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Investigation of Antidiabetic and Antioxidant Potential of Annona Squamosa Extract: In Vitro and In Vivo Evaluation with Phytochemical Profiling

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ABSTRACT:

Background: Diabetes mellitus affects over 537 million adults worldwide, with type 2 diabetes characterized by insulin resistance and β-cell dysfunction. Current therapies have limitations, necessitating safer natural alternatives. Annona squamosa, a medicinal plant with traditional antidiabetic use, requires systematic evaluation.

Objective: To investigate the antidiabetic and antioxidant potential of A. squamosa hydroalcoholic extract through integrated in vitro and in vivo approaches.

Methods: The extract was prepared by Soxhlet extraction (70% ethanol) and characterized by HPTLC. Antioxidant activity was evaluated via DPPH and nitric oxide scavenging assays, while antidiabetic potential was assessed through α-amylase and α-glucosidase inhibition. Type 2 diabetes was induced in Sprague-Dawley rats using high-fat diet followed by low-dose streptozotocin (35 mg/kg). Diabetic rats received A. squamosa extract (100, 200, 400 mg/kg) or glibenclamide (5 mg/kg) orally for 4 weeks. Fasting blood glucose, glucose tolerance, insulin sensitivity, lipid profile, serum insulin, and pancreatic histopathology were evaluated.

Results: HPTLC profiling indicated the presence of rutin, quercetin, kaempferol and minor peak corresponding to gallic acid. The extract showed potent antioxidant (DPPH IC_{50}: 50.40 µg/mL; NO IC_{50}: 34.38 µg/mL) and enzyme inhibitory activities (α-amylase IC_{50}: 240.89 µg/mL; α-glucosidase IC_{50}: 218.27 µg/mL). At 400 mg/kg, the extract significantly reduced fasting blood glucose (26.3%), improved glucose-lowering response to exogenous insulin (27.7% AUC reduction), increased serum insulin (9.8%), improved dyslipidemia (50.1% LDL reduction; 88.9% HDL increase), and improved pancreatic architecture.

Conclusion: A. squamosa extract exhibits significant antihyperglycemic and antioxidant activities, supporting its traditional use and potential as a promising natural candidate for further investigation in type 2 diabetes.

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from defects in insulin secretion, insulin action, or both. It affects approximately 537 million adults worldwide, with projections estimating a rise to 783 million by 2045 (IDF, 2021). Type 2 diabetes mellitus (T2DM), which accounts for the vast majority of diabetes cases, is primarily driven by insulin resistance and progressive pancreatic β-cell dysfunction (DeFronzo et al., 2015). Prolonged hyperglycemia triggers oxidative stress and low-grade systemic inflammation, which play central roles in the development of serious microvascular and macrovascular complications, including diabetic nephropathy, retinopathy, neuropathy, and cardiovascular disease (Brownlee, 2001).

Current pharmacotherapeutic options for T2DM, such as metformin, sulfonylureas, DPP-IV inhibitors, SGLT-2 inhibitors, and GLP-1 receptor agonists, provide effective glycemic control but are often associated with adverse effects including gastrointestinal disturbances, weight gain, hypoglycemia, and potential cardiovascular risks. Moreover, their high cost and limited accessibility in low- and middle-income countries further restrict their widespread use (Inzucchi et al., 2015, Chaudhury et al., 2017). These limitations have intensified the global search for safer, more affordable, and effective alternatives, particularly from natural sources. The World Health Organization has advocated for the integration of traditional medicine into healthcare systems, particularly where evidence supports its safety and efficacy (WHO, 2013).

The Annonaceae family, comprising about 120 genera and over 2,300 species distributed mainly in tropical and subtropical regions, represents one of the richest yet relatively underexplored sources of bioactive compounds (Chatrou et al., 2012). Plants belonging to this family have been traditionally used for the management of parasitic infections, inflammatory conditions, and metabolic disorders, including diabetes. Phytochemical studies have revealed the presence of unique classes of compounds such as annonaceous acetogenins, aporphine alkaloids, flavonoids, terpenoids, and cyclopeptides, which contribute to their diverse pharmacological activities (Pandey & Barve, 2011, Al Kazman et al., 2022).

Among the Annonaceae family, Annona squamosa Linn. (commonly known as sugar apple or custard apple) holds significant therapeutic promise. This small tropical tree is widely cultivated in India, Brazil, China, and Southeast Asia (Orwa et al., 2009). In the Ayurvedic system of medicine, various parts of the plant—particularly the leaves, fruits, seeds, and bark— used as tonic, digestive, and antidiabetic agents. Traditional uses also include the treatment of gastritis, diarrhea, fever, and parasitic infestations (Sarmah et al., 2021).

Extensive Phytochemical investigations of *A. squamosa* have identified a rich profile of bioactive constituents, including annonaceous acetogenins, alkaloids, flavonoids (quercetin, kaempferol, rutin), terpenoids, and cyclopeptides (Yang et al., 2008, Chen et al., 2012). The plant contains polyphenolic compounds that may contribute to its antioxidant, anti-inflammatory, and antidiabetic activities. Several previous studies have demonstrated the antidiabetic potential of Annona squamosa. Aqueous leaf extract showed significant antihyperglycemic activity in streptozotocin-nicotinamide-induced type 2 diabetic rats (Shirwaikar et al., 2004). Ethanolic and methanolic extracts of seeds and leaves reduced blood glucose levels in alloxan-induced diabetic models (Singala et al., 2011), while hydroalcoholic leaf extract improved glycemic control, lipid profile, and insulin sensitivity in streptozotocin-induced diabetic rats (Tomar, 2012). Mechanistic studies have reported that *A. squamosa* extracts may stimulate insulin secretion, enhance glucose handling, and exert antioxidant effects (Davis et al., 2012; Ofori et al., 2022). In addition, some reports have indicated anti-inflammatory and antioxidant activities of the plant extracts in diabetic complications (Almalki et al., 2024).

Despite this evidence, the majority of existing reports have examined either antidiabetic or antioxidant activities in isolation, with limited integration of these complementary aspects in the evaluation of *A. squamosa* (Donath & Shoelson, 2011). This fragmented approach fails to capture the potential multi-targeted mechanism of action that is characteristic of plant-derived phytocompounds, which often exert synergistic effects simultaneously on glycemic control, inflammatory mediators, and redox balance (Hotamisligil, 2017).

Another significant limitation of prior research is the lack of correlation between in vitro antioxidant activity (e.g., DPPH, nitric oxide scavenging) and in vivo pharmacological efficacy. While many studies report free radical scavenging activity in vitro, establishing whether such activity translates into meaningful protection against hyperglycemia-induced oxidative damage in target organs such as the pancreas, liver, or kidneys remains an important area for further investigation. Establishing such correlations is essential to identify reliable biomarkers for quality control and to predict in vivo performance from in vitro data.

Moreover, some previous *in vivo* studies have employed single-dose or short-duration treatment protocols, which may provide limited information on sustained effects during diabetes management. Extended treatment periods (e.g., 28 days) can provide additional information on glycemic control, lipid profiles, body weight, and potential delayed toxicity (King, 2012). Therefore, the present study was designed to address these identified gaps through a comprehensive, integrated approach.

MATERIALS AND METHODS

Materials

Collection and Authentication of Plant Material

Fresh, healthy bark of Annona squamosa L. was collected from Nagpur, Maharashtra, India (November–February). The plant was taxonomically authenticated (Authentication No. 254) by Dr. D.S. Dongarwar, Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, and a herbarium specimen was deposited in the departmental herbarium.

Chemicals and Kits

Streptozotocin (STZ; 18883-66-4) was procured from Sigma-Aldrich. Serum glucose, lipid profile (LDL, HDL, total cholesterol, triglycerides), and total protein were estimated using commercial kits (Coral Clinical System; catalog nos. 1102113150, 1102170040, 1102150040, 1102050075, 1101201150, and 1102230020, respectively). Rat insulin levels were quantified using ELISA kits (Elabscience; catalog nos. E-EL-R0015, E-EL-R2856, and E-EL-R2466, respectively).

Instruments

Biochemical parameters were analyzed using an autoanalyzer (CoralLab), blood glucose was monitored using a glucometer (OneTouch), insulin levels were determined using an ELISA plate reader (Bio-Rad iMark), tissue imaging was performed using a fluorescence microscope (Nikon E1), and controlled perfusion was carried out using a perfusion pump (Kent, USA).

Animals

Adult male Sprague-Dawley rats (200–220 g) were procured from a CPCSEA-registered breeder and housed individually in polypropylene cages under controlled conditions (22 ± 2 °C; 70 ± 5% RH; 12 h light/dark cycle) with free access to food and water. After one week of acclimatization, all procedures were approved by the Institutional Animal Ethics Committee and conducted per CPCSEA guidelines (CPCSEA, 2018).

Methods

Preparation of Extract

The powdered plant material was defatted with n-hexane (1:3 w/v) at room temperature for 24 h with occasional stirring. The mixture was filtered, and the residue was air-dried. The defatted powder was then subjected to Soxhlet extraction using 70% ethanol (ethanol:water = 70:30, v/v) at 60–65 °C for 12–24 h with a solvent-to-solid ratio of 10:1 (500 mL per 50 g) (Handa et al., 2008). The extraction was continued until the siphon arm became colourless. The solvent was evaporated under reduced pressure using a rotary evaporator at ≤45 °C to obtain a crude 70% hydroalcoholic extract (yield: 7.2% w/w). The hydroalcoholic extract was subsequently concentrated and maintained as a tincture at 4 °C. The tincture was used as the test preparation for the in vitro and in vivo studies.

Phytochemical / Bioactive Characterization

The 70% hydroalcoholic extract of *Annona squamosa* was subjected to HPTLC for phytochemical profiling (Wagner & Bladt, 1996). Chromatography was performed on pre-coated silica gel 60 F_{254} plates (10 × 10 cm) using a mobile phase consisting of toluene:ethyl acetate:formic acid (5.4:3.8:0.8, v/v/v). Quercetin, gallic acid, kaempferol, and rutin were used as reference standards. Densitometric scanning was performed using a CAMAG TLC Scanner at 254 nm in absorption/remission mode. The phytoconstituents were tentatively identified by comparing the Rf values of the sample peaks with those of the reference standards.

In vitro antioxidant and antidiabetic activity

The tincture prepared from the 70% hydroalcoholic extract of *Annona squamosa* was evaluated for DPPH radical scavenging and nitric oxide scavenging activity at 20–100 µg/ml, with ascorbic acid as standard. Absorbance was recorded at 517 nm and 546 nm, respectively, and IC_{50} values were calculated from dose-response curves (Athavale et al., 2012, Gulcin & Alwasel, 2023). For nitric oxide, the extract was incubated with sodium nitroprusside and Griess reagent, following absorbance measurement at 546 nm (Sreejayan & Rao, 1997, Baliga et al., 2003). Antidiabetic activity was assessed via α-amylase inhibition (100–500 µg/ml) using the DNSA method at 540 nm, and α-glucosidase inhibition (1–1000 µg/ml) using pNPG substrate at 405 nm, with acarbose as positive control in both assays (Pistia-Brueggeman & Hollingsworth, 2001, Alema et al., 2020, Mechchate et al., 2021).

Acute Oral Toxicity Study

Acute oral toxicity of the A. squamosa tincture was assessed in female Sprague-Dawley rats (180–220 g) according to OECD 423 (OECD, 2001). Animals received single oral doses of 300 or 2000 mg/kg (n = 3/group) and were observed for 14 days for mortality, clinical signs, and body weight changes. At the end of the study, vital organs from animals receiving 2000 mg/kg were examined histopathologically.

Preparation of Extract for Dosing

The 70% hydroalcoholic extract was concentrated and stored as a tincture at 4 °C. The tincture was used as the test preparation for the in vitro and in vivo studies. For in vivo administration, the tincture was diluted appropriately with 0.5% carboxymethylcellulose (CMC) immediately before dosing to achieve the desired doses of 100, 200, and 400 mg/kg body weight. The freshly prepared formulations were administered orally using a gastric gavage needle, with the dosing volume maintained at 0.5–1 mL per animal.

Induction of Type 2 Diabetes Mellitus

Type 2 diabetes was induced using a high-fat diet (HFD) followed by low-dose streptozotocin (STZ) (Reed et al., 2000). After acclimatization, rats were fed an HFD (composition: cholesterol 1%, lard 10%, egg yolk powder 10%, milk powder 6%, carbohydrate 21%, fat 40%, and protein 12%) for 4 weeks (Srinivasan et al., 2005). At the end of the fourth week, animals were fasted overnight and administered a single intraperitoneal injection of STZ (35 mg/kg), freshly prepared in ice-cold 0.1 M citrate buffer (pH 4.5). To prevent hypoglycemic mortality, 5% glucose solution was provided for 24 h following STZ administration. After 72 h, fasting blood glucose was measured, and rats with fasting blood glucose levels ≥250 mg/dL were considered diabetic and included in the study. The HFD was continued throughout the treatment period until the end of the experimental study.

Experimental Grouping and Drug Treatment

Following confirmation of diabetes, animals were randomly divided into six groups (n = 5): normal control (vehicle), diabetic control (vehicle), diabetic rats treated with A. squamosa tincture (100, 200, and 400 mg/kg, p.o.), and standard control (glibenclamide 5 mg/kg in 0.5% CMC, p.o.) (Grover et al., 2002). The doses were selected based on the acute oral toxicity study (OECD 423) and previous literature reports demonstrating antidiabetic activity of Annona species within this dose range. All treatments were administered orally once daily from week 6 to week 9, with the dosing volume maintained at 0.5–1 mL. Body weight and fasting blood glucose were monitored weekly after a 5 h fast using tail-vein blood samples and a commercial glucose kit (Coral Clinical System). In the second treatment week, an intraperitoneal glucose tolerance test (2 g/kg glucose) and insulin tolerance test (0.75 U/kg insulin) were performed, with blood glucose measured at 0, 15, 30, 60, 90, and 120 min after administration. At the end of the study, blood was collected via retro-orbital plexus puncture under anaesthesia; serum was separated by centrifugation (2500 rpm, 15 min) and stored at −20 °C for further biochemical analysis (Parasuraman et al., 2010).

Statistical Calculations

Data were expressed as mean ± SEM. Statistical differences between groups were analyzed using two-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparisons test. A value of $p < 0.05$ was considered statistically significant.

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RESULTS AND DISCUSSION

Preliminary Phytochemical Analysis

HPTLC fingerprinting of the 70% hydroalcoholic extract of *Annona squamosa* indicated the presence of several phytoconstituents. Based on comparison of retention factor (Rf) values with reference standards, prominent peaks corresponding to rutin (Rf 0.02), quercetin (Rf 0.65–0.72), and kaempferol (Rf 0.83–0.88) were observed. A minor peak corresponding to gallic acid (Rf 0.30–0.34) was also detected. These findings indicate the presence of these flavonoid and phenolic compounds in the extract.

In vitro antioxidant and antidiabetic activity

The *A. squamosa* tincture showed dose-dependent free-radical scavenging activity in the DPPH and nitric oxide assays (Fig. 1). DPPH inhibition ranged from 40.66% at 20 µg/mL to 61.42% at 100 µg/mL (IC_{50} = 50.40 µg/mL), while nitric oxide scavenging ranged from 44.43% to 65.68% (IC_{50} = 34.38 µg/mL). Ascorbic acid exhibited higher activity in both assays (IC_{50} = 8.50 and 5.98 µg/mL, respectively), indicating that the tincture possesses antioxidant activity, although it was less potent than the standard.

The tincture also inhibited carbohydrate-digesting enzymes in a dose-dependent manner. α-Amylase inhibition ranged from 40.58% at 100 µg/mL to 67.30% at 500 µg/mL (IC_{50} = 240.89 µg/mL), while α-glucosidase inhibition ranged from 38.67% to 75.19% (IC_{50} = 218.27 µg/mL). Acarbose showed stronger inhibition in both assays (IC_{50} = 107.07 and 118.27 µg/mL, respectively), indicating that the tincture exhibited inhibitory activity against α-amylase and α-glucosidase, although it was less potent than the standard (Pistia-Brueggeman & Hollingsworth, 2001; Alema et al., 2020; Mechchate et al., 2021).

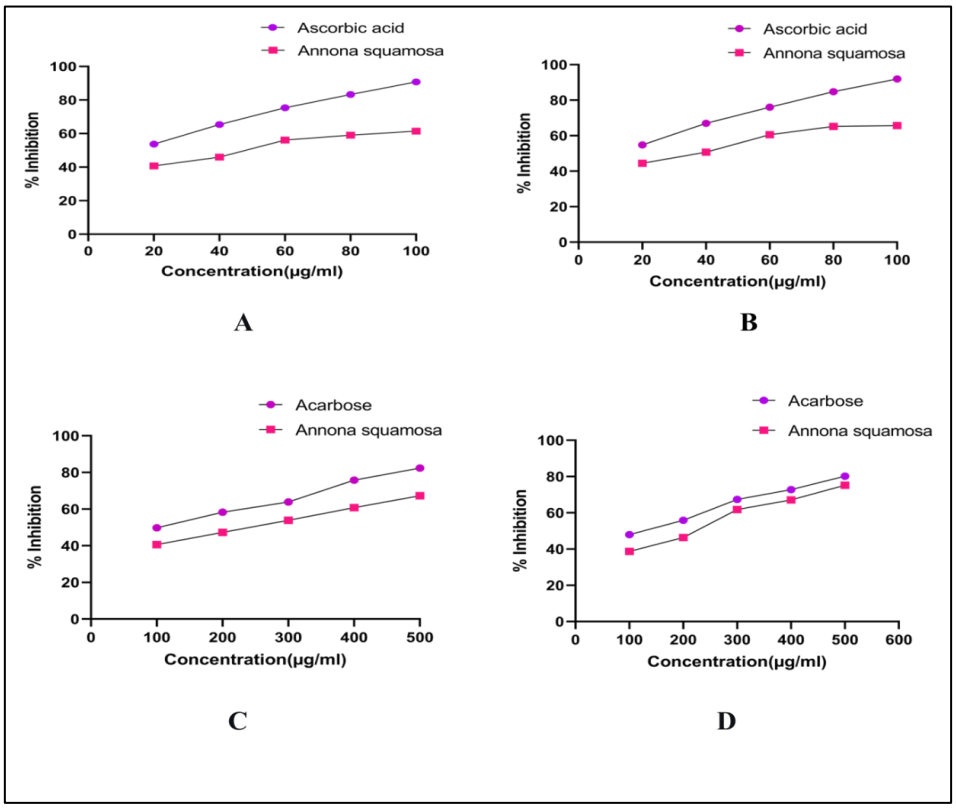

Figure 1: *In vitro* antioxidant and antidiabetic activities of the of *Annona squamosa* tincture. (A) DPPH radical scavenging activity, (B) nitric oxide scavenging activity, (C) α-amylase inhibition, and (D) α-glucosidase inhibition. Values are presented as mean ± SD (n=3). Ascorbic acid and acarbose were used as reference standards for antioxidant and antidiabetic assays, respectively.

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Acute Oral Toxicity Study

The acute oral toxicity study revealed no treatment-related mortality or observable toxic reactions following administration of the *A. squamosa* tincture. No mortality or overt signs of toxicity were observed at the maximum tested dose of 2000 mg/kg during the 14-day observation period.

Effect of Annona squamosa Tincture on Fasting Blood Glucose and Body Weight in Diabetic Rats

The diabetic control group exhibited a progressive and sustained elevation in FBG throughout the study period, increasing from 128.8 ± 6.30 mg/dL at week 1 to 401.6 ± 9.24 mg/dL at week 10, representing a 211.8% increase from baseline. The sustained elevation in FBG indicates persistent hyperglycemia in the untreated diabetic animals (Reed et al., 2000; King, 2012).

Administration of *A. squamosa* tincture reduced FBG in the treated groups (Table 1). The 100 mg/kg group showed an 11.0% reduction in FBG from peak values (378.4 ± 31.95 to 336.8 ± 18.54 mg/dL). The 200 mg/kg group demonstrated a 19.0% reduction (387.0 ± 24.77 to 313.4 ± 22.64 mg/dL). The greatest reduction at the final assessment was observed in the 400 mg/kg group, where FBG declined from 363.8 ± 5.93 mg/dL to 296.0 ± 12.12 mg/dL (18.6% reduction from peak and 26.3% lower than the diabetic control at week 10). Glibenclamide demonstrated a greater reduction, with FBG declining from 362.6 ± 23.62 to 247.4 ± 10.21 mg/dL (31.8% reduction from peak and 38.4% lower than the diabetic control at week 10). Glibenclamide demonstrated superior efficacy, reducing FBG from 362.6 ± 23.62 to 247.4 ± 10.21 mg/dL (31.8% reduction from peak; 38.4% reduction compared to diabetic control).

The decline in FBG following treatment initiation (weeks 6–9) suggests an improvement in glycemic control following administration of *A. squamosa* tincture. The observed effect may be associated with mechanisms involving insulin secretion and/or peripheral glucose utilization; however, these mechanisms were not directly investigated in the present study. The findings are consistent with previous reports describing insulin-releasing and antidiabetic effects of *A. squamosa* constituents (Shirwaikar et al., 2004; Ofori et al., 2022) and reported PTP1B inhibitory activity of *A. squamosa* extract (Davis et al., 2012).

Table 1: Effect of A. squamosa Tincture on Fasting Blood Glucose in STZ-Induced Diabetic Rats

Group	Week 1 (Baseline)	Peak FBG (Week 6-8)	Week 10 (Final)	% Reduction from Peak	% vs Diabetic Control (Week 10)
Diabetic Control	128.8 ± 6.30	401.6 ± 9.24 (Wk 10)	401.6 ± 9.24	-	-
A. squamosa 100 mg/kg	126.4 ± 12.62	378.4 ± 31.95 (Wk 6)	336.8 ± 18.54	11.0%	16.1% lower
A. squamosa 200 mg/kg	119.2 ± 12.62	387.0 ± 24.77 (Wk 7)	313.4 ± 22.64	19.0%	22.0% lower
A. squamosa 400 mg/kg	119.2 ± 12.62	363.8 ± 5.93 (Wk 6)	296.0 ± 12.12	18.6%	26.3% lower
Glibenclamide	126.6 ± 14.33	362.6 ± 23.62 (Wk 6)	247.4 ± 10.21	**31.8%**	**38.4% lower**

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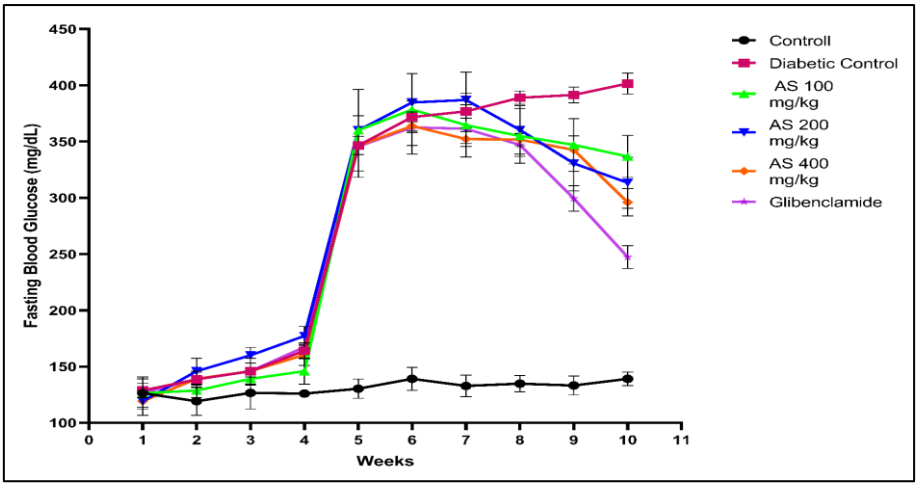

Figure 2. Effect of *A. squamosa* Tincture on fasting blood glucose (FBG) levels in streptozotocin (STZ)-induced **diabetic rats** over 10 weeks**.** Data are presented as mean ± SEM (n = 5). Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test. $p < 0.05$, **p** < 0.01, and ***p*** < 0.001 compared with the diabetic control group.

Body Weight.

The diabetic control group showed substantial weight gain, increasing from 204.0 ± 4.47 to 294.0 ± 4.47 g (44.1% increase). Treatment with *A. squamosa* tincture resulted in a comparatively lower increase in body weight (Fig. 3). The 400 mg/kg group showed the most favorable outcome, with body weight reaching 248.0 ± 4.47 g, closely approximating the normal control (244.4 ± 3.85 g) and glibenclamide group (240.2 ± 4.21 g). The comparatively lower body weight gain in the treated groups, particularly at 400 mg/kg, occurred alongside improved glycemic control, although food intake was not assessed in the present study.

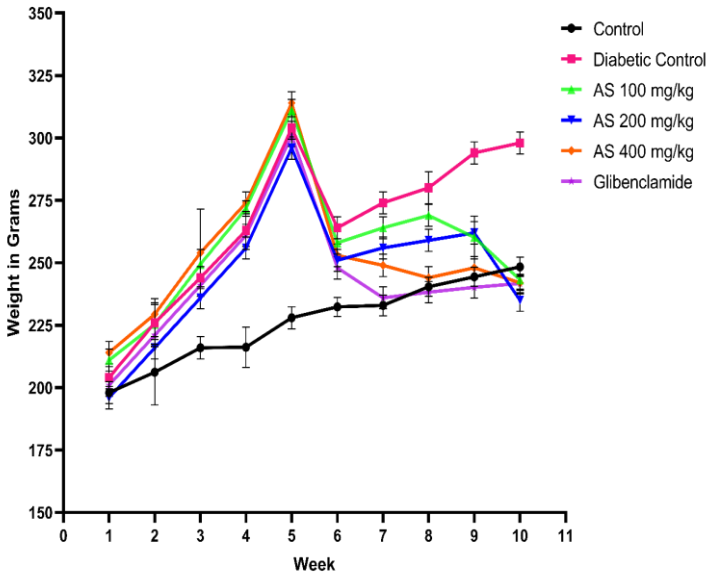

Figure 3. Effect of *AS* Tincture on body weight levels in streptozotocin (STZ)-induced diabetic rats over 10 weeks. Data are presented as mean ± SEM (n = 5). Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test. $p < 0.05$, **p** < 0.01, and ***p*** < 0.001 compared with the diabetic control group.

The OGTT was performed during the second treatment week to evaluate the effect of the *A. squamosa* tincture on glucose tolerance (Fig. 4). In the diabetic control group, blood glucose peaked at 460.4 ± 14.77 mg/dL at 30 min and remained elevated at 413.2 ± 15.02 mg/dL at 120 min, indicating impaired glucose tolerance (DeFronzo et al., 2015).

Administration of *A. squamosa* tincture improved the glucose response during the OGTT. The 100 mg/kg group showed a peak of 410.4 ± 7.77 mg/dL at 30 min, declining to 362.8 ± 5.63 mg/dL at 120 min. The 200 mg/kg group peaked at 392.8 ± 26.94 mg/dL and declined to 342.6 ± 13.22 mg/dL at 120 min. The 400 mg/kg group demonstrated the greatest improvement, with a peak of 355.4 ± 7.20 mg/dL at 30 min and a decline to 301.0 ± 10.77 mg/dL at 120 min. Glibenclamide showed a greater improvement in glucose handling, with blood glucose peaking at 362.6 ± 23.94 mg/dL at 15 min and declining to 266.2 ± 8.35 mg/dL at 120 min.

The area under the curve (AUC) for the OGTT was calculated using the trapezoidal rule. The diabetic control group showed the highest AUC (42,152 mg·min/dL), indicating poorer glucose tolerance. The *A. squamosa* 400 mg/kg group significantly reduced the AUC to 32,438 mg·min/dL, representing a 23.0% reduction compared with the diabetic control, while glibenclamide produced the greatest reduction, to 29,639 mg·min/dL (29.7% reduction). These findings indicate that *A. squamosa* tincture improved glucose tolerance and reduced overall glucose exposure during the OGTT, consistent with previously reported antidiabetic effects of *A. squamosa* (Shirwaikar et al., 2004; Ofori et al., 2022).

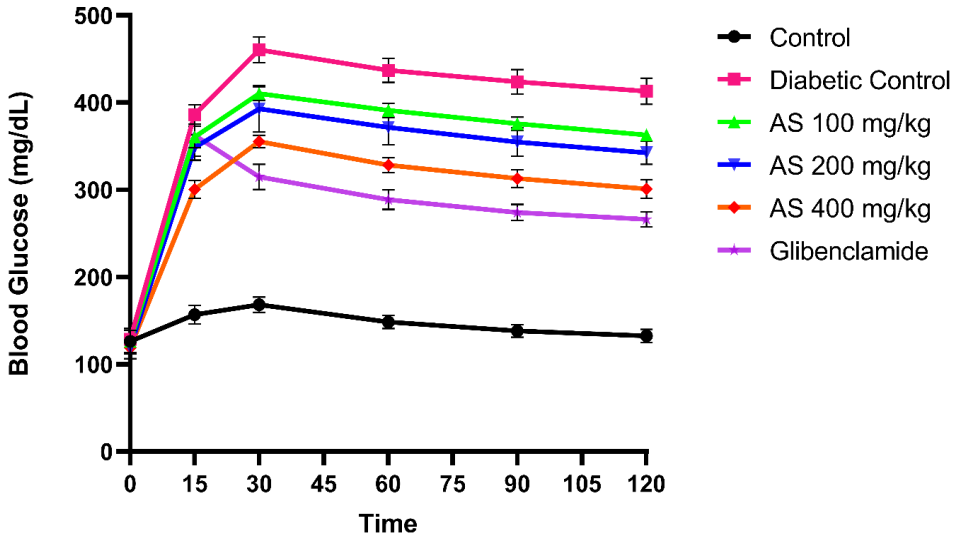

Figure 4: Effect of *A. squamosa* Tincture on oral glucose tolerance test (OGTT) in STZ-induced diabetic rats. Data are presented as mean ± SEM (n = 5). ***p < 0.001, **p < 0.01, *p < 0.05 vs. diabetic control (two-way ANOVA with Bonferroni's post-hoc test).

Effect of *A. squamosa* Tincture on Insulin Tolerance Test (ITT)

The ITT was performed to evaluate the effect of *A. squamosa* tincture on glucose response to exogenous insulin (Fig. 5). In the diabetic control group, blood glucose declined from 401.6 ± 8.96 mg/dL at 0 min to a nadir of 138.6 ± 7.09 mg/dL at 60 min (65.5% reduction) and remained relatively low at 146.0 ± 11.20 mg/dL at 120 min. The glucose response to exogenous insulin indicated that the diabetic animals retained a measurable insulin-mediated glucose-lowering response.

Administration of *A. squamosa* tincture resulted in a reduction in blood glucose during the ITT. The 100 mg/kg group showed a decline from 372.2 ± 8.35 to 150.8 ± 6.87 mg/dL at 60 min (59.5% reduction). The 200 mg/kg group showed a decline from 313.4 ± 31.48 to 119.2 ± 12.09 mg/dL at 60 min (62.0% reduction). The 400 mg/kg group demonstrated a decline from 304.0 ± 19.91 to 119.2 ± 12.09 mg/dL at 60 min (60.8% reduction), with blood glucose remaining at 120.0 ± 8.40 mg/dL at 120 min. Glibenclamide produced a greater overall glucose-lowering response, declining from 247.4 ± 10.06 mg/dL at 0 min to 105.0 ± 5.10 mg/dL at 60 min (57.6% reduction) and 87.2 ± 4.02 mg/dL at 120 min.

The area under the curve (AUC) for the ITT was calculated using the trapezoidal rule (Fig. 5). The diabetic control group showed an AUC of 31,613 mg·min/dL. The *A. squamosa* 400 mg/kg group reduced the AUC to 22,865 mg·min/dL (27.7% reduction compared with the diabetic control), while glibenclamide produced the greatest reduction, to 19,320 mg·min/dL (38.9% reduction). These findings indicate an improved glucose-lowering response to exogenous insulin in the *A. squamosa*-treated groups, particularly at 400 mg/kg. This observation is consistent with previous reports of antidiabetic activity and PTP1B inhibitory effects of *A. squamosa* extracts (Davis et al., 2012). However, specific mechanisms involving PTP1B inhibition, GLUT-4 upregulation, or other changes in insulin signaling were not directly investigated in the present study.

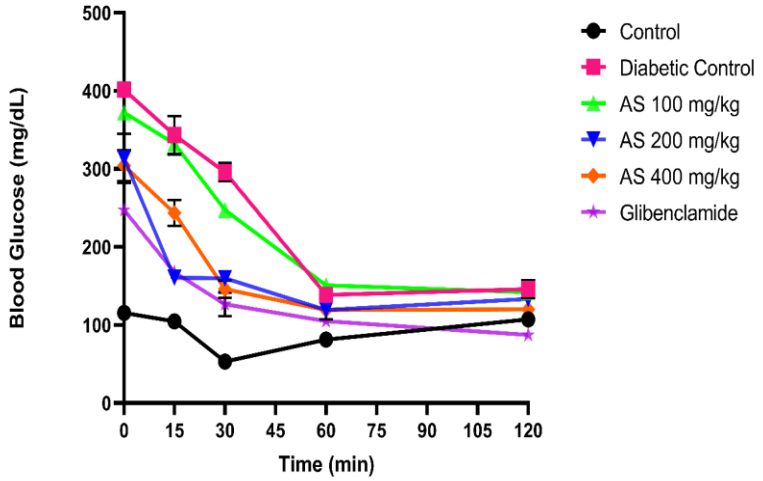

Figure 5: Effect of *A. squamosa* tincture on insulin tolerance test (ITT) in STZ-induced diabetic rats. Data are presented as mean ± SEM (n = 5). ***p < 0.001, **p < 0.01, *p < 0.05 vs. diabetic control (two-way ANOVA with Bonferroni's post-hoc test).

Correlation between OGTT, ITT, and FBG

The 400 mg/kg group showed the most favorable overall response across the measured glycemic parameters, with FBG of 296.0 ± 12.12 mg/dL, OGTT AUC of 32,438 mg·min/dL, and ITT AUC of 22,865 mg·min/dL. The concurrent improvement in FBG, OGTT response, and ITT response suggests an overall improvement in glycemic control following treatment with A. squamosa tincture. The reduction in serum glucose during the ITT, together with the increase in serum insulin observed at 400 mg/kg, may indicate improved glucose regulation; however, the specific contributions of insulin secretion and peripheral insulin sensitivity were not directly established in the present study. These findings are consistent with the reported antidiabetic effects of A. squamosa and its potential effects on insulin-related pathways (Shirwaikar et al., 2004; Ofori et al., 2022).

Effect of *A. squamosa* Tincture on Serum Biochemical Parameters

Lipid Profile

Diabetic dyslipidemia, characterized by elevated total cholesterol (TC), triglycerides (TG), and low-density lipoprotein (LDL), together with reduced high-density lipoprotein (HDL), is commonly associated with diabetes and increased cardiovascular risk (Brownlee, 2001; IDF, 2021). In the present study, diabetic control rats exhibited marked dyslipidemia, with TC (386.91 ± 11.24 mg/dL), TG (475.57 ± 13.77 mg/dL), and LDL (67.92 ± 2.54 mg/dL) increased and HDL (23.57 ± 1.27 mg/dL) reduced compared with normal controls (TC: 70.20 ± 3.40; TG: 70.20 ± 3.40; LDL: 22.38 ± 1.50; HDL: 42.75 ± 3.51 mg/dL). Treatment with A. squamosa tincture was associated with improvement in the lipid profile. The 100 mg/kg group showed LDL, HDL, TG, and TC values of 56.90 ± 1.85, 26.90 ± 1.10, 397.00 ± 5.04, and 305.75 ± 8.89 mg/dL, respectively, while the 200 mg/kg group showed values of 47.25 ± 1.94, 35.05 ± 1.48, 305.75 ± 8.89, and 269.88 ± 10.12 mg/dL, respectively. The 400 mg/kg group showed the most favorable lipid profile, with LDL of 33.88 ± 2.03 mg/dL, HDL of 44.52 ± 1.30 mg/dL, TG of 175.05 ± 9.51 mg/dL, and TC of 160.70 ± 9.88 mg/dL. Compared with the diabetic control, the 400 mg/kg group showed a 50.1% reduction in LDL and an 88.9% increase in HDL. LDL at 400 mg/kg was comparable to that observed with glibenclamide (33.05 ± 2.07 mg/dL), while HDL was higher than that in the glibenclamide group (38.12 ± 2.51 mg/dL). These

findings indicate that A. squamosa tincture was associated with an improved lipid profile, particularly at 400 mg/kg. Previous studies have suggested that polyphenolic constituents of medicinal plants may contribute to improvements in lipid metabolism; however, the specific mechanisms responsible for the observed effects were not investigated in the present study.

Total Protein

Serum total protein reflects aspects of nutritional and metabolic status (Brownlee, 2001). Diabetic control rats showed reduced total protein (5.38 ± 0.20 g/dL) compared with normal controls (7.22 ± 0.17 g/dL). Treatment with A. squamosa tincture was associated with an increase in serum total protein, with values of 5.83 ± 0.17, 6.25 ± 0.27, and 6.85 ± 0.13 g/dL at doses of 100, 200, and 400 mg/kg, respectively. The 400 mg/kg group approached the value observed in the normal control and glibenclamide groups (7.08 ± 0.14 g/dL). The 400 mg/kg treatment represented a 27.3% increase compared with the diabetic control. These findings suggest an improvement in the altered protein status associated with diabetes following treatment with A. squamosa tincture.

Serum Insulin

Serum insulin levels were reduced in the diabetic control group (1317.25 ± 34.78 pg/mL) compared with the normal control group (1575.75 ± 15.72 pg/mL). Treatment with A. squamosa tincture increased serum insulin levels, with values of 1316.50 ± 29.97, 1346.25 ± 31.27, and 1446.50 ± 29.40 pg/mL at doses of 100, 200, and 400 mg/kg, respectively. The 400 mg/kg dose produced a 9.8% increase in serum insulin compared with the diabetic control, while glibenclamide showed the highest insulin level (1500.00 ± 11.53 pg/mL). The increase in serum insulin at 400 mg/kg, together with the improved FBG and OGTT response, suggests a possible improvement in glycemic regulation following treatment with A. squamosa tincture. This finding is consistent with previous reports describing insulin-releasing effects of A. squamosa preparations (Shirwaikar et al., 2004; Ofori et al., 2022). Ofori et al. (2022) further reported insulin-secretory activity of A. squamosa polyphenolic constituents in BRIN-BD11 cells and isolated mouse islets.

Table 2: Effect of ***AS*** on Biochemical Parameters in STZ-Induced Diabetic Rats

Experimental Group	LDL (mg/dL)	HDL (mg/dL)	TG (mg/dL)	TC (mg/dL)	Total Protein (g/dL)	Insulin (pg/mL)
Control	22.38 ± 1.50	42.75 ± 3.51	70.20 ± 3.40	70.20 ± 3.40	7.22 ± 0.17	1575.75 ± 15.72
Diabetic Control	67.92 ± 2.54	23.57 ± 1.27	475.57 ± 13.77	386.91 ± 11.24	5.38 ± 0.20	1317.25 ± 34.78
AS (100 mg/kg)	56.90 ± 1.85	26.90 ± 1.10	397.00 ± 5.04	305.75 ± 8.89	5.83 ± 0.17	1316.50 ± 29.97
AS (200 mg/kg)	47.25 ± 1.94	35.05 ± 1.48	305.75 ± 8.89	269.88 ± 10.12	6.25 ± 0.27	1346.25 ± 31.27
AS (400 mg/kg)	33.88 ± 2.03	44.52 ± 1.30	175.05 ± 9.51	160.70 ± 9.88	6.85 ± 0.13	1446.50 ± 29.40
Glibenclamide	33.05 ± 2.07	38.12 ± 2.51	103.45 ± 9.83	103.45 ± 9.83	7.08 ± 0.14	1500.00 ± 11.53

Values expressed as Mean ± SEM (n = 5); AS = Annona squamosa; TG = Triglycerides; TC = Total Cholesterol

Overall Interpretation

The integrated findings indicate that *A. squamosa* tincture at 400 mg/kg produced the most favorable overall improvement in glycemic parameters, including FBG, OGTT, and ITT responses, together with an increase in serum insulin levels. The 400 mg/kg treatment was associated with a 9.8% increase in serum insulin, a 23.0% reduction in OGTT AUC, and a 27.7% reduction in ITT AUC compared with the diabetic control group. These findings suggest an overall improvement in glycemic regulation following treatment with *A. squamosa* tincture; however, the specific contributions of enhanced insulin secretion and

improved insulin sensitivity were not directly established in the present study. The treatment also improved the lipid profile, with reductions in LDL and increases in HDL, and increased serum total protein levels compared with the diabetic control group. These findings suggest a beneficial effect of *A. squamosa* tincture on several metabolic abnormalities associated with diabetes. The 400 mg/kg dose produced improvements in several parameters, although glibenclamide generally showed greater glycemic efficacy. The higher HDL level observed with the 400 mg/kg tincture compared with glibenclamide indicates a favorable lipid-profile response; however, cardiovascular protection was not directly evaluated in the present study.

HISTOPATHOLOGICAL EXAMINATION

Pancreas

Histopathological examination of pancreatic tissue demonstrated normal architecture in the control group, characterized by intact islets of Langerhans and well-organized acinar cells. In contrast, the diabetic control group exhibited marked pathological alterations, including degeneration of pancreatic cellular architecture, inflammatory cell infiltration, acinar cell damage, and distortion of the pancreatic tissue.

Treatment with the standard drug significantly improved pancreatic morphology, showing restoration of islet architecture with minimal inflammatory changes. Administration of *Annona squamosa* tincture at 400 mg/kg also produced noticeable improvement in pancreatic morphology, evidenced by reduced cellular degeneration, decreased inflammatory infiltration, and partial restoration of pancreatic tissue architecture.

Comparative evaluation demonstrated that treatment with *A. squamosa* tincture produced measurable improvement compared with the diabetic control group, while the standard drug showed the greatest degree of morphological restoration.

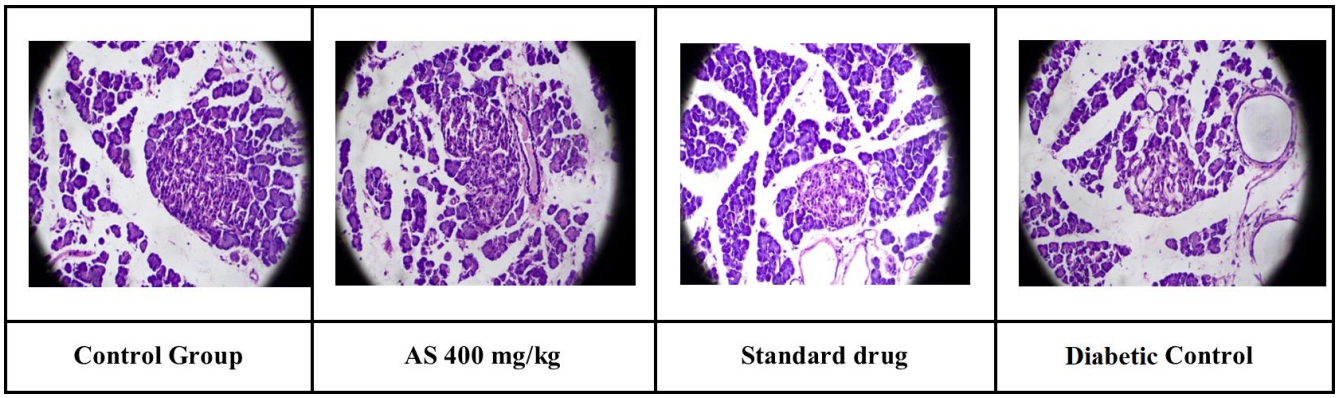

Figure 6. Histopathological evaluation of pancreatic tissues in different experimental groups. (H&E staining, 400x magnification).

CONCLUSION:

Annona squamosa tincture demonstrated antidiabetic potential by improving glycemic parameters, including fasting blood glucose, glucose tolerance, and the response to exogenous insulin, together with an increase in serum insulin levels. The tincture also improved the lipid profile and serum total protein levels and was associated with improved pancreatic morphology. Among the tested doses, 400 mg/kg produced the most favorable overall response, although glibenclamide generally showed greater glycemic efficacy. These findings support *A. squamosa* tincture as a promising natural candidate for further investigation in the management of type 2 diabetes.

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