methanol. The sample solution was filtered using a 0.2 μm nylon membrane, then the filtered sample was injected into the column for GC-MS analysis. The study was conducted using a Clarus 600 GC model from Perkin-Elmer Technologies, Inc., Wilmington, DE, which we linked to a mass spectrometer on a Perkin-Elmer workstation. An Elite-5MS capillary tube was used (30 m $\times$ 0.25 mm, with a film thickness of 0.25 μm) in the following conditions. The device features a ramp program that raises the temperature from $6^\circ\text{C}/\text{min}$ to 310°C for 3 min, followed by another 3 min of isothermal holding, with a starting oven temperature of 55°C . Helium (He) was used as the carrier gas, and the flow rate split ratios were 10:1. The samples were injected in 2 L

quantities and the injector's temperature was maintained at 250 °C.

2.16. *In silico* study

2.16.1. Molecular docking

The docking study shows how the bioactive compounds from *C. viscosa* interact with some markers from the heart and blood in this study. It may also give us more information to back up the results of the *in vitro* experiments. The phytochemicals from *C. viscosa* FD-40 were used as ligands, and enzymes like SOD, LDH, CK-MB, and XOD were used as targets. The ligand Protein Data Bank (PDB) files were generated using the online smile translator tool (<https://cactus.nci.nih.gov/translate/>), and the MMFF94x force field was applied for pre-optimization. The crystal structures of target proteins like SOD (5YTO), LDH (4QO8), CK-MB (1I0E), and XOD (1N5X) from the PDB (<http://www.rcsb.org/>) were retrieved. All co-crystallized ligands and other heteroatoms were removed before initiating the docking simulation, retaining only the required locations. Docking experiments were performed using two docking servers: PatchDock and FireDock[27]. PatchDock (<https://bioinfo3d.cs.tau.ac.il/PatchDock/>) conducted the initial screening. The PatchDock server receives all the protein and ligand candidates uploaded in pdb format. We selected a clustering RMSD of 4.0 for analysis, and we set the complex type to the default parameters. The PatchDock server submitted the best-docked conformations for refinement to the FireDock server. The FireDock method used global energy (kcal/mol) as the primary criteria to select the best-docked solutions. PyMOL has conducted the visual analysis of protein-ligand complexes.

2.16.2. Physico-chemical and ADMET study

The *C. viscosa* bioactive compounds were further studied for their ADMET and physicochemical properties (log P, MW, TPSA, rotatable bonds, hydrogen bond donors, and acceptors) as per the Rule of Five. For the ADMET of compounds, a preADMET server (<http://preadmet.bmdrc.org/>) was used.

2.17. Statistical analysis

All experimental data were expressed as mean±standard error (SE) using SPSS version 16.0 and one-way ANOVA analysis, and Tukey's *post-hoc* and Duncan's multiple range tests were used to analyze the results. The dose-response curves were created using GraphPad Prism (Version 5.1), plotting the percentage of scavenging activity versus concentrations and the IC₅₀ values obtained from a linear regression analysis.

2.18. Ethical statement

This animal study was approved by the Institutional Animal Ethics Committee (1889/GO/Re/S/16/CPCSEA SKU/ZOO/02/2018) of Sri Krishnadevaraya University, Andhra Pradesh, India.

3. Results

3.1. Total phenolic and flavonoid contents in different crude extracts obtained from *C. viscosa*

The total phenolic contents of methanolic extract (MeCV), aqueous extract (AqCV), ethyl acetate extract (EaCV), and *n*-hexane extract of *C. viscosa* (NhCV) were (86.20±2.31), (81.90±1.98), (22.29±0.52), and (17.78±0.63) mg GAE/g, respectively. Moreover, total flavonoid contents of these crude extracts were (67.39±0.81), (65.90±0.25), (13.30±0.15), and (11.48±0.04) mg QRE/g, respectively. MeCV exhibited the highest levels compared to the other crude extracts. Overall, the total phenolic content was higher than the total flavonoid content in all crude extracts.

3.2. Effect of different crude extracts from *C. viscosa* on ABTS, DPPH, H₂O₂ radical scavenging, and FRAP

The ABTS, DPPH, and H₂O₂ radical scavenging activities of four different solvent crude extracts are illustrated in Figure 1, with their IC₅₀ values provided in Table 1. MeCV demonstrated more potent radical scavenging activities against DPPH, ABTS, and H₂O₂ in a concentration-dependent manner compared to the AqCV, EaCV, and NhCV.

The four distinct solvent crude extracts all exhibited significant FRAP activity. The highest FRAP activity was observed for MeCV (156.71±3.53) µmol AAE/g DW among the four extracts, followed by AqCV (156.05±3.41) µmol AAE/g DW, EaCV (109.11±0.20) µmol AAE/g DW, and NhCV (81.25±2.53) µmol AAE/g DW. The FRAP activity of NhCV was significantly lower ($P < 0.05$) compared to the other extracts. Based on the total phenolic and flavonoid contents, *in vitro* antioxidant and antidiabetic activities[11], MeCV was identified as the bioactive extract among the four studied crude extracts. Therefore, MeCV was selected for further isolation and fractionation.

Table 1. IC₅₀ values of different extracts of *Cleome viscosa* (*C. viscosa*) for ABTS, DPPH, and H₂O₂ radical scavenging activities (µg/mL).

Plant extract	ABTS radical scavenging activity	DPPH radical scavenging activity	H ₂ O ₂ scavenging activity
MeCV	31.39±0.58 ^a	82.45±1.08 ^a	57.82±0.94 ^a
AqCV	37.83±0.58 ^b	84.01±1.21 ^a	62.54±0.74 ^b
EaCV	49.17±0.75 ^c	119.31±1.53 ^b	87.12±1.45 ^c
NhCV	55.71±0.88 ^d	124.47±1.78 ^b	92.05±1.06 ^d
ASA	28.87±0.15 ^a	-	31.27±0.65 ^e
GAA	-	34.47±0.81 ^c	-

The data are expressed as mean ± standard error (SE) and analyzed by one-way ANOVA followed by Tukey's *post hoc* analysis. Different letters indicate a significant difference at $P < 0.05$. ASA: ascorbic acid; GAA: gallic acid; MeCV: methanolic extract of *C. viscosa*; AqCV: aqueous extract of *C. viscosa*; EaCV: ethyl acetate extract of *C. viscosa*; NhCV: *n*-hexane extract of *C. viscosa*.

3.3. Fractionation of bioactive MeCV

Column chromatography analysis was utilized to obtain a total of 16 purified fractions during the separation and purification of MeCV. All collected fractions were concentrated at 40 °C using rotary evaporation. The retention factor (R_f) values of each of the 16 fractions were shown in a TLC chromatogram. The comparable fractions were divided into four major categories (FA, FB, FC, and FD) based on their R_f values[11]. Using STZ-induced diabetic rats, the FA, FB, FC, and FD fractions were tested for *in vivo* antihyperglycemic efficacy.

3.4. Effect of a single oral administration of isolated fractions of MeCV on blood glucose levels in STZ-induced diabetic rats

The study used four isolated pooled fractions, each with two doses (FA, FB, FC, and FD fractions at 20 mg/kg b.w. and 40 mg/kg b.w.). Supplementary Figure 3 demonstrates the effect of oral administration of these fractions at doses of 20 mg/kg b.w. and 40 mg/kg b.w. on diabetic rats. Glibenclamide (20 mg/kg b.w.) was used as a positive

control for this study. A significant ($P<0.05$) decline in FBGL was observed in fractions-treated rats in a dose-dependent manner from 1 h to 7 h. At 5 h, all fractions showed a gradual decline in FBGL at both doses. When compared to other fractions at all time points, 40 mg/kg FD (FD-40) showed the lowest FBGL.

3.5. Effect of 4-week administration of FD-40 on FBGL and body weight in STZ-induced diabetic rats

As shown in Figure 2A, STZ significantly increased FBGL throughout the experiment ($P<0.05$). On the 28th day, FD-40 treatment significantly decreased FBGL ($P<0.05$). No difference in FBGL levels was observed between the FD-40 treated control and the normal control.

After 28 days, the body weight of STZ-induced diabetic rats was considerably ($P<0.05$) lower than that of normal control rats (Figure 2B). In addition, treatment with FD-40 improved body weight when compared to diabetic control rats ($P<0.05$). Furthermore, no changes were observed in body weights of FD-40-treated normal rats when compared with normal control rats.

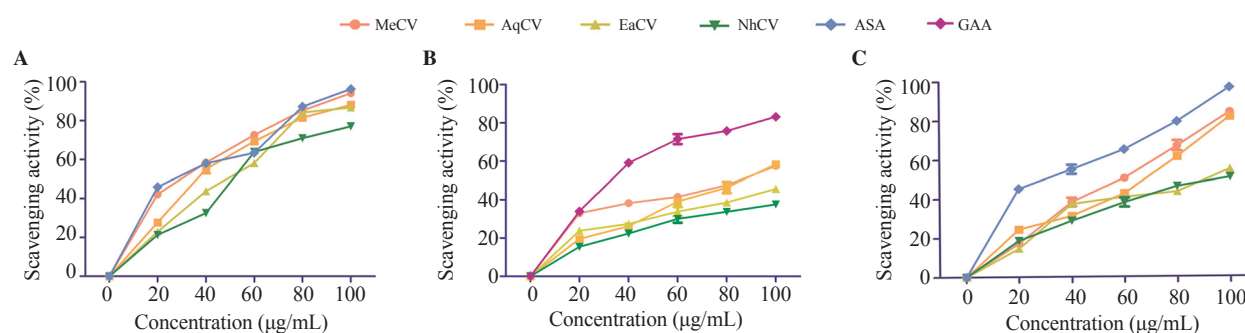


Figure 1. Free radical scavenging activity of different crude extracts obtained from *Cleome viscosa* (*C. viscosa*) L. (A) ABTS radical scavenging activity; (B) DPPH radical scavenging activity; (C) H_2O_2 scavenging activity. ASA: ascorbic acid; GAA: gallic acid; MeCV: methanolic extract of *C. viscosa*; AqCV: aqueous extract of *C. viscosa*; EaCV: ethyl acetate extract of *C. viscosa*; NhCV: *n*-hexane extract of *C. viscosa*.

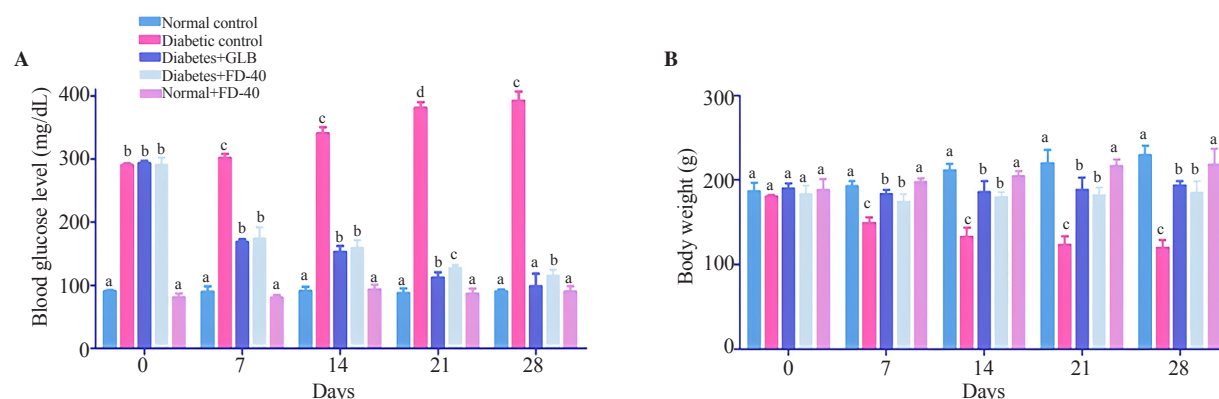


Figure 2. Effect of FD-40 on fasting blood glucose level and body weight of streptozotocin-induced diabetic rats. The results are shown as mean $\pm$ SE ($n = 6$). Different letters indicate a significant difference at $P<0.05$ by one-way ANOVA followed by Duncan's multiple range test. FD-40: 40 mg/kg b.w. of FD fraction from *Cleome viscosa* methanolic crude extract; GLB: glibenclamide.

3.6. Effect of administration of FD-40 on serum markers in diabetic rats

3.6.1. Effect of administration of FD-40 on insulin level, β -cell function, insulin resistance, and QUICKI

As demonstrated in Figure 3A, STZ administration significantly lowered blood insulin levels ($P<0.05$). FD-40 markedly raised insulin level of diabetic rats ($P<0.05$). No changes were found in normal rats treated with FD-40.

Moreover, there was a significant increase in HOMA-IR in STZ-induced diabetic rats as compared to normal rats ($P<0.05$). Treatment with FD-40 effectively reduced insulin resistance compared to diabetic rats ($P<0.05$) (Figure 3B). The levels of HOMA- β and QUICKI were also significantly decreased in diabetic rats compared to normal rats ($P<0.05$) (Figure 3C-D). Treatment with FD-40 significantly increased HOMA- β and QUICKI as compared to the diabetic control ($P<0.05$). Further, FD-40 treatment in normal rats did not affect basal levels of HOMA-IR, HOMA- β , and QUICKI.

3.6.2. Effect of administration of FD-40 on HbA1c level in diabetic rats

In Figure 3G, the blood HbA1c level was notably elevated in STZ-induced diabetic rats ($P<0.05$). In contrast, administration of FD-40 significantly decreased blood HbA1c levels ($P<0.05$). FD-40 treatment showed no significant change in HbA1c level of normal rats.

3.7. Effect of administration of FD-40 on cardiac markers in STZ-induced diabetic rats

Figure 3E-F shows that the levels of CK-MB and LDH were markedly increased in STZ-induced diabetic rats ($P<0.05$).

Treatment with FD-40 significantly lowered the levels of CK-MB and LDH in diabetic rats ($P<0.05$). Furthermore, there were no significant changes in both markers after treatment with FD-40 in normal rats.

3.8. Effect of administration of FD-40 on enzymatic and non-enzymatic antioxidants in STZ-induced diabetic rats

3.8.1. Effect of administration of FD-40 on enzymatic antioxidants

Diabetic rats showed significantly lower activities of SOD, catalase, and GST compared with normal rats ($P<0.05$). Treatment with FD-40 for 28 d significantly increased SOD, catalase, and GST activities in diabetic rats ($P<0.05$). In addition, there was no significant difference in the antioxidant levels of cardiac tissue between normal control rats and normal rats treated with FD-40 (Table 2).

3.8.2. Effect of administration of FD-40 on non-enzymatic antioxidants

As compared to normal rats, the amount of ascorbic acid and GSH was decreased significantly ($P<0.05$) in diabetic control rats. The administration of FD-40 restored STZ-induced diminished levels of ascorbic acid and GSH ($P<0.05$) (Table 2). The levels of these antioxidants in the heart tissue of normal control rats and normal rats treated with FD-40 did not differ significantly.

3.9. Effect of administration of FD-40 on oxidative stress markers

A significant increase in lipid peroxidation and a decrease in XOD inhibition were found in the heart tissue of diabetic rats compared to normal rats ($P<0.05$). After four weeks of treatment with FD-

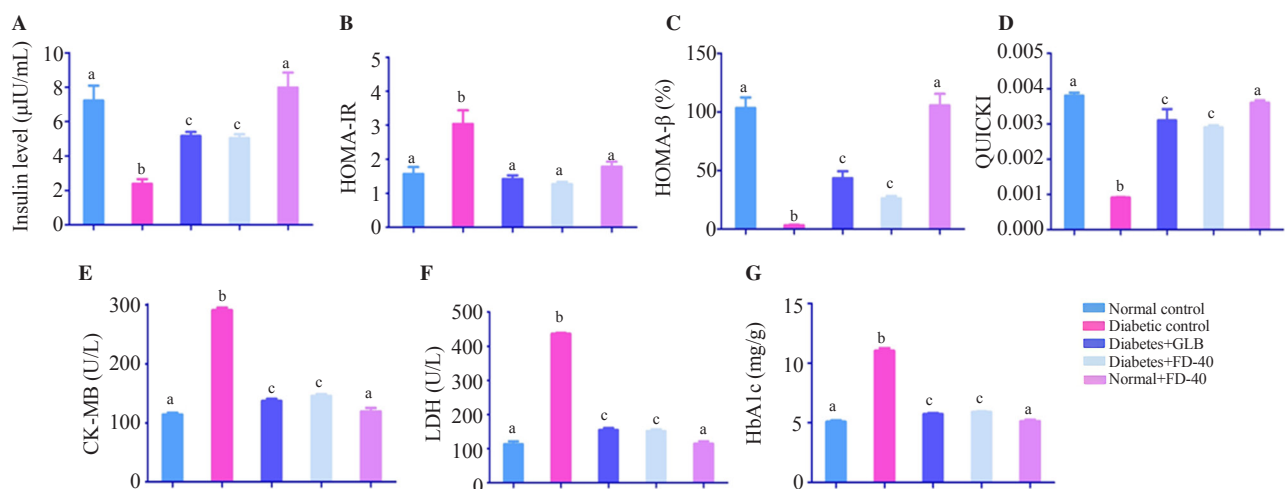


Figure 3. Effect of administration of FD-40 on insulin levels, HOMA-IR, HOMA- β , QUICKI, CK-MB, LDH and blood HbA1c in streptozotocin-induced diabetic rats. The results are shown as mean $\pm$ SE ($n = 6$). Different letters indicate a significant difference at $P<0.05$ by one-way ANOVA followed by Duncan's multiple range test. HOMA-IR: homeostatic model assessment for insulin resistance; HOMA- β : homeostasis model assessment of β -cell function; QUICKI: quantitative insulin sensitivity check index; CK-MB: creatine kinase-MB; LDH: lactate dehydrogenase.

40, MDA levels dropped significantly and XOD inhibition rose in diabetic rats ($P<0.05$) (Table 2).

3.10. Effect of administration of FD-40 on histological changes in STZ-induced diabetic rats

Cardiac tissue from normal rats exhibited normal structure (Figure 4A). STZ-induced diabetic rats showed cardiomyocyte vacuolization, vascular congestion, inflammatory cell infiltration, myocardial tissue necrosis with separation, and focal or diffuse fibrosis (Supplementary Table 1 and Figure 4B). Treatment with FD-40 improved STZ-induced changes in cardiac tissue, as evidenced by a reduction in leukocyte infiltration, myofibril fragmentation, and intracellular gaps inside the heart (Figure 4D). The cytoarchitecture of the myocardium in FD-40-treated normal rats closely resembled that of normal rats (Figure 4E).

3.11. Phytochemicals identified in FD fraction by GC–MS

The GC-MS spectrum confirmed the presence of various components with different retention times using the database of the National Institute of Standards and Technology (Supplementary Figures 4 and 5 and Table 3). Results reveal that seven components isolated from FD-40 of MeCV were identified. The confirmed compounds in the FD-40 fraction of MeCV were acetamide, N,N'-carbonylbis, 2-methyl-5-(1,2,2-trimethylcyclopentyl) phenol, 2-bromododecane, hexadecanoic acid methyl ester, 6-vinyl-7-methoxy-2,2-dimethyl chromene, hexose (5TMS), and 4-hydroxy-6-(4-methoxyphenyl)-2-pyranone.

3.12. Docking study

The docking study showed that FD-40 compounds had atomic interactions with active site residues of the target proteins. For all

Table 2. Effect of administration of FD-40 on enzymatic and non-enzymatic antioxidants and oxidative stress markers in diabetic rats.

Group	SOD (inhibit chromogen produced by 50%/ min/mg of protein)	Catalase (μmol of H_2O_2 decomposed/min/mg protein)	GST (μmol of CDNB conjugate formed/mg of protein)	GSH (μg /mg of tissue)	Ascorbic acid (μg /mg of protein)	MDA (μmol /L)	XOD inhibition (%)
Normal control	1.980 $\pm$ 0.005 ^a	9.54 $\pm$ 0.16 ^a	768.75 $\pm$ 40.26 ^a	17.76 $\pm$ 0.33 ^a	88.18 $\pm$ 7.24 ^a	0.610 $\pm$ 0.008 ^a	77.07 $\pm$ 0.42 ^a
Diabetes	0.520 $\pm$ 0.030 ^b	4.70 $\pm$ 0.08 ^b	206.25 $\pm$ 28.98 ^b	11.51 $\pm$ 0.21 ^b	39.39 $\pm$ 3.71 ^b	1.450 $\pm$ 0.030 ^b	23.32 $\pm$ 0.46 ^b
Diabetes+GLB	1.860 $\pm$ 0.004 ^c	7.22 $\pm$ 0.11 ^c	443.75 $\pm$ 53.50 ^c	14.72 $\pm$ 0.48 ^c	66.87 $\pm$ 3.22 ^c	0.870 $\pm$ 0.020 ^c	65.92 $\pm$ 0.52 ^c
Diabetes+FD-40	1.820 $\pm$ 0.003 ^c	6.70 $\pm$ 0.09 ^d	418.75 $\pm$ 48.00 ^c	13.60 $\pm$ 0.23 ^c	66.75 $\pm$ 2.28 ^c	0.870 $\pm$ 0.020 ^c	60.75 $\pm$ 0.35 ^d
Normal+FD-40	1.980 $\pm$ 0.002 ^a	8.83 $\pm$ 0.12 ^c	775.00 $\pm$ 45.71 ^a	17.78 $\pm$ 0.68 ^a	85.63 $\pm$ 4.18 ^a	0.620 $\pm$ 0.004 ^a	76.59 $\pm$ 0.45 ^a

The data are expressed as mean $\pm$ standard error (SE) and analyzed by one-way ANOVA followed by Duncan's multiple range test. Different letters indicate a significant difference at $P<0.05$. GLB: glibenclamide; FD-40: 40 mg/kg b.w. of FD fraction from *C. viscosa* methanolic crude extract.

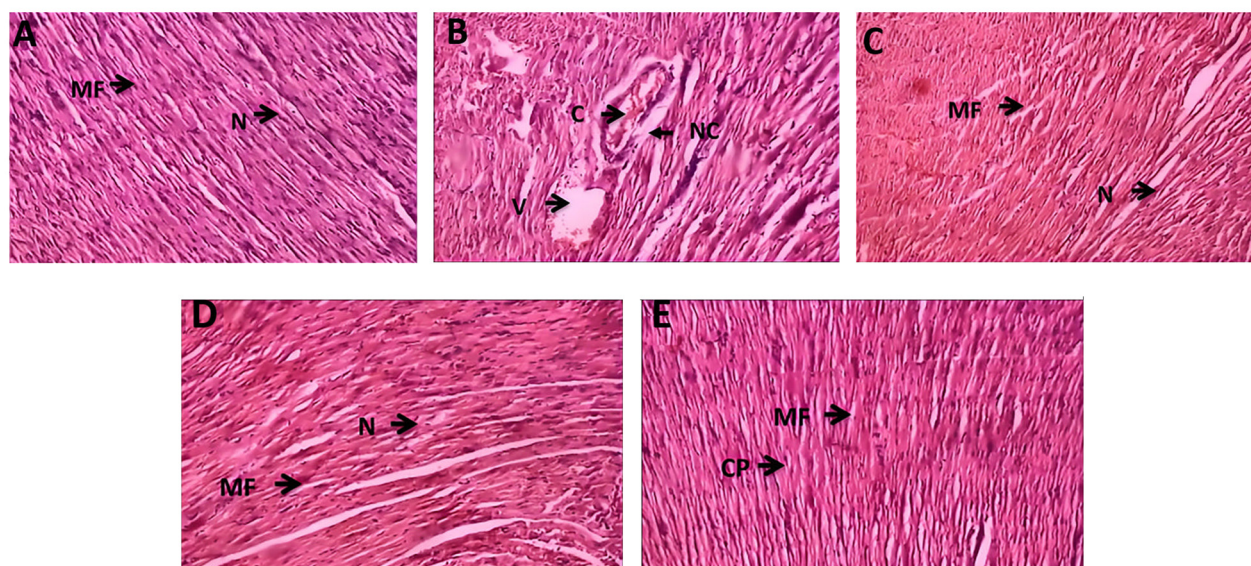


Figure 4. Effect of FD-40 on histological changes in cardiac tissue of diabetic rats (H&E staining; scale bar: 50 μm ; magnification: 10 $\times$). (A) The normal control group shows normal architecture of myocardial tissue. (B) The diabetic control group shows structural changes (degeneration of myocardial tissue, congestion, necrotic changes and formation of vacuolization). (C) The glibenclamide-treated diabetic group demonstrates normal myocardial tissue. (D) The FD-40 treated diabetic group shows significant improvement in cardiac architecture. (E) The FD-40 treated normal group shows normal architecture. C: congestion; CP: capillary; MF: myocardial fiber; N: nuclei; NC: necrotic change; V: vacuolization.

Table 3. Phytocomponents isolated and identified in the FD fraction of *C. viscosa* methanolic extract by GC-MS.

Peak no	Retention time (min)	Area%	Compound	Molecular formula	Molecular weight
1	3.05	65.98	Acetamide,N,N'-carbonylbis	C ₅ H ₈ N ₂ O ₃	144.12
2	8.51	22.18	2-Methyl-5-(1,2,2-tri methyl cyclopentyl) phenol	C ₁₅ H ₂₂ O	218.00
3	15.16	36.89	2-Bromododecane	C ₁₂ H ₂₅ BR	249.23
4	18.47	47.19	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.00
5	21.39	48.08	6-Vinyl-7-methoxy-2,2-dimethyl chromene	C ₁₄ H ₁₆ O ₂	216.28
6	27.79	98.04	Hexose (5TMS)	C ₂₁ H ₅₂ O ₆ Si ₅	540.00
7	29.08	92.12	4-Hydroxyl-6-(4-methoxyl phenyl)-2-pyranone	C ₁₂ H ₁₀ O ₄	218.00

Table 4. Binding energy of *C. viscosa* bioactive compounds docked against CK-MB, LDH, SOD, and XOD.

Compound	CK-MB		LDH		SOD		XOD	
	Docking	Docking energy	Docking	Docking energy	Docking	Docking energy	Docking	Docking energy
	score	(kcal/mol)	score	(kcal/mol)	score	(kcal/mol)	score	(kcal/mol)
Acetamide,N,N'-carbonylbis	2506	-22.88	2268	-20.71	2096	-23.62	2878	-26.90
2-Methyl-5-(1,2,2-tri methyl cyclopentyl) phenol	3712	-40.24 ^y	3414	-29.75	2894	-28.30	4250	-38.32
2-Bromododecane	3302	-28.25	3478	-26.88	3402	-28.64	4492	-32.93
Hexadecanoic acid, methyl ester	4144	-36.55	4592	-29.12	4700	-36.84 ^y	5122	-42.59
6-Vinyl-7-methoxy-2,2-dimethyl chromene	3450	-32.30	3360	-29.86	2908	-29.36	4110	-40.30
Hexose (5TMS)	5352	-37.36	5928	-47.24 ^y	4274	-29.33	6438	-50.90 ^y
4-Hydroxyl-6-(4-methoxyl phenyl)-2-pyranone	3170	-38.71	3108	-33.07	2810	-28.68	3720	-38.22

^yBest docking energy. CK-MB: creatine kinase-MB; LDH: lactate dehydrogenase; SOD: superoxide dismutase; XOD: xanthine oxidase.

targets, the interactive residues within 4Å of each ligand have been selected. The docking energy (kcal/mol) and hydrogen bonding interactions indicated that the compound has a high binding affinity, which depicts the global energy (kcal/mol) of FD-40 compounds (Supplementary Figure 6 and Table 4). The best docking energies of SOD, LDH, CK-MB, and XOD were determined to be -36.84 kcal/mol (hexadecanoic acid, methyl ester), -47.24 kcal/mol [hexose (5TMS)], -40.24 kcal/mol [2-methyl-5-(1,2,2-tri methyl cyclopentyl) phenol], and -50.90 kcal/mol [hexose (5TMS)], respectively. Among all enzymes, XOD exhibited the highest docking energy with hexose (5TMS) indicating that hexose (5TMS) is a potential inhibitor of XOD. The residues of FD-40 that interact with target enzymes are depicted in Supplementary Table 2.

The physico-chemical properties of bioactive compounds of *C. viscosa* L. are summarized in Supplementary Table 3. According to the data, all phytocompounds of FD-40 satisfied Lipinski's rule. However, 2-bromododecane, hexadecanoic acid, methyl ester, and hexose (5TMS) had log P values greater than 5, according to the rule of thumb. Using the Pre-ADMET server, the ADMET analyses of seven phytocompounds were predicted. All of these phytochemicals exhibited satisfactory ADMET outcomes (Supplementary Table 4). Except for acetamide,N,N'-carbonylbis, all compounds demonstrated excellent intestinal absorption and modest Caco-2 cell permeability. In addition, the results showed that compounds 2-methyl-5-(1,2,2-tri methyl cyclopentyl) phenol, 2-bromododecane, hexadecanoic acid, methyl ester, 6-vinyl-7-methoxy-2,2-dimethyl chromene, and hexose (5TMS) strongly bind to plasma protein, while acetamide,N,N'-carbonylbis and 4-hydroxyl-6-(4-methoxyl

phenyl)-2-pyranone weakly bind. Except for acetamide,N,N'-carbonylbis, all compounds exhibited moderate permeability for *in vitro* MDCK cells. Acetamide,N,N'-carbonylbis and 4-hydroxyl-6-(4-methoxyl phenyl)-2-pyranone showed limited blood-brain barrier penetration *in vivo*, while the remaining compounds demonstrated good absorption into the central nervous system.

4. Discussion

The development of antidiabetic therapies without side effects remains a formidable challenge for scientists. Researchers are continuously investigating medicinal plants to develop a relatively safe and effective antidiabetic treatment, either alone or in combination with other medications. Numerous plant species, including the green fruit of *Ficus racemosa*, *Gynura procumbens*[28], lemon balm of *Melissa officinalis*, *Mentha*, and piperita have been investigated for their antidiabetic properties, but no effective herbal treatment or drug has been approved. In animal models, the antidiabetic and cardioprotective potential of *C. viscosa* has not yet been investigated. This study examined the antidiabetic effects of *C. viscosa* extracts and bioassay-guided fractions with a focus on cardiac and antioxidant markers. For the selection of the best active extract from *C. viscosa*, we have screened MeCV, AqCV, EaCV, and NhCV extracts using standard *in vitro* antioxidant assays including ABTS, DPPH, H₂O₂, and FRAP.

Among the tested crude extracts, MeCV showed high ABTS, DPPH, FRAP, and H₂O₂ scavenging action. The presence of free

radical-scavenging phytochemicals in MeCV is one potential explanation for the high antioxidant effect of the substance. Based on the above results, we selected MeCV for further studies, including bioassay-guided fractionation and GC-MS characterization of compounds in fractions. STZ is used to induce diabetes in rats because it has a higher β -cell selectivity and lower toxicity than other diabetogens. STZ-induced diabetes resulted in increased serum glucose levels and HbA1c, and a significant drop in serum insulin. The hyperglycemia may have contributed to the abnormal insulin action and secretion, which in turn led to the lowered insulin secretion. Hyperglycemia in diabetes is associated with glucose intolerance, hyperinsulinemia, and insulin resistance[29].

This study examined the dose-dependent antihyperglycemic effect of MeCV-isolated pooled fractions in STZ-induced diabetes. The fractions at doses of 20 and 40 mg/kg b.w. decreased FBGL in diabetic rats. Until the end of the seventh hour, the examined fractions demonstrated a drop in FBGL. A significant decline in FBGL was observed at the 5th hour following a dose of 40 mg/kg b.w. of the FD fraction. Kifle and Enyew[30] reported similar findings regarding *Bersama abyssinica* in STZ-induced diabetic rats.

In STZ-induced diabetic rats, islet loss, β -cell degeneration, and elevated blood glucose levels were seen. Administering FD-40 to diabetic rats reversed these changes. Similar results were found by Gilani *et al.*[31], who reported a substantial decrease in glucose levels in diabetic rats treated with 50 and 100 mg/kg b.w. of the chloroform fraction of *Prosopis cineraria* stem. According to the findings of Moharir *et al.*[32], STZ-induced diabetic rats treated with plant-based vitamin D demonstrated better blood insulin levels. Studies on medicinal plant extracts have demonstrated that they can stimulate the body's β -cells, which can generate insulin. The anti-hyperglycemic effects may be exerted *via* stimulating the insulin signaling system in β -cells or by rebuilding injured β -cells through various mechanisms[33]. Furthermore, body weight of rats gradually decreased after STZ administration. Insulin insufficiency induces protein and fat catabolism, which could result in weight reduction[34].

Diabetes mellitus can be divided into two categories based on insulin resistance and pancreatic beta-cell dysfunction. Important indicators for detecting insulin resistance and evaluating β -cell function are HOMA-IR and HOMA- β . These two important parameters were improved by treatment with FD-40, as shown by increased insulin sensitivity and production. Given that polyphenols have been demonstrated to support the regeneration of pancreatic β -cells and improve insulin production in diabetic rats, it would be plausible to attribute this action to the high polyphenol content of FD-40 of MeCV. The findings of earlier publications from our laboratory are consistent with those of this investigation[11]. STZ encourages the selective destruction of pancreatic β -cells; more free radicals are produced as a result of the oxidation of extra glucose, non-enzymatic protein glycation, and oxidative degradation of glycated proteins, which raises HbA1c levels[18]. Other researchers

have reported similar results in rats with STZ-induced diabetes. Our study found that administering FD-40 to diabetic rats dramatically lowered HbA1c levels. This may be because FD-40 has a high concentration of phytochemicals and has antioxidant properties[11].

In this study, the cardioprotective effect of isolated FD-40 was also evaluated in STZ-induced diabetic rats. Cardio-specific proteins are often present in serum at low concentrations. When the layers are damaged, enzymes such as CK-MB and LDH are released into blood and their levels rise, providing information on the extent of myocardial damage caused by lipid peroxidation[35]. Elevated levels of LDH and CK-MB served as indicators of inadequate blood flow, cerebrovascular stroke, heart attack, tissue injury, and severe dystrophy. Following administration of STZ, the serum concentrations of LDH and CK-MB increased. Treatment with FD-40 substantially decreased serum LDH and CK-MB levels. This potential activity of FD-40 may be attributed to the active compounds present in the fraction. Polyphenols are believed to have an inverse association with cardiovascular disease[36]. However, further studies are needed to confirm it.

Oxidative stress can actively harm macromolecules, including lipids, proteins, and nucleic acids in diabetes. According to previous studies, high blood glucose levels increase the generation of reactive species as a result of oxidative stress, which lowers the levels of enzymatic antioxidants including SOD, catalase, and GST[37]. GSH, a non-enzymatic antioxidant, guards against oxidative cell damage. Furthermore, higher blood glucose and ROS levels are associated with increased MDA activation. By causing auto-oxidation and non-enzymatic glycation of proteins, glucose can enhance the production of ROS. Oxidative stress may result from the fructosamines' oxidative breakdown in hyperglycemia[38]. In this study, diabetic rats demonstrated elevated serum MDA levels along with lower SOD, catalase, and GST activities. FD-40 treatment could reverse these alternations and enhance antioxidant levels, thus decreasing oxidative stress.

The non-enzymatic antioxidant system is known to include ascorbic acid and GSH. Vitamin supplementation affects heart tissue differently. In the heart tissue, FD-40 raised levels of GSH and ascorbic acid. Ascorbic acid and GSH levels were lower in diabetic individuals, which may be a sign of oxidative stress[39]. The levels of vitamin C and GSH are closely correlated with the metabolism of GSH, which takes part in the processes catalyzed by glutathione peroxidase and reductase[40]. This supports the finding that ascorbic acid and GSH activities increased, as well as the overall antioxidant enhancement in this investigation.

Lipid peroxidation is a sign of oxidative cell death brought on by ROS. The extraordinary sensitivity of diabetics to oxidative stress was taken into account. Nitric oxide, a free radical oxidant generated by STZ in diabetic mice, causes an increase in blood lipid peroxide levels due to cell oxidation. FD-40 treatment reduced the generation of lipid peroxides in diabetic rats. These findings reveal that FD-

40 protects against STZ-caused cell damage by preventing lipid peroxidation, perhaps through the antioxidant system[41].

XOD is a major source of superoxide ($O_2^{\cdot-}$) in living organisms[42]. XOD catalyzes the conversion of hypoxanthine to xanthine and then to uric acid, generating both superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) as byproducts. The superoxide and hydrogen peroxide can be further converted into highly reactive hydroxyl radicals ($\bullet OH$), leading to oxidative stress. In the current examination, XOD was increased in cardiac tissue of diabetic rats. This outcome offers support for the recently revealed diabetes-induced cardiac stress. Treatment with FD-40 markedly reduced XOD levels. This may indicate a decrease in adenosine triphosphate degradation, leading to downregulation of purine metabolism, which reduces xanthine and hypoxanthine levels, both critical for XOD activity. Similar outcomes were observed in diabetic rats treated with bioassay-guided isolated fractions from the leaves of *Perilla frutescens*[42].

Based on the above results, further studies were done to identify active compounds of FD-40. The GC-MS analysis revealed seven peaks of various phytoconstituents. Acetamide, N,N'-carbonylbis, hexose (5TMS), and 4-hydroxyl-6-(4-methoxyl phenyl)-2-pyrone were present in very high concentrations in the isolated FD-40. It may be thought that the high concentration of main chemicals is a component of the plant's defense mechanism. Our GC-MS findings were compared to earlier research where acetamide, N,N'-carbonylbis was obtained from the methanol extract of *Clerodendrum viscosum* leaves and hexadecanoic acid, methyl ester is a well-known molecule having antioxidant, antibacterial[43], and anticancer action that was synthesized from diverse plant extracts. They are a part of a large class of protective compounds called "phytoanticipins" or "phytoprotectants" that have been discovered in this plant[44]. The FD-40 chemical components that have been found may be significant ecologically. Most of the chemical components found in FD-40 of MeCV have not yet been disclosed.

Furthermore, an *in silico* study was carried out to delve into how the phytocompounds found in FD-40 interact with target proteins. Hexose (5TMS) showed great affinity for LDH and XOD proteins; its strong interaction might be the reason for FD-40's protective function. The other compounds from FD-40 have shown moderate interaction with target proteins.

ADMET analysis showed that these compounds have a good absorption capacity based on their absorption and plasma binding properties, which might be helpful to the development of phytocompound-based antidiabetic drugs in the future. Although the study demonstrates a strong cardioprotective effect of *C. viscosa*, certain limitations should be considered. While molecular docking provided valuable insights into the interactions between the phytocompounds and cardiac markers, further experimental studies are needed to validate these findings and clarify the underlying

mechanisms. Additionally, the docking simulations were performed under ideal conditions, which may not fully reflect complex biological environments. Future research should focus on validating these interactions through *in vitro* and *in vivo* experiments to confirm the therapeutic potential of *C. viscosa*.

The histology of the myocardial tissue in FD-40-treated rats also demonstrated a significant improvement in cardiac tissue while STZ-induced diabetic rats showed severe necrosis and mild myocyte shrinkage. The same findings were observed in a recent antidiabetic trial[45].

In conclusion, the methanolic extract (FD-40) of *C. viscosa* demonstrated beneficial effects on STZ-induced diabetic rats, as supported by biochemical and histological evidence. The cardioprotective effects observed may be attributed to the presence of active phytochemicals, which potentially regulate insulin, HbA1c, CK-MB, LDH, and antioxidant levels. Molecular docking studies suggested that hexose (5TMS) showed notable binding interactions with XOD and LDH, while 2-methyl-5-(1,2,2-tri methyl cyclopentyl) phenol and hexadecanoic acid, methyl ester interacted with CK-MB and SOD, respectively. These results suggest that FD-40's cardioprotective action may involve synergistic effects of multiple phytocompounds. Further studies are required to validate these interactions and preclinical validation and clinical trials are necessary to explore its efficacy and safety in diabetes management.

Conflict of interest statement

All authors declared that they have no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

SY participated in conceptualizing the study and designing the study, conducted the research, optimized methods, and contributed to original draft, data curation, validation, and revising the manuscript. LT participated in conceptualizing the study and drafting and revising the manuscript. SC helped in conducting research methods, molecular docking study, entering data, and drafting and revising the manuscript. VRN participated in conceptualizing and supervising the study, revising the manuscript, editing and reviewing. RRS conceptualized and designed the study, drafted and revised the manuscript, analyzed data, and supervised the research work, review, and editing. All authors had perused and approved the final edition of the manuscript and consented to be responsible for all parts of the work in guaranteeing that questions related to the work are properly examined and resolved.

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Running title: Cardioprotective effect of bioassay-guided fractions of *Cleome viscosa*

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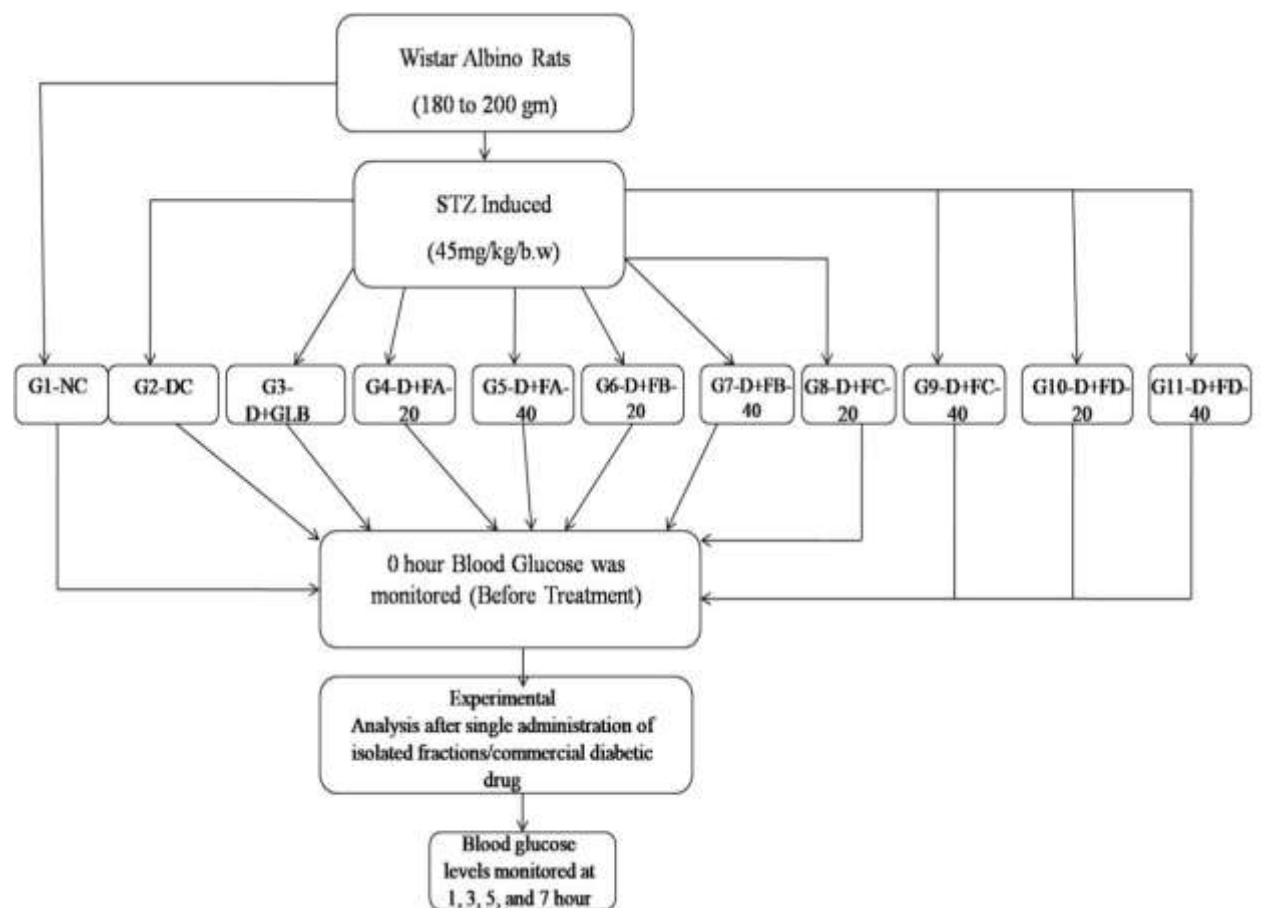
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Supplementary Figure 1. Schematic diagram of study design and treatment of acute antihyperglycemic activity of isolated fractions. G1-11: groups; DC: diabetic control;

D: diabetic; GLB: glibenclamide. FA: Fraction-A; FB: Fraction-B; FC: Fraction-C;
FD: Fraction-D.

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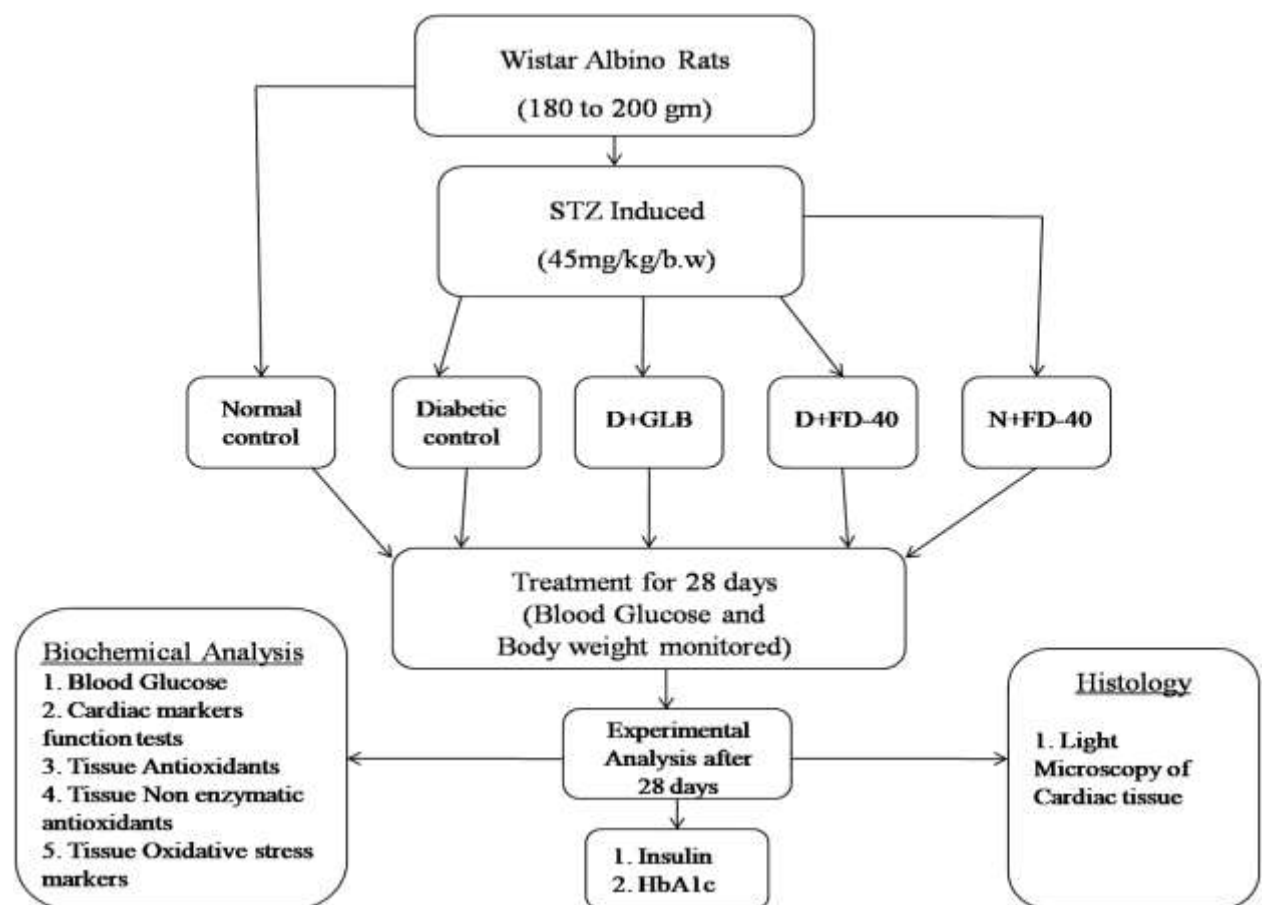
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Supplementary Figure 2. Schematic diagram of study design and treatment of antihyperglycemic and cardioprotective activity of FD-40 fraction.

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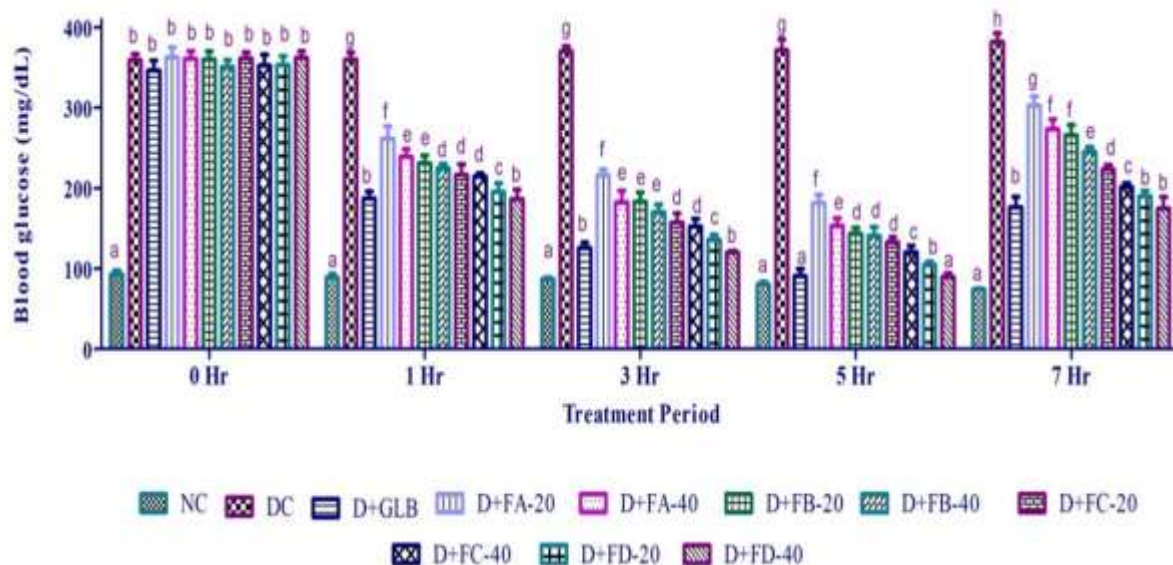
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Supplementary Figure 3. Acute antihyperglycemic activity of isolated pooled fractions of MeCV in streptozotocin-induced diabetic rats. The results are represented as mean $\pm$ SE (n = 6). Different alphabets indicate a significant difference at $P < 0.05$ by one-way ANOVA followed by Duncan's multiple range test.

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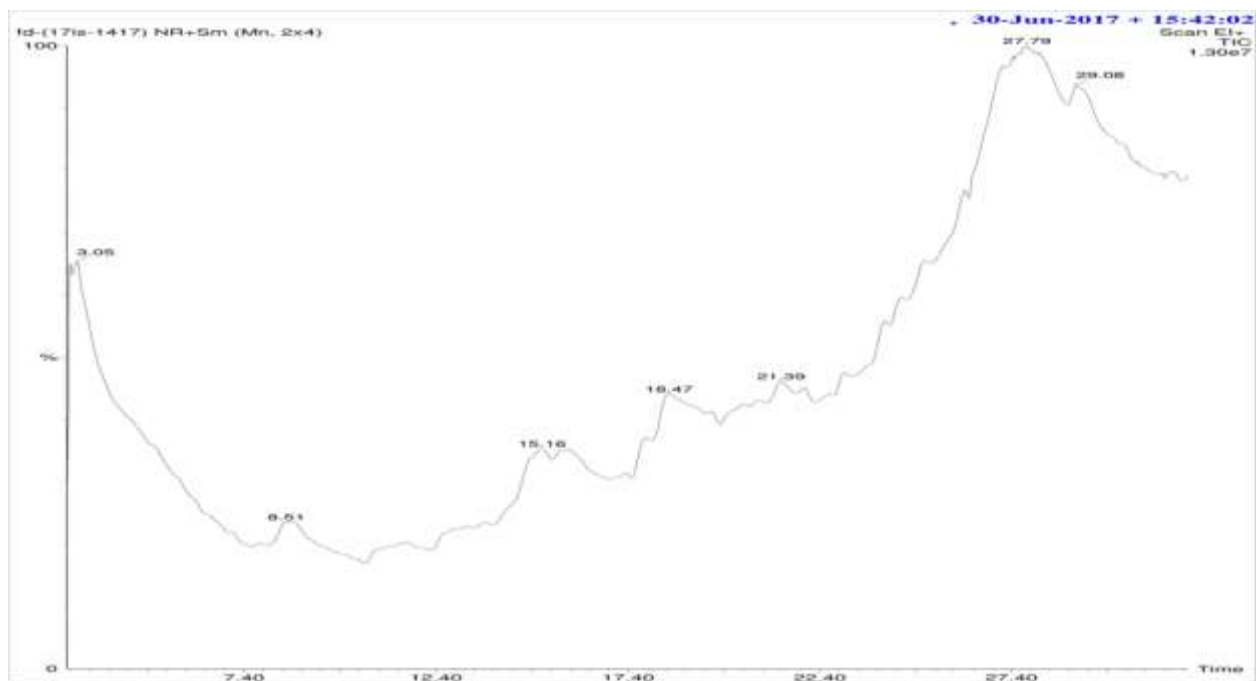
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Supplementary Figure 4. GC-MS chromatograph of bioactive FD-40 fraction isolated from *Cleome viscosa* methanolic extract.

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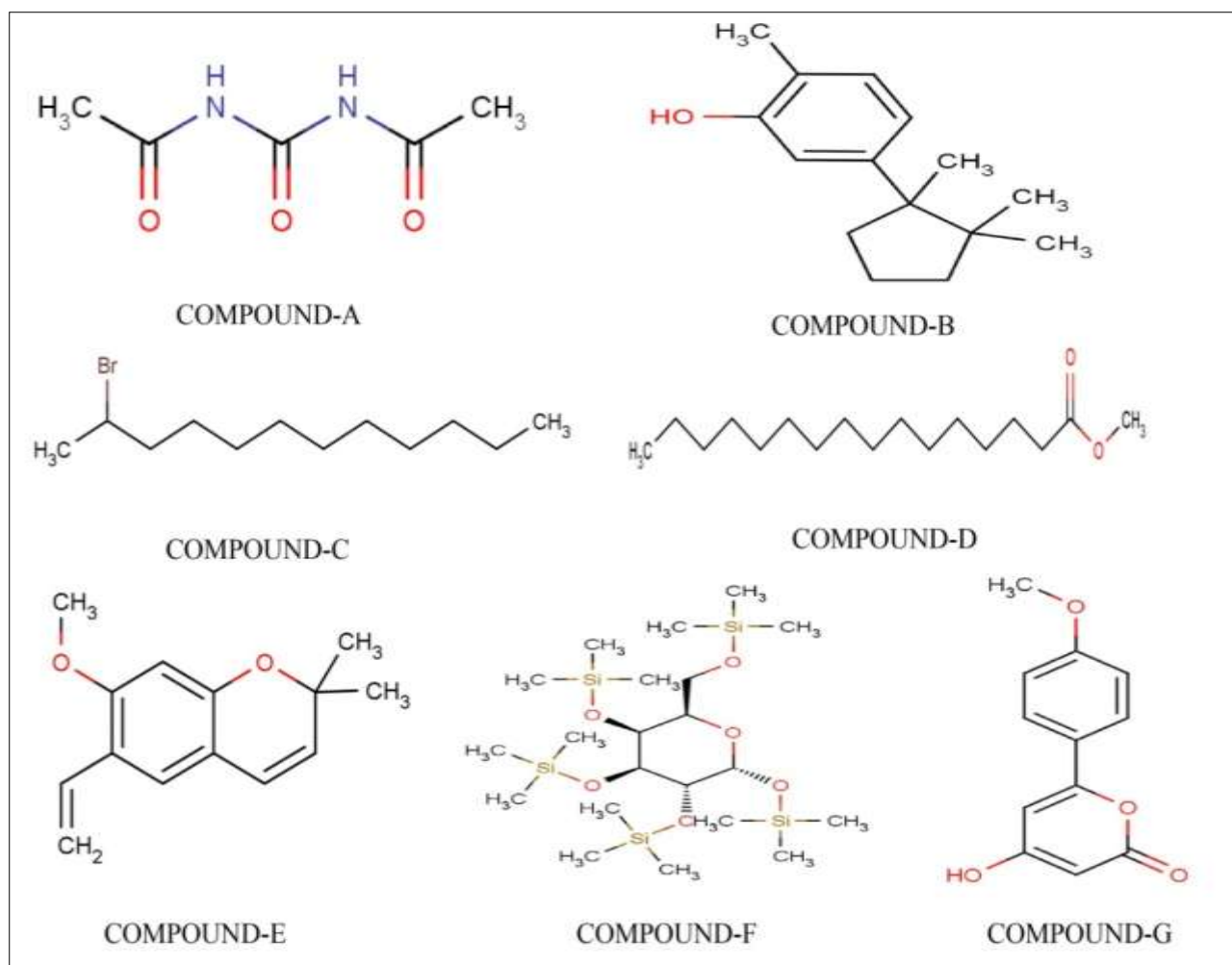
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Supplementary Figure 5. Structure of Compound A (acetamide,N,N'-carbonylbis), Compound B [2-methyl-5-(1,2,2-tri methyl cyclopentyl) phenol], Compound C (2-bromododecane), Compound D (hexadecanoic acid, methyl Ester), Compound E (6-vinyl-7-methoxy-2,2-dimethyl chromene), Compound F [hexose (5TMS)] and Compound G (4-hydroxyl-6-(4-methoxyl phenyl)-2-pyrone) isolated from FD-40 fraction of *Cleome viscosa* methanolic extract.

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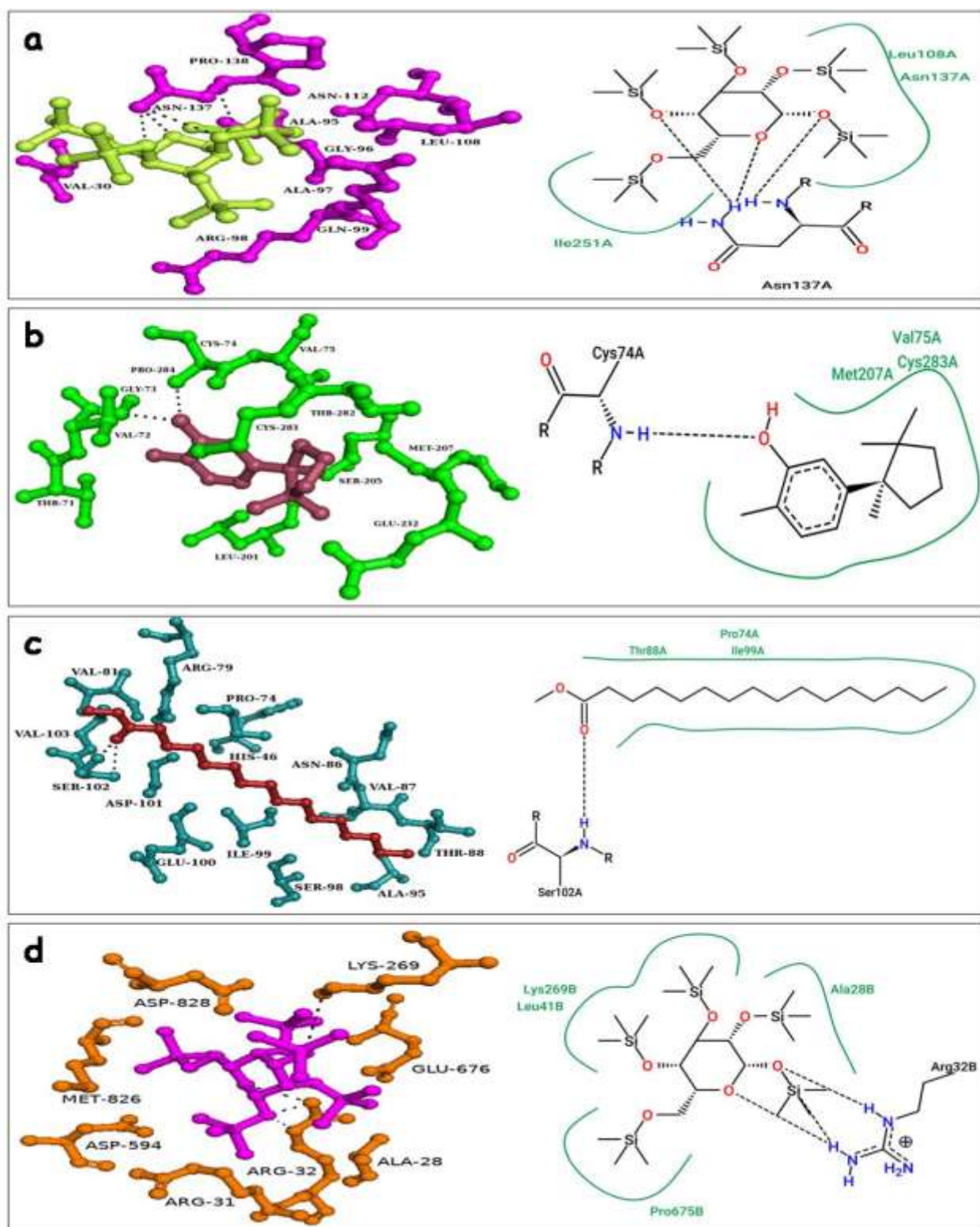
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Supplementary Figure 6. Interactive analysis of protein-ligand binding interfaces by Autodock (left) and Proteins Plus server (right) for CK-MB, LDH, SOD and XOD.

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Supplementary Table 1. Histological injury score of cardiac tissue of diabetic rats treated with FD-40.

Groups	Necrosis	Infiltration of inflammatory cells	Vessel congestion	Focal or diffuse fibrosis	Fibrotic area
NC	0	0	0	0	0
DC	3	1	2	2	2
D+GLB	0	0	0	0	0
D+FD	0	0	0	0	0
N+FD	0	0	0	0	0
Cardiac tissues are scored for histological injury via light microscopy with score 0= no visible cell damage; score-1= injure <30% of tissue; score-2= injure in 30%-50% of tissue; score-3= broad, lesions >50%; score-4= total necrosis.					

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Supplementary Table 2. Interactive residues of docked protein-ligand complexes.

Protein	Best docked compound	Interactive residues
CK-MB	2-Methyl-5-(1,2,2-trimethyl cyclopentyl) phenol	Thr 71, Val 72, Gly 73*, Cys 74*, Val 75, Glu 232, Thr 282, Cys 283, Pro 284
LDH	Hexose (5TMS)	Ala 95, Gly 96, Gln 99, Val 135, Ser 136, Asn137*, Ile 251
SOD	Hexadecanoic acid, methyl ester	Pro 74, Asn 86, Thr 88, Ile 99, Asp 101, Ser 102*, Val 103*
XOD	Hexose (5TMS)	Thr 25, Ala 28, Arg 32*, Cys 269, Asp 594, Pro675, Glu 676, Glu 679

*Amino acids participated in H-bond formation.

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Supplementary Table 3. Physicochemical properties of compounds.

Compound	log <i>P</i>	TPSA	<i>n</i> atoms	MW	<i>n</i> ON	<i>n</i> OHNH	<i>n</i> violations	<i>n</i> rotate	Volume
C1	-1.53	75.27	10	144.13	5	2	0	0	127.51
C2	4.96	20.23	16	218.34	1	1	0	1	230.88
C3	6.39	0.00	13	249.24	0	0	1	9	231.69
C4	7.37	26.30	19	270.46	2	0	1	15	308.95
C5	3.80	18.47	16	216.28	2	0	0	2	212.51
C6	9.49	55.40	32	541.07	6	0	2	11	552.80
C7	2.13	59.67	16	218.21	4	1	0	2	189.57

TPSA: topological polar surface area; MW: molecular weight; C1: acetamide,N,N'-carbonylbis; C2: 2-methyl-5-(1,2,2-tri methyl cyclopentyl) phenol, C3: 2-bromododecane; C4: hexadecanoic acid, methyl Ester, C5: 6-vinyl-7-methoxy-2,2-dimethyl chromene, C6: hexose (5TMS); C7: 4-hydroxyl-6-(4-methoxyl phenyl)-2-pyranone.

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Supplementary Table 4. ADMET predicted profile.

Compound	^a Human intestinal absorption (%)	^b <i>In vitro</i> Caco-2 cell permeability (nm/s)	^c <i>In vitro</i> MDCK cell permeability (nm/s)	^d <i>In vitro</i> plasma protein binding (%)	^e <i>In vivo</i> blood brain barrier penetration (C.brain/C.blood)	
C1		65.93	19.81	1.21	0.17	0.28
C2		100	40.09	127.71	100	10.33
C3		100	48.96	30.77	100	22.82
C4		100	45.83	64.61	100	13.94
C5		100	45.94	89.24	93.22	1.16
C6		98.36	50.79	68.01	100	13.10
C7		95.05	24.33	36.84	80.29	0.64

^aHuman intestinal absorption: 0-20% (poor absorption), 20-70% (moderate absorption), 70-100% (good absorption).

^b*In vitro* Caco-2 cell permeability (nm/s): The values are <4: low permeability, 4-70: middle permeability, >70: high permeability.

^c*In vitro* MDCK cell permeability (nm/s): The values are <25: low permeability, 25-500: middle permeability, >500: high permeability.

^d*In vitro* plasma protein binding (%): The values <90%: weak binding and >90%: strong binding.

^e*In vivo* blood-brain barrier penetration (C.brain/C.blood): The values <0.1: low absorption, 0.1-2.0: middle

absorption, >2.0: higher absorption.

C1: acetamide,N,N'-carbonylbis; C2: 2-methyl-5-(1,2,2-tri methyl cyclopentyl) phenol, C3: 2-bromododecane; C4- hexadecanoic acid, methyl ester,

C5: 6-vinyl-7-methoxy-2,2-dimethyl chromene, C6: hexose (5TMS); C7: 4-hydroxyl-6-(4-methoxyl phenyl)-2-pyranone.