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Phenolic enriched fraction of *Clerodendrum glandulosum* Lindl. leaf extract ameliorates hyperglycemia and oxidative stress in streptozotocin-nicotinamide induced diabetic rats

Puspanjali Khound [a,b,1], Prashanta Kumar Deb [a,c,1], Swarnali Bhattacharjee [a,b], Karla Damián Medina [d], Partha Pratim Sarma [a], Biswatrish Sarkar [c], Rajlakshmi Devi [a,b,*]

[a] *Life Sciences Division, Institute of Advanced Study in Science and Technology, Guwahati, Assam, 781035, India*
[b] *Department of Zoology, Gauhati University, Jalukbari, Guwahati, Assam, 781014, India*
[c] *Department of Pharmaceutical Sciences & Technology, Birla Institute of Technology, Mesra, Jharkhand, 835215, India*
[d] *Center for Research and Assistance in Technology and Design of the State of Jalisco, A.C. (CIATEJ), Food Technology Unit, 45019, Jalisco, Mexico*

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ABSTRACT

Background: Clerodendrum glandulosum Lindl. is an important ethnomedicinal shrub of Northeast India, used by traditional healers to control various ailments like diabetes, hypertension, arthritis, etc.
Objectives: The present study was conducted to explore the anti-hyperglycemic and antioxidative effects of the polyphenol-rich fraction (PRF) of *C. glandulosum* leaf extract and identification of its major bioactive compounds. Further, an *in-silico* molecular docking study was also performed to understand the molecular interactions of the identified major compounds with some target proteins associated with diabetic complications.
Materials and methods: PRF was purified from the hydromethanolic (80% MeOH) extract of leaves and subjected to assessment of *in-vitro* antioxidant and anti-diabetic properties. It was also subjected to evaluate the ameliorative effect during streptozotocin-nicotinamide-induced hyperglycemia in Wistar albino rats. An *in-silico* molecular docking study was also performed to complement the *in-vitro/in-vivo* studies.
Results: Chemical analysis of PRF showed the presence of phenolics like caffeic acid, verbascoside, isoverbascoside, and apigenin, of which verbascoside (598.14 ± 1.24 mg/g) was found to be the principal compound. *In-vitro* studies showed potent antioxidant (IC_{50} of DPPH:32.45 ± 2.16 μg/mL; ABTS:39.08 ± 0.53 μg/mL) properties and excellent aldose reductase inhibition potential (IC_{50} 2.18 ± 0.10 μg/mL). Treatment with PRF showed reduced blood glucose levels and increased plasma insulin levels. The results also indicate an improvement of endogenous antioxidants and suppression of inflammatory cytokines (IL-6 and TNF-α) comparable to the standard. Molecular docking studies predicted promising interactions between the identified molecules and the crucial amino acid residues of the enzymes involved in the development of hyperglycemia.
Conclusion: This study revealed the antihyperglycemic and antioxidant potential of partially purified fraction PRF of *C. glandulosum* leaves.

1. Introduction

Diabetes is a common metabolic disorder with an ever-increasing global footprint with high morbidity and mortality rates if left untreated. Currently, more than 450 million people worldwide are suffering from pre-diabetic to diabetic conditions, including hyperglycemia and associated clinical complications [1]. Hyperglycemia is characterized by high blood glucose, with or without insulin resistance, and impaired glucose homeostasis [2]. The destruction of pancreatic β-cells due to cellular oxidative stress and impaired endogenous antioxidant defense triggers the pathogenesis of hyperglycemia [2,3]. Management of high blood sugar levels and improvement of pancreatic β-cell count are the primary focus of most antidiabetic therapies. Currently, various strategies have been developed to suppress the

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* Corresponding author. Life Sciences Division, Institute of Advanced Study in Science & Technology, Guwahati, 781035, India.
E-mail address: rajiasst@gmail.com (R. Devi).
[1] Authors contributed equally.

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elevated blood glucose level in the circulation. However, the complexity of the disease mechanism and the malfunctioning of multiple vital organs complicate the procedure of choosing the right therapeutic strategy [4,5]. Although numerous classes of synthetic medications are available in the existing therapy, the major issues are unwanted side effects and toxicity, which may occur due to the off-target binding [6]. Thus, the search for newer, effective, and safe therapeutic strategies to overcome the complications of diabetes around the globe is constant. In this context, medicinal plants and natural products provide numerous opportunities to develop antidiabetic drug leads and newer therapeutic strategies [7]. Several active plant metabolites, like berberine, phlorizine, anthocyanins, phenolic acids, etc., have been investigated extensively as effective alternative treatments for diabetes with fewer or no side effects [7,8]. Similarly, dietary herbs enriched with phenolics and flavonoids have been experimentally proven for their antioxidant, anti-inflammatory, and protective properties during oxidative stress-triggered diseases [9]. Overall, a rising demand for the consumption of polyphenol-rich herbal health products and functional foods for the management of diabetes and metabolic syndrome owing to the versatile antioxidant properties of polyphenols has been noticed globally [10,11].

Clerodendrum glandulosum Lindl. (Synonym: *C. colebrookianum* Walp., Family: Lamiaceae) is an ethnopharmacologically important evergreen plant abundantly available in India's North-East region (NER) [12]. It is consumed as a traditional food/medicine to cure metabolic disorders, hyperglycemia, hyperlipidemia, and hypertension by the people of NER. Different parts of this plant are used to cure several other ailments, such as helminthic infections, diarrhea, indigestion, asthma, bronchitis, and fever [13]. According to literature reports, the leaf extract of *C. glandulosum* exhibited many potential pharmacological properties, including cardioprotective, antidiabetic, anti-hypertensive, antioxidant, etc., in various experimental models [13,14]. The literature revealed that other allied species of Genus *Clerodendrum* also exhibited potential antioxidant and anti-hyperglycemic properties in various experimental animal models of oxidative stress and metabolic disorders [15–19]. Some important phytochemicals like verbascoside, isoverbascoside, caffeic acid, colebroside A-E, oleanolic acid, and colebrin, have been identified so far from the leaf extract of *C. glandulosum* [13,20]. An earlier study by Deb et al. discussed verbascoside as the major phenolic compound present in the various parts of *C. glandulosum*. The same study also reported the high phenolic content and promising α-amylase and α-glucosidase inhibition properties of the crude extract of *C. glandulosum* leaves [13].

The antihyperglycemic potential and the phytochemicals in *C. glandulosum,* responsible for the acclaimed ethnomedicinal benefit, are unexplored. In the present study, the phenolic-rich fraction (PRF) of *C. glandulosum* leaves is being investigated for its anti-diabetic and anti-hyperglycemic potentials. Bioactive compounds present in the PRF were also identified, and the *in-silico* molecular interaction of the identified molecules with the crucial amino acid residues of the various target enzymes involved in the development of diabetes was analyzed to complement and correlate the hypoglycemic effects.

2. Materials and methods

2.1. Chemical and reagents used

All the chemicals were procured from Sigma Aldrich (USA). Kits for biochemical analysis were procured from Tulip Diagnostics (Tulip Group, Coral Clinical System, India). ELISA kits were purchased from Abchem (USA) for IL-6 and TNF-α. The serum insulin estimation ELISA kit was from Cayman (USA). HPLC-grade solvents and other reagents were purchased from Merck.

2.2. Plant material collection and preparation of PRF

Fresh leaves of *C. glandulosum* Lindl. were collected from Guwahati, India (GPS location: N 26° 8′36.5928" - E 91°40′34.8163″) and identified by Dr. Chaya Deori, Scientist, Botanical Survey of India (BSI), Shillong, India. A voucher specimen (BSI/ERC/Tech/2019/601) was submitted to BSI for future reference.

Approximately 500 g of powdered leaves (# 40 mesh size) were extracted with 2.5 L (1: 5 w/v) of water-methanol (80% methanol) by cold maceration at room temperature (25 ± 2 °C) for seven days. The procedure was repeated three times with fresh solvent each time. The crude extract was concentrated under reduced pressure at 37 ± 2 °C in a vacuum rotary evaporator (Buchi R210, Switzerland).

A portion (50 g) of hydromethanolic extract was dissolved in 500 mL of distilled water and sequentially fractioned with an equal volume of hexane (500 mL × 3), followed by chloroform (500 mL × 3). The residual brown aqueous phase was considered the PRF which was concentrated and dried in a freeze dryer.

2.3. Phytochemical analysis of PRF

2.3.1. Qualitative and quantitative analysis of phytochemicals

The presence of phenolic compounds was confirmed by proximate qualitative analysis following a well-established method [21]. Total phenolic content (TPC) was measured by using the Folin-ciocalteu method [22] and expressed as mg of gallic acid equivalent (GAE mg/g).

2.3.2. HPLC-PDA analysis of PRF

PRF's qualitative and quantitative chemical profiling was performed using the HPLC-PDA system (Waters 1525 pump, 2998 PDA detector, USA) with a C_{18} column (4.6 × 250 mm, 5 μm). Briefly, PRF was dissolved in a 1:1 ratio of methanol: H_2O and filtered through a 0.22 μm syringe filter (Merck, Millipore). The PRF solution (1 mg/mL) was injected using a Hamilton syringe (Waters, USA). The mobile phase [0.1% aqueous formic acid (solvent A) and methanol (solvent B)], gradient programme [0–10 min 25%–65% B; 10–15 min 65%–85% B; 15–20 min 85%–95% B]. Quantification of the major compounds was achieved from the calibration curve of analytical standards.

2.4. Assessment of in-vitro antioxidant potential of PRF

In-vitro antioxidant activity of PRF was estimated by using 1,-1-diphenyl-2-picrylhydrazyl (DPPH) assay following earlier reported method [23] and 2,2-amino-di (2-ethylbenzothiazoline sulfonic acid-6) ammonium salt (ABTS) radical scavenging assay as per previously established method [24].

2.5. Assessment of in-vitro antidiabetic activity of PRF

PRF was screened for *in-vitro* antidiabetic potential by performing an α-amylase inhibition assay as per the reported method [13,25] and an α-glucosidase enzyme inhibition assay was performed as per the established method [13,26]. The aldose reductase inhibition capacity was measured following the established method [27], and antiglycation potential was also studied according to the established method [28].

2.6. Assessment of in-vivo antidiabetic activity of PRF

2.6.1. Experimental animals

Wistar albino rats weighing 180–200 g were maintained in an air-conditioned environment (25 ± 2 °C, 55 ± 5% humidity) and a 12-hr light/dark cycle. The animals received purified water and a normal laboratory chow diet *ad libitum*. All the experiments were carried out during the light phase, and ethical clearance was approved by the Institutional Animal Ethics Committee (Protocol No. IASST/IAEC/2016/-17/01-B dated 23.01.17).

2.6.2. Acute toxicity study

A single-dose oral acute toxicity assessment was performed per the OECD 425 guidelines [29]. All the animals were fasted overnight, and a single dose (2000 mg/kg, p.o.) of PRF was administered to five female rats, and gross behavioral changes were observed after 4 h. The animals were further monitored for clinical signs and mortality for 14 days.

2.6.3. Induction of hyperglycemia

Animals 12 weeks of age weighing between 180 and 200 g were used in this study. After acclimatization for a week, experimental hyperglycemia was induced in the overnight-fasted male Wistar rats by a single dose intraperitoneal injection (i.p) of STZ (60 mg/kg) prepared in 0.01 M citrate buffer (pH 4.5). Before STZ administration, glucose solution was administered to all the animals. Immediately after STZ administration, all the animals were given 120 mg/kg nicotinamide (i.p.) to prevent drastic damage to β-cells. After 72 h of STZ administration, rats with blood glucose levels≥ 300 mg/dL were considered diabetic [30]. Blood was collected from the tail vein, and blood sugar level was estimated using a glucometer (AccuCheck, Roche Diabetes Care, Germany).

2.6.4. Experimental design

Animals were randomly divided into five experimental groups (n = 6), and the treatment interventions were as follows:

i. Normal control (NC): 1 mL/kg of vehicle (0.5% CMC in normal saline), p.o.
ii. Diabetic control (DC): STZ-NA + 1 mL/kg of vehicle, p.o.
iii. PRF_{100}: STZ-NA + PRF 100 mg/kg (p.o.) dissolved in vehicle
iv. PRF_{200}: STZ-NA + PRF 200 mg/kg (p.o.) was dissolved in vehicle
v. GLB: STZ-NA + glibenclamide 10 mg/kg (p.o.) dissolved in vehicle

All rats were fed a standard diet during the treatment period. The experimental dose of PRF was chosen as 1/20th and 1/10th of the dose used in the acute toxicity study. All treatments were administered orally as a suspension in 0.5% carboxymethylcellulose prepared in normal saline. Treatment with PRF and the standard antidiabetic compound glibenclamide (GLB) was continued for 14 days. Fasting blood glucose levels were monitored on the 0^{th}, 7th' and 15th day. The final body weight of all animals was noted on the 15th day. At the end of the treatment period, all the animals were sacrificed by Ketamine-xylazine (87:3). Blood was collected by heart puncture, and vital organs such as the liver, pancreas, and kidney were collected for histopathological examination. A schematic indicating the different groups used for the animal study and the study paradigm used to induce hyperglycemia is shown in Fig. 1.

2.6.5. Oral glucose tolerance test (OGTT)

The glucose-tolerating ability of animals in different treatment groups was examined by performing the oral glucose tolerance test (OGTT) on the 10th day. A single dose of glucose solution (2 g/kg) was administered orally to all the animals (male Wistar rats) after overnight fasting condition, and blood glucose concentration (mg/dL) was estimated at various time points (0, 30, 60, 90, and 120 min) after glucose loading.

2.6.6. Serum biochemical analysis

Blood was collected by cardiac puncture and centrifuged at 3000 rpm for 10 min to obtain serum. Serum insulin levels were estimated by ELISA assay using insulin (rat) ELISA kits (Cayman, USA). Liver biochemical markers (AST, ALT, and ALP) and lipid profiles (TG, TC, HDL-C, and LDL-C) were estimated according to the manufacturer's protocol using standard diagnostic kits.

2.6.7. Preparation of tissue homogenate and analysis of oxidative stress markers

Excised livers were immediately washed with ice-cold potassium phosphate buffer saline (PBS 0.01 M, pH 7.4). Next, 10% (w/v) tissue homogenate was prepared in ice-cold PBS (7.4), and the homogenate was centrifuged at 5000 rpm for 15 min at 4 °C to obtain a clear supernatant. The total protein in the supernatant was estimated using a standard protocol reported by Bradford [31]. Endogenous antioxidant status was monitored by estimation of glutathione (GSH) level as per Ellman's method [32], superoxide dismutase (SOD) level as per reported method [33], catalase level as per the method of Góth [34], and TBARS level was measured as per the established method [35].

Expression of inflammatory markers in the liver homogenate (IL-6 and TNF-α) was quantified using ELISA kits per the manufacturer's

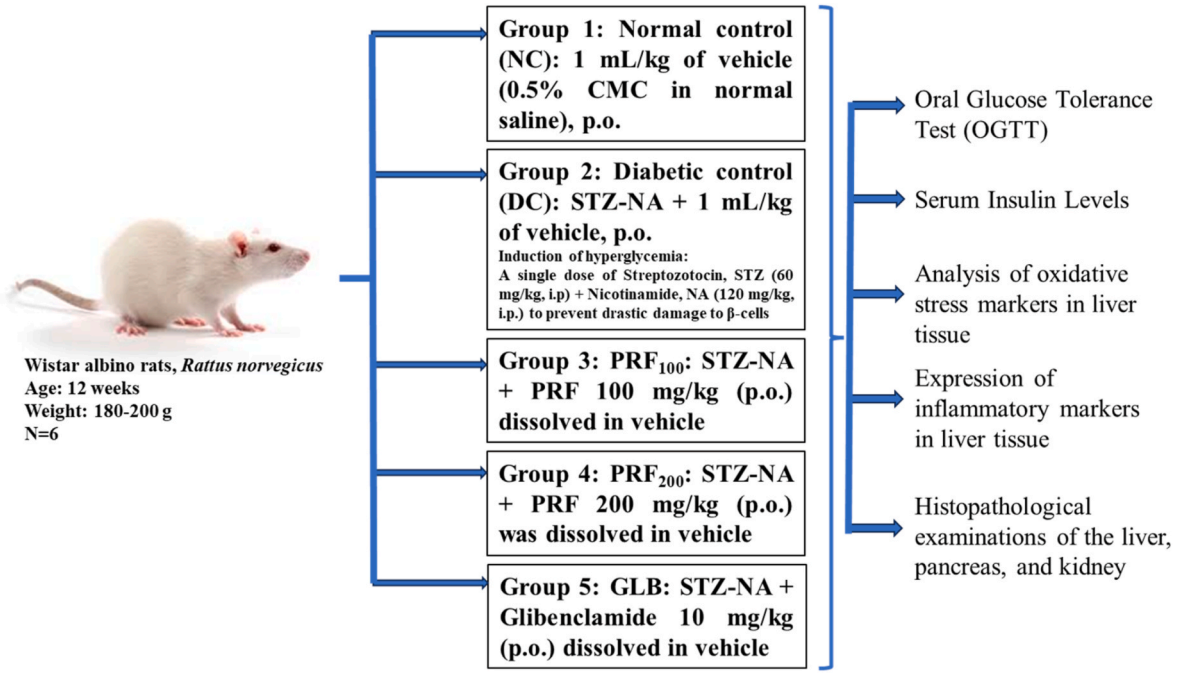


Fig. 1. A schematic indicating the different groups used for the animal study and the study paradigm used to induce hyperglycemia.

protocol (Abchem, USA).

2.6.8. Histopathological examinations

Histopathological examinations of the liver, pancreas, and kidney were performed. A part of the liver, kidney, and pancreas was gently washed with 0.9% normal saline solution to remove the blood and preserved in a 10% buffered formalin solution (prepared in PBS, pH 7.4) to fix the tissue. Then, the tissue sections were dehydrated in xylene solution, followed by paraffin-embedded sections (5 μm) prepared in an automatic tissue processor and further stained with hematoxylin-eosin (HE) solution to observe histopathological changes.

2.7. Molecular docking study of the major identified compounds

The probable relationship between the proteins involved in developing hyperglycemia with the major compounds (apigenin, caffeic acid, isoverbascoside, and verbascoside) identified by HPLC-PDA in the PRF was investigated using molecular docking experiments. Three proteins (1US0, 1HXO, and 2QMJ) were selected, and their 3D structures were downloaded from the Protein Data Bank (https://www.rcsb.org/). The 3D structures of the ligands were retrieved from the PubChem open database (https://pubchem.ncbi.nlm.nih.gov/). Acarbose was used as a standard inhibitor of α-amylase and α-glucosidase, whereas sorbinil was used as a standard inhibitor of aldose reductase enzyme. Docking calculations were carried out using AutoDock Vina [36] following the previously reported protocols [18,37,38]. Visualization and interaction analysis of protein-ligand complexes were performed using BIOVIA Discovery Studio 2017 (http://www.3dsbiovia.com/).

3. Statistical analysis

The results of the study were represented as mean ± SD. A one-way analysis of variance (ANOVA) was performed, followed by Tukey's and Dunnett's post hoc tests. GraphPad Prism Software Version 5.0 (San Diego, CA, USA) was used for statistical analysis.

4. Results

4.1. Chemical analysis of PRF of C. glandulosum Lindl

The percentage yield of crude extract was approximately 20.4% (w/w) of the powdered leaves used for the extraction. The final yield of the PRF was estimated at 67.7% (w/w) of the total crude extract. PRF was observed as a light brown powdered mass soluble in water and exerted a characteristic organic odor. Qualitative chemical analysis of PRF positively confirmed the presence of phenolic compounds in high abundance. Total phenolic content (TPC) in PRF was found to be 347.33 ± 4.19 mg GAE/g, which was significantly ($p < 0.05$) higher than the TPC present in the crude extract (301.59 ± 3.92 mg GAE/g).

Chemical profiling of PRF was performed using HPLC-PDA, revealing four major phenolic constituents (Fig. 2). The phenolic compounds found in PRF were identified as caffeic acid (RT: 7.88, Conc.: 36.20 ± 0.53 mg/g), verbascoside (RT: 8.82, Conc.: 598.14 ± 1.24 mg/g), isoverbascoside (RT: 10.77, Conc.: 214.12 ± 0.91 mg/g), and apigenin (RT: 15.57, Conc.: 22.49 ± 0.56 mg/g). Individual constituents were identified by comparing their retention time and UV spectrum (Fig. S1) with those reported in the literature. As per the chromatogram, peak 1 (RT: 7.88) exhibited UV shifts of 217.2, 240.8, and 323.9 nm (Figs. S1–A). Peak 2 (RT: 8.82) and peak 3 (RT: 10.77) exhibited UV shift at 331.1 nm (Figure S1-B and S1-C), supporting the assumed identity of the compounds as verbascoside and isoverbascoside, respectively. Peak 4 (RT: 15.57) was tentatively identified as apigenin, exhibiting UV shifts of 206.6, 266.8, and 338.3 (Figs. S1–D).

4.2. In-vitro antioxidant activity of PRF of C. glandulosum Lindl

The antioxidant potential of PRF was assessed based on the quenching capacity of PRF against stable free radicals such as DPPH and ABTS (Table 1). PRF was found to be capable of quenching free radicals with IC_{50} of 32.45 ± 2.16 μg/mL and 39.08 ± 0.53 μg/mL against DPPH and ABTS free radicals, respectively.

4.3. In-vitro antidiabetic activity of PRF of C. glandulosum Lindl

An array of *in-vitro* antidiabetic assays was performed to assess the anti-hyperglycemic and antidiabetic potential of PRF (Table 2). It was observed that PRF (IC_{50}: 371.98 ± 4.93 μg/mL) and its principal compound verbascoside (IC_{50}: 337.4 ± 6.58 μg/mL) exhibited a considerable α-amylase inhibition potential as compared to standard inhibitor acarbose (IC_{50}: 363.29 ± 2.68 μg/mL). No statistically significant ($p < 0.05$) difference between PRF and verbascoside was found. PRF (IC_{50}: 77.18 ± 3.08 μg/mL) alongside verbascoside (IC_{50}: 42.28 ± 0.17 μg/mL) also showed significantly ($p < 0.05$) better inhibition of

Table 1
In-vitro antioxidant capacity of phenolic rich fraction (PRF) and major compound of *C. glandulosum* Lindl. Leaves

Samples	IC_{50} (μg/mL)	
	DPPH assay	ABTS assay
PRF	32.45 ± 2.16[a]	39.08 ± 0.53[a]
Verbascoside	7.74 ± 0.06[b]	7.86 ± 0.14[b]
Ascorbic acid	12.85 ± 0.44[c]	12.03 ± 0.14[c]

Data are represented as the mean ± SD (n = 3) of three independent experiments.
Different superscripted letters in each column indicate significant ($p < 0.05$) differences when compared to each other by one-way ANOVA followed by Tukey's post hoc test.

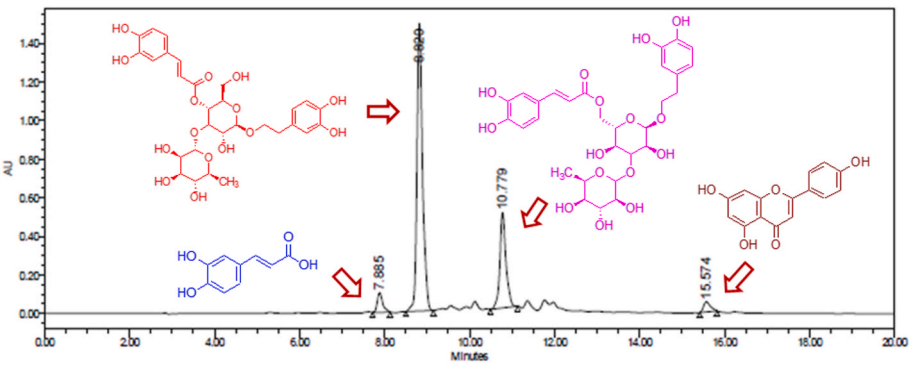


Fig. 2. Chemical analysis of phenolic-rich fraction of *C. glandulosum* Lindl. Chromatogram obtained at 331 nm (λ_{max}).
RT: 7.88: Caffeic acid, RT: 8.82: Verbascoside, RT: 10.779: Isoverbascoside, RT: 15.57: Apigenin.

Table 2
In-vitro antidiabetic properties of phenolic rich fraction (PRF) and major compound of *C. glandulosum* Lindl. Leaves

Samples	IC_{50} (μg/mL)			
	α-amylase	α-glucosidase	Aldose reductase	Antiglycation
PRF	371.98 ± 4.93[a]	77.18 ± 3.08[a]	2.18 ± 0.10[a]	71.79 ± 2.07[a]
Verbascoside	337.4 ± 6.58[c]	42.28 ± 0.17[b]	0.73 ± 0.07[b]	68.31 ± 3.14[a]
Acarbose	363.29 ± 2.68[ab]	122.07 ± 4.23[c]	–	–
Sorbinil			0.59 ± 0.04[b]	–
Aminoguanidine				53.04 ± 7.98[b]

Data are represented as the mean ± SD (n = 3) of three independent experiments.
Different superscripted letters in each column indicate significant ($p < 0.05$) differences when compared to each other by one-way ANOVA followed by Tukey's post hoc test.

α-glucosidase, in comparison to standard inhibitor acarbose (IC_{50}: 122.07 ± 4.23 μg/mL). Likewise, PRF and verbascoside also exhibited significant ($p < 0.05$) inhibition of aldose reductase enzyme with IC_{50} values of 2.18 ± 0.10 μg/mL and 0.73 ± 0.07 μg/mL, respectively. Similarly, PRF and verbascoside inhibited the formation of advanced glycation end products (AGEs), yielding IC_{50} values of 71.79 ± 2.07 μg/mL and 68.31 ± 3.14 μg/mL with no significant difference ($p < 0.05$) between each other. However, the standard antiglycation agent aminoguanidine showed slightly better activity than PRF and verbascoside, with an IC_{50} value of 53.04 ± 7.98 μg/mL.

4.4. *In-vivo antidiabetic potential of PRF*

4.4.1. *Acute toxicity and experimental dose selection*

An oral acute toxicity study with a single high dose of PRF (2000 mg/kg) revealed no mortality and noticeable abnormal behavioral changes in the female Wistar rats during and after 4 h of administration. It was also safe over the two-week observation period with no abnormal clinical signs. As we found that 2000 mg/kg is safe in all the animals, the lower dose (1/20th and 1/10th) of the acute toxicity study dose was chosen to evaluate the antihyperglycemic and antioxidative effects.

4.4.2. *Effect of PRF on oral glucose tolerance (OGTT)*

As illustrated in Fig. 3A, oral glucose administration (2 g/kg) substantially elevated blood glucose levels at 30 min in all experimental animals and peaked at 60 min. The DC group exhibited a noticeable rise in blood glucose levels during the observation period (120 min) compared to the NC group. Furthermore, it did not return to the initial level (0 min) even at the end of the study period (120 min). On the other hand, PRF_{100} and PRF_{200} showed a declining trend in blood glucose levels with increasing time. PRF_{200} exhibited a substantial decline in blood glucose levels, similar to that of GLB.

4.4.3. *Effect of PRF on body weight changes*

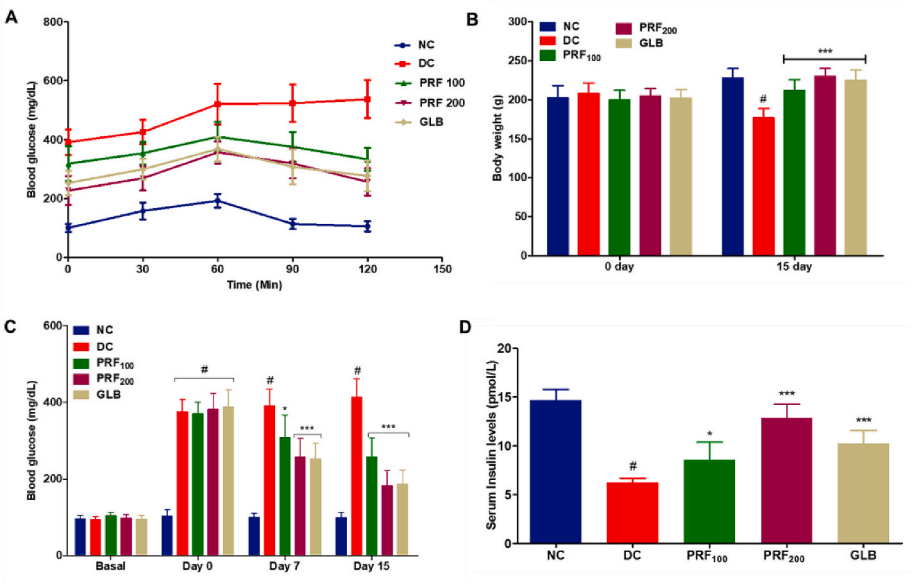
Changes in body weight during the STZ-NA-induced hyperglycemia and after the intervention period are given in Fig. 3B. The body weight of the DC group was found to increase significantly ($p < 0.05$) reduced after 14 days of i.p. injection of STZ compared to their initial body weight. Meanwhile,PRF_{100} and PRF_{200} showed slightly increased body weight, similar to the NC. The body weights of PRF_{100} and PRF_{200} showed significant improvement compared to the DC group, which was similar to GLB.

4.4.4. *Effect of PRF on fasting blood glucose level*

The effect of PRF administration on STZ-NA-induced hyperglycemic animals is shown in Fig. 3C. The results showed that i.p. injection of STZ (60 mg/kg) significantly ($p < 0.001$) induced hyperglycemia in all the animals within 72 h. All the animals' fasting blood glucose level was significantly ($p < 0.001$) higher than that of the NC. Diabetic animals (DC) treated with PRF_{100}, PRF_{200}, and GLB exhibited a time-and dose-dependent reduction in fasting blood glucose levels during the 14-day intervention period.

4.4.5. *Effect of PRF on serum insulin level*

As shown in Fig. 3D, STZ administration significantly ($p < 0.001$) lowered the serum insulin level in the DC compared to the NC. The animals treated with PRF_{100} and PRF_{200} as well as GLB for 14 days significantly ($p < 0.001$) improved serum insulin concentration compared to the DC, which was found to be similar to the NC. The animals treated with PRF_{100} and PRF_{200} showed higher serum insulin concentration, which was similar to GLB with no significant ($p < 0.001$)



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statistical difference.

4.4.6. Effect of PRF on liver enzymes

As shown in Table 3, the serum AST, ALT, and ALP levels of the DC were significantly ($p < 0.001$) higher than those of the NC. PRF_{100} and PRF_{200} treatment for 14 days significantly ($p < 0.001$) lowered elevated levels of AST, ALT, and ALP in the serum. The effect of PRF_{100} and PRF_{200} on the liver biochemical profile of STZ-induced diabetic (DC) animals was found to be significantly ($p < 0.001$) better than the standard drug glibenclamide (5 mg/kg).

4.4.7. Effect of PRF on serum lipid profile

The serum levels of total cholesterol, triacylglycerides, and LDL-C were significantly ($p < 0.001$) higher in the DC compared to the NC group. HDL level was significantly ($p < 0.001$) lower in the DC. Treatment with PRF_{100} and PRF_{200} for 14 days substantially lowers serum TC, TG, LDL, and elevated HDL levels (Table 4).

4.4.8. Effect of PRF on the oxidative stress and inflammatory cytokines

As illustrated in Table 5, elevated levels of lipid peroxidation and decreased levels of antioxidant enzymes (GSH, SOD, and catalase) were observed in the liver of DC when compared to the NC. On the contrary, PRF_{100} and PRF_{200} exhibited significant improvement in the GSH, SOD, and catalase activities compared to the DC. Lipid peroxidation levels were significantly reduced in the PRF_{100} and PRF_{200} as in GLB-treated groups compared to the DC. The effect of PRF treatment on the endogenous antioxidant status of diabetic animals was found to be better than that of the GLB.

Inflammatory markers (TNF-α and IL-6) associated with oxidative stress in the liver were found to be significantly ($p < 0.01$, $p < 0.001$) increased in the DC compared to the NC. On the contrary, PRF_{100} and PRF_{200} substantially down-regulated IL-6 in a dose-dependent manner. The TNF-α level was reduced after PRF treatment, equivalent to GLB. (Fig. 4).

4.4.9. Histopathological examination of the liver, pancreas, and kidney

Histological observation of the liver tissue sections indicated that NC contained many hepatic cells that were compactly arranged and uniformly disposed of throughout the sections. Sinusoids appear as light areas between hepatic cells. Dilated portal veins were also observed in the NC. Distortion of the normal architecture of hepatocytes with mild inflammation and sinusoidal dilatation was observed in the liver sections of the DC. Cellular degeneration, along with shrinkage of the portal vein and vascular congestion, were also visible. In contrast, restoration of near-normal hepatic architecture was evident in PRF_{100}, PRF_{200}, and GLB-treated diabetic animals (Fig. 5).

Tissue sections of the pancreas of DC exhibited substantial injury and necrotic changes in the acinar cells compared with the NC group. Histological examination of the pancreas of PRF_{100} and PRF_{200} showed noticeable improvement in the pancreatic acinar cells, as observed in GLB (Fig. 5).

Histopathological investigations of the kidney of NC revealed a normal glomerulus microenvironment with distinct renal corpuscles (RC) surrounding the proximal and distal convoluted tubules and Bowman's capsule. Kidney sections of the DC showed marked tubular damage and inflammation. In addition, the overall change in renal architecture was evident with shrinkage of glomerular capillaries and cloudy changes in tubular cells. Treatment with PRF_{100} and PRF_{200}, as well as GLB, substantially restored the renal architecture of the STZ-treated animals, which is identical to the tissue histopathology of NC (Fig. 5).

Table 3
Effects of phenolic rich fraction (PRF) of *C. glandulosum* Lindl. on liver profile during STZ-NA-induced hyperglycemia.

Groups	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
NC	39.96 ± 3.94	22.11 ± 4.36	79.75 ± 17.92
DC	149.38 ± 19.24$^{\#}$	77.60 ± 9.20$^{\#}$	233.75 ± 38.89$^{\#}$
PRF_{100}	91.82 ± 17.40***	39.77 ± 10.79***	140.93 ± 20.62***
PRF_{200}	76.14 ± 19.03***	32.98 ± 11.18***	79.75 ± 22.59***
GLB	68.74 ± 31.45***	30.55 ± 4.60***	90.75 ± 28.51***

NC: normal control; DC: diabetic control; PRF_{100} and PRF_{200}: Phenolic rich fractions (100 and 200 mg/kg); GLB: glibenclamide (5 mg/kg); AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase.
Data are presented as mean ± SD (n = 6). $^{\#}$ indicates significant ($p < 0.001$) difference compared to NC group; *($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicates significant difference compared to DC group. One-way ANOVA followed by Dunnett post hoc test was performed for comparison between the groups.

Table 4
Effects of phenolic rich fraction (PRF) of *C. glandulosum* Lindl. on metabolic parameters during STZ-NA-induced hyperglycemia.

Groups	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
NC	92.38 ± 1.95	115.55 ± 3.72	46.39 ± 4.31	38.55 ± 1.79
DC	112.32 ± 4.81$^{\#}$	165.84 ± 12.35$^{\#}$	36.56 ± 6.35$^{\#}$	59.04 ± 3.04$^{\#}$
PRF_{100}	100.91 ± 3.19***	129.87 ± 8.93***	49.02 ± 4.16**	44.67 ± 2.13***
PRF_{200}	97.29 ± 2.80***	119.99 ± 7.30***	53.56 ± 5.59***	39.97 ± 2.81***
GLB	104.87 ± 3.04**	126.41 ± 9.63***	54.97 ± 5.48***	41.49 ± 4.32***

NC: normal control; DC: diabetic control; PRF_{100} and PRF_{200}: Phenolic rich fractions (100 and 200 mg/kg); GLB: glibenclamide (5 mg/kg); TC: Total cholesterol; TG: Triglycerides; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein-cholesterol; Data are presented as mean ± SD (n = 6). $^{\#}$ indicates significant ($p < 0.001$) difference compared to NC group; *($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicates significant difference compared to DC group.
One-way ANOVA followed by Dunnett test was performed for comparison between the groups.

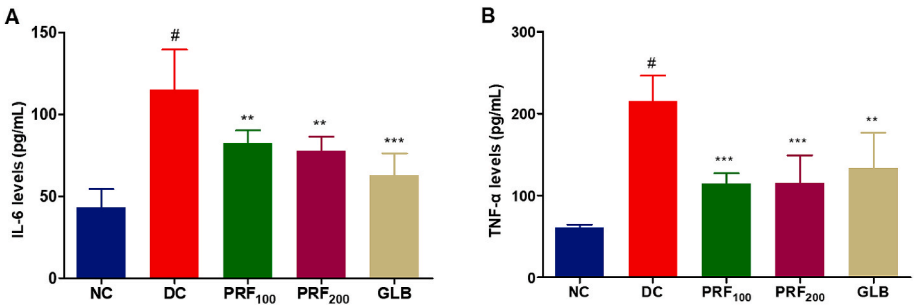
Table 5
Effects of phenolic rich fraction (PRF) of *C. glandulosum* Lindl. on oxidative stress parameters during STZ-NA-induced hyperglycemia.

Groups	GSH (nM/g tissue)	SOD (U/mg protein)	Catalase (U/mg protein)	TBARS (nM/mg tissue)
NC	2.65 ± 0.35	58.79 ± 4.86	81.33 ± 23.14	36.79 ± 7.03
DC	1.77 ± 0.15$^{\#}$	42.5 ± 3.48$^{\#}$	40.72 ± 11.47$^{\#}$	73.22 ± 17.68$^{\#}$
PRF_{100}	2.30 ± 0.48*	63.88 ± 6.25***	49.75 ± 15.72	41.74 ± 3.86***
PRF_{200}	2.54 ± 0.34**	70.83 ± 5.89***	78.73 ± 37.21*	39.22 ± 3.31***
GLB	2.44 ± 0.20**	50.52 ± 4.69*	69.27 ± 24.43	44.59 ± 8.87***

NC: normal control; DC: diabetic control; PRF_{100} and PRF_{200}: Phenolic rich fractions (100 and 200 mg/kg); GLB: glibenclamide (5 mg/kg); GSH: Glutathione S-transferase; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances.
Data are presented as mean ± SD (n = 6). # indicates significant ($p < 0.001$) difference compared to NC group; *($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicates significant difference compared to DC group. One-way ANOVA followed by Dunnett test was performed for comparison between the groups.

4.5. In-silico interactions of the main compounds in PRF with antidiabetic drug targets

Molecular docking analysis of the main constituents of PRF was carried out with the three key enzymes involved in the progression of diabetes. The binding free energies (docking score) and binding interactions between the key amino acid residues and ligands are presented in Table 6.



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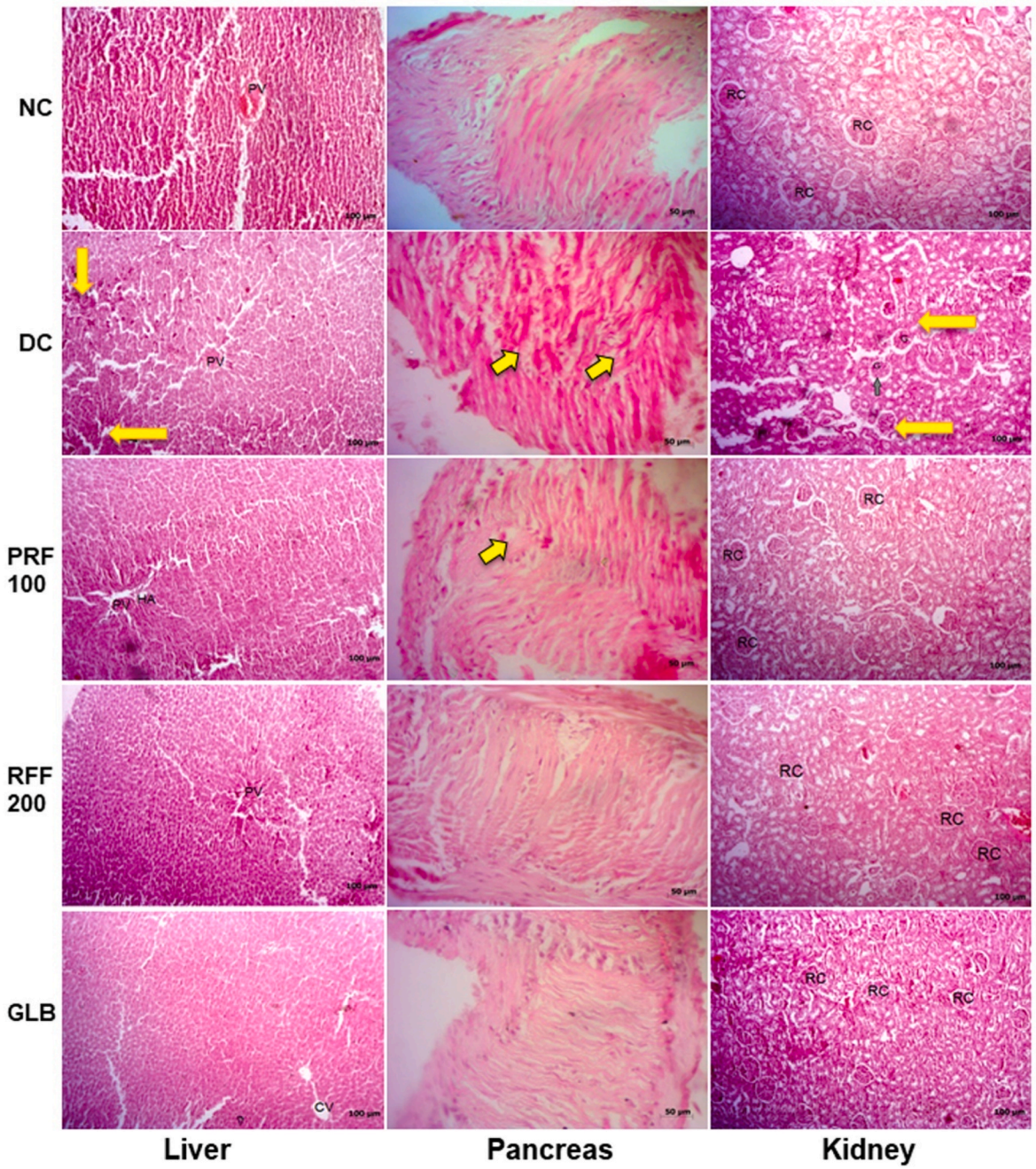

Fig. 5. Histopathology of liver, pancreas, and kidney stained with hematoxylin-eosin. Damaged areas were highlighted with yellow-coloured arrows. [PV: portal vein; CV: central vein; RC: Renal corpuscles; NC: Normal control; DC: Disease control; PRF_{100} and PRF_{200}: Phenolic rich fraction (100 and 200 mg/kg); GLB: Glibenclamide (5 mg/kg)].

Table 6
Summary of the free binding energy and key amino acid residue interaction of each protein with the respective ligands.

Protein (PDB ID)	Ligand	Docking score	Hydrogen bonds	Hydrophobic interactions
α-amylase (1HXO)	Verbascoside	−8.8	TRP^{59}, HIS^{305}, HIS^{201}, GLU^{240}	ALA^{307}, ALA^{235}, TRP^{59}
	Isoverbascoside	−8.8	SER^{105}, GLY^{106}	LEU^{162}, TYR^{62}
	Apigenin	−9.1	HIS^{299}, ASP^{197}, GLN^{63}	TYR^{62}, TRP^{59}, VAL^{163}
	Caffeic acid	−6.6	GLN^{63}, TRP^{59}, GLU^{233}	TYR^{62}, ASP^{197}
	Acarbose	−7.7	VAL^{163}, GLN^{63}, ASP^{300}, TYR^{62}, GLY^{306}, HIS^{201}	–
α-glucosidase (2QMJ)	Verbascoside	−8.8	ASP^{327}, ASP^{542}, THR^{205}, ASN^{207}	TYR^{299}, PHE^{575}, ALA^{576}, LEU^{473}
	Isoverbascoside	−8.2	GLN^{603}, ASP^{443}	ALA^{576}, TYR^{605}, TYR^{299}, PHE^{575}
	Apigenin	−7.4	ASP^{542}	TYR^{299}, PHE^{575}, TRP^{406}
	Caffeic acid	−6.3	ASP^{327}, ASP^{542}, HIS^{600}	TYR^{299}, PHE^{575}
	Acarbose	−8.0	ASP^{327}, ASP^{443}, ASP^{542}, HIS^{600}, THR^{205}	TYR^{299}, PHE^{575}, TRP^{406}
Aldose reductase (1US0)	Verbascoside	−11.9	HIS^{312}, ARG^{296}, ASN^{294}, GLU^{193}, GLN^{192}	LEU^{190}, LEU^{195}
	Isoverbascoside	−11.7	TYR^{309}, HIS^{163}, ASN^{294}, THR^{191}	ARG^{296}, LYS^{194}
	Apigenin	−11.5	THR^{191}, GLU^{193}, HIS^{312}, HIS^{163}, PRO^{310}	LEU^{195}, LYS^{194}
	Caffeic acid	−8.9	HIS^{163}, GLU^{193}, GLN^{192}, ASN^{292}	LYS^{194}
	Sorbinil	−11.0	ARG^{296}, LYS^{194}, GLU^{193}	LEU^{195}

When evaluating the effect of the bioactive compounds identified in PRF, we observed that verbascoside was the most effective molecule (lowest docking score and maximum interactions with amino acid residues) out of the four phenolic compounds against all three target proteins studied. Verbascoside being the major phenolic compound of *C. glandulosum* exhibited strong and stable interactions (docking score −8.8) with α-amylase by binding with the TRP^{59}, HIS^{305}, HIS^{201}, and GLU^{240} amino acid residues with α-glucosidase (docking score −8.8) via hydrogen bond formation with ASP^{327}, ASP^{542}, THR^{205}, ASN^{207} amino acid residues (Fig. 6A and B). Similarly, verbascoside also showed the highest efficacy (docking score −11.9) against aldose reductase enzyme by interacting with HIS^{312}, ARG^{296}, ASN^{294}, GLU^{193}, and GLN^{192} amino acid residues (Fig. 6C). Likewise, isoverbascoside, a functional isomer of verbascoside, also exhibited good binding interactions with all three enzymes studied (Table 6). On the other hand, as expected, the standard inhibitors, acarbose and sorbinil, showed good binding affinities by interacting with key amino acid residues in the respective protein targets. Acarbose interacts with α-amylase by forming hydrogen bonds via VAL^{163}, GLN^{63}, ASP^{300}, TYR^{62}, GLY^{306}, and HIS^{201} residues and interacts with α-glucosidase via the formation of hydrogen bonds through ASP^{327}, ASP^{443}, ASP^{542}, HIS^{600}, and THR^{205} residues. Nevertheless, the *in-silico* study reflects considerably better efficacy of the main phenolic compounds than the standard enzyme inhibitors. The results obtained from the *in-silico* study also aligned with the *in-vitro* enzyme inhibition potential of PRF and its main compound, verbascoside.

5. Discussion

The therapeutic effects of medicinal plants may be attributed to the occurrence of phenolic compounds. Phenolic compounds (such as apigenin, caffeic acid, naringenin, quercetin, etc.) are considered to be responsible for their therapeutic response [9,11,39]. Recently, phenolic compounds from dietary herbs and medicinal plants have been addressed globally in managing oxidative stress-induced hyperglycemia and metabolic syndrome due to their multitargeted pharmacological actions [10,11,40,41].

C. glandulosum, an endemic herb to NER, has been studied against metabolic syndrome and chemical/diet-induced oxidative stress in animal models [13,14,39,42]. However, this is the first study to investigate the PRF of *C. glandulosum* leaves in amelioration of hyperglycemia and oxidative stress with an attempt to explore the major phytochemicals associated with the effect.

The phenolic content was significantly higher in the PRF than in the crude extract. It was evident that the purification process removed the non-phenolic phytochemicals from the matrix and enhanced the phenolic content in the purified fraction. HPLC-PDA analysis of PRF confirmed the presence of verbascoside as the principal phenolic compound, which corroborates our findings with the earlier reports [13,43]. Verbascoside and isoverbascoside are phenylethanoid phenolics that exhibit remarkable antidiabetic and antioxidant potential in animal models [44,45]. These compounds are also abundantly present in many important Verbanaceae/Lamiaceae family herbs, which are globally consumed as phytopharmaceuticals or nutraceuticals [45,46]. Caffeic acid and apigenin, which have previously been reported as antidiabetic and antioxidants, were also identified in the PRF and also demonstrated a substantial modulatory effect on metabolic disorders and oxidative stress [47,48].

α-amylase and α-glucosidase are involved in the enzymatic breakdown of carbohydrates and are the potential targets for controlling elevated sugar levels in the circulation. Many plant-derived phenolic compounds have been reported as excellent inhibitors of these enzymes [49]. Our study found that PRF and its major compound, verbascoside, inhibited both enzymes.

Aldose reductase, which is responsible for converting glucose into sorbitol, is another target for controlling diabetic complications. Because of hyperglycemia during diabetes mellitus, excessive sugar efflux allows faster production and increased sorbitol accumulation in blood vessels, lenses, nerves, retina, and kidneys, leading to altered membrane permeability and impaired cellular pathology [50]. In our study, PRF and its marker compound, verbascoside, displayed efficient inhibition of AGEs and aldose reductase enzyme with an appreciable IC_{50} value compared to the respective standard inhibitors. A similar effect has been demonstrated by other plant extracts, where verbascoside was present as one of the major phenolic compounds [51,52].

In the present study, it was observed that the body weight of the animals was reduced following STZ-induced hyperglycemia. This phenomenon is due to enhanced catabolic processes, including protein degradation, lipid breakdown, altered hepatic metabolism of

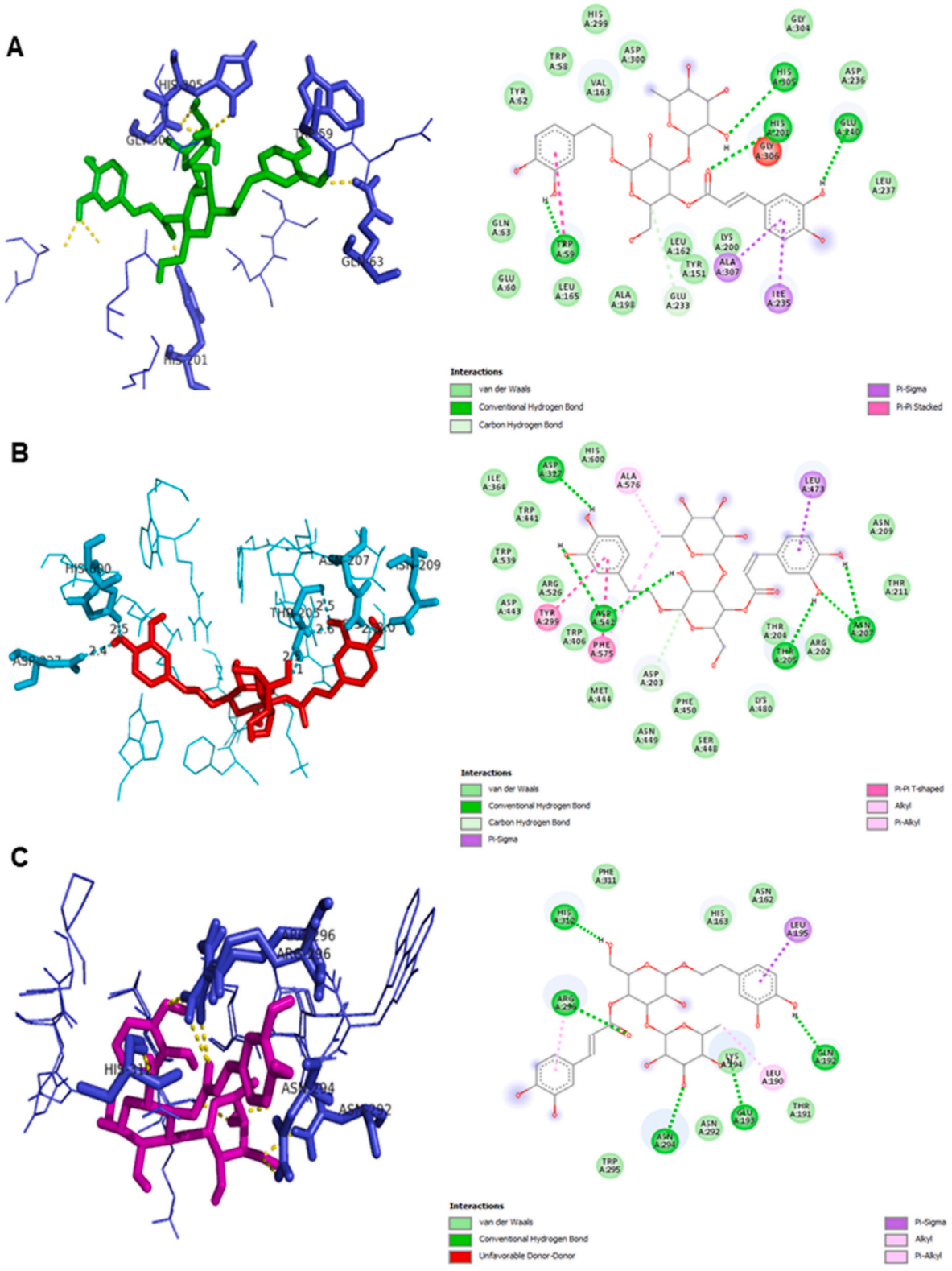

Fig. 6. Molecular docking studies showing key amino acid residue interaction of each protein with the respective ligands.
(A) Verbascoside - α-amylase protein-ligand complex; (B) Verbascoside - α-glucosidase protein-ligand complex; (C) Verbascoside - Aldose reductase protein-ligand complex.

carbohydrates, and increased inflammation. Rapid and severe apoptosis of pancreatic β-cells in STZ-injected animals triggered elevated blood glucose levels and reduced serum insulin levels [53]. The malfunction of the glucose homeostatic mechanism was also observed with an aborted glucose tolerance capacity during a glucose overload study in animals [1,2]. On the other hand, PRF-treated animals showed a significant improvement in body weight compared to the untreated diabetic animals, along with their body's ability to regulate blood glucose and

plasma insulin levels. Phenolic-rich plant extracts have been found to possess protective and regenerative effects on pancreatic β-cells. Phenolic compounds have been shown to stimulate insulin secretion by β-cells [49]. Therefore, the ameliorative effect of PRF justifies the beneficial role of its extract against diabetes and oxidative stress-related diseases reported earlier [54].

STZ generates harmful free radicals (methyl nitrosourea) in the liver, which causes significant damage to hepatocytes, resulting in elevated levels of AST, ALT, and ALP in the serum [55]. Impaired metabolism due to the malfunction of hepatocytes augments the abnormal rise of triglycerides, cholesterol, LDL-C, and reduction of HDL-C in diabetic animals, as observed in this study. The combined effect of β-cell apoptosis and oxidative damage of hepatocytes due to STZ-induced oxidative stress resulted in a severe depletion of endogenous antioxidant status (GSH, SOD, and catalase) and high lipid peroxidation [53]. This depletion of endogenous antioxidants leads to malfunction of the redox balance and activates inflammatory pathways [56]. An elevated level of IL-6 and TNF-α have been observed in the STZ-NA-treated animals, indicating oxidative stress and inflammation. However, intervention with PRF showed substantial improvement in the hepatic enzyme profile and serum lipid profile, comparable to the standard antidiabetic drug, glibenclamide. Moreover, in our study, PRF treatment significantly counters oxidative stress in the animals by improving the liver antioxidant status (GSH, SOD) and suppressing inflammatory cytokines (IL-6 and TNF-α). It has been reported that the PI3K/AKT/GSK3β signaling pathway is one of the master regulatory pathways in managing hyperglycemia and insulin resistance in diabetic conditions. Previously, intervention with 10-hydroxy-2-decenoic acid (10-HAD), a principal active ingredient of royal jelly, has been reported to enhance hepatic SOD, catalase, and GSH activities and decreased IL-6 and TNF-α content in diabetic mouse liver. This report also suggested that hypoglycemic effects are exerted via the PI3K/AKT/GSK3β signaling pathway [57]. Similarly, PRF might also act via this signaling pathway. This indicated that the phytochemicals present in the PRF exert hepatoprotective and anti-inflammatory properties. The literature also supports these observations indicating the potential hepatoprotective and anti-inflammatory action of verbascoside and other phenolic compounds present in PRF [45,58,59]. An overall protective effect of PRF was observed from the improved antioxidant status and reduced inflammatory markers in the liver. The phytoconstituents of PRF *viz.*, verbascoside, isoverbascoside, caffeic acid, and apigenin have been reported to work via the NF-κB/TNF-α/IL-1β pathway [45,58–60]. Verbascoside has been shown to suppress proinflammatory cytokines TNF-α and IL-6 and, thus, the activation of NFκB signaling in a dose-dependent manner [60]. In addition, histological observations of the liver, pancreas, and kidney also strongly support the beneficial effect of PRF during STZ-induced oxidative damage.

Plant extracts with a high content of antioxidants, such as polyphenols, are often used to counter diabetes and other metabolic syndromes because of their ability to neutralize free radical activities or activate cellular antioxidant signaling pathways. Antioxidant phytochemicals, mostly phenolic and flavonoids, have been proven to protect and regenerate β-cells by activating endogenous antioxidant pathways like the Nrf2-KEAP1 signaling pathway [9,49]. In this study, chemical analysis of PRF indicates the occurrence of antioxidant phytochemicals in certain concentrations, which might be attributed to the antioxidant and ameliorative effects during STZ-induced hyperglycemia. Other authors have also reported that verbascoside substantially regenerates pancreatic β-cells and exhibits notable suppression of inflammatory cytokines [61]. Similar findings have also been reported on isoverbascoside and the non-glycosides (*viz.*, apigenin and caffeic acid) [45,48,62]. These phytochemicals are responsible for lowering the proinflammatory cytokines (TNF-α and IL-6) and elevating the antioxidant enzyme profile (SOD, GST, CAT) and thus lowering hyperglycemia-induced ROS.

Molecular docking studies suggested that verbascoside and isoverbascoside strongly interacted in the inhibitor binding pocket of the studied proteins. Interestingly, the effect of phytochemicals in PRF was better than that of the standard inhibitors, represented by the lowest docking score. It indicates that these phytochemicals could block the enzyme's active site by binding tightly to the catalytic amino acid residues, which could inhibit specific enzyme activity. Hence, the *in-silico* assessment portrays complementary support alongside the *in-vitro/in-vivo* to the ameliorative effect of PRF in controlling hyperglycemia during STZ-induced diabetic animal models.

Our findings suggested the ameliorative effect of the purified fraction, PRF, against hyperglycemia, which can be attributed to the presence of potent phytoconstituents characterized in this study. Further controlled and random clinical trials are required to determine this PRF's dietary and phytopharmaceutical use for managing diabetes and its oxidative and inflammatory complications.

6. Conclusion

This study revealed the antihyperglycemic and antioxidant potential of partially purified phenolic enriched fraction (PRF) of *C. glandulosum* leaves. Further, an *in-vivo* antidiabetic study confirms the lowering effect of blood glucose and reversal of oxidative stress and inflammation during chemical (STZ) induced hyperglycemia. Verbascoside was found to be the main phenolic compound that may largely contribute to PRF's antihyperglycemic, antioxidant, and anti-inflammatory properties. However, the in-depth mechanism of the antidiabetic action of PRF remains unclear. Further investigation with a large cohort study in diverse strata and molecular pathway evaluations is required to substantiate the current findings.

Author contribution

Puspanjali Khound: Methodology, Validation, Investigation, Resources, Writing – review and editing, Funding acquisition; Prashant Kumar Deb: Conceptualization, Study design, Software, Validation, Formal Analysis, Investigation, Writing – original draft, Visualization, Project administration; Swarnali Bhattacharjee: Methodology, Validation, Investigation; Karla Damián Medina: Software, Investigation; Partha Pratim Sarma: Validation, Investigation; Biswatrish Sarkar: Validation, Writing – review and editing, Visualization; Rajlakshmi Devi: Methodology, Validation, Writing – review and editing, Visualization, Supervision, Project administration, Funding acquisition.

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Declaration on use of generative AI in scientific writing

No AI and AI-assisted technologies have been used in the writing of this research article.

Conflict of Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rajlakshmi Devi reports financial support and administrative support were provided by Institute of Advanced Study in Science and Technology. Rajlakshmi Devi reports a relationship with Institute of Advanced Study in Science and Technology that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2024.100906.

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