



In vitro Anti-diabetic and Anti-oxidative Evaluation of Hydro-methanol Bark Extract of *Bauhinia acuminata* (L.)

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ABSTRACT

Diabetes mellitus (DM) is a multifactorial disease of significant public health concern globally. This study aimed to assess the *in-vitro* effect of hydro-methanol (3:2) *Bauhinia acuminata* stem bark extract on diabetes and diabetes-linked oxidative stress to develop an herbal-based product against diabetic complications. Qualitative analysis, TLC, and LC-MS were performed to determine the phyto molecule(s) in the extract. Diabetes was induced in rats by a single intramuscular injection of streptozotocin. The liver, kidney, small intestine, heart, and skeletal muscle were dissected from the control and diabetic groups on the 29th day of streptozotocin injection and placed immediately into the *in-vitro* media and dosed with the extract except for the control and untreated diabetic groups. Carbohydrate metabolic enzymes, anti-oxidative markers, and general toxicity sensors were evaluated. Dosing of extract at 10, 20, and 40 mg/10 mL of *in-vitro* media reduced the activity of glucose-6-phosphatase significantly ($p < 0.05$), but hexokinase activity in liver, skeletal, and cardiac muscle were unchanged compared with the untreated diabetic group. Inhibition in enteric alpha-glucosidase activity was noted. The activities of antioxidant enzymes and thiobarbituric acid reactive substances level were recovered significantly ($p < 0.05$) in the hepatic and renal tissues after extract treatment compared to the untreated diabetic group. Glutamate oxaloacetate transaminase and glutamate pyruvate transaminase activities were reduced in the tissues of treated groups compared to the untreated diabetic group. This study showed that at 1mg/ml, the extract exhibited significant effects on the metabolic function of diabetic rat tissues with an increase in endogenous antioxidant levels.

Keywords: *Bauhinia acuminata* (L), Hydro-methanol extract, Anti-diabetic, Anti-oxidative, Liquid chromatography-mass spectrophotometry

Introduction

Diabetes Mellitus (DM) is a multifactorial growing disease cum syndrome throughout the world.¹ Genetic factors combined with environmental factors, lifestyle, and food style changes are the major causes of the increasing prevalence of DM.² Globally, out of 534 million diabetic people, India has reported about 72.4 million diabetes and obtained the second rank after China.³ Type I Diabetes is a major autoimmune endocrine disorder that developed due to the destruction of pancreatic beta cells, which alters carbohydrate, protein, and fat metabolism.⁴ Low or lack of insulin secretion or resistance to insulin causes continual high blood glucose levels with multiple common symptoms like polydipsia, polyphagia, polyuria, and chronic infection.⁵ DM is associated with persistent hyperglycemia that stimulates the generation of excessive amounts of Reactive Oxygen [O_2^- , OH, O=O] Species (ROS) by activating a variety of metabolic pathways, including glucose auto-oxidation, the nonenzymatic glycation end products generation process, and the polyol pathway.⁶

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Oxidative stress-induced apoptosis is caused by an increased concentration of highly reactive unpaired radicals that govern the formation of chronic macro and microvascular complications, including retinopathy, nephropathy, neuropathy, cardiovascular issues, and skin ulcers.⁷

The treatment of DM involves oral anti-diabetic drugs and insulin injections with different pharmacokinetic and pharmacodynamic properties, ensuring glucose utilisation at the cellular level. However, long-term use of these drugs is associated with various side effects like oedema, lactic acidosis, constipation, and drug resistance, which are very common.⁸ Considering these reasons, people are seeking alternative drug therapies, including medicinal herbs or shrubs or plants, which are less toxic, cheap, and have little or no side effects compared to synthetic oral anti-diabetic drugs. *Bauhinia acuminata* (L) (Shet Kanchan) is a herbal plant that belongs to the Fabaceae family, generally grows all over India in mildly acidic soil, have potent pharmacological properties including anti-bacterial, anti-diarrhoeal and membrane stabilising activity.⁹ It has been stated that the methanol extract of *B. acuminata* bark has promising anti-diabetic activity, tested on Swiss albino female mice, considering only the blood glucose parameter, but no extensive study in this line is available.¹⁰ No study has yet focused on its direct effect on carbohydrate metabolic enzymes in diabetic rats. Diabetes-associated carbohydrate metabolic enzyme and oxidative stress management using phyto molecules as safe therapeutic agents could be a pivotal strategy to control raised blood glucose levels in diabetes.

This study investigated the *in-vitro* effects of hydro-methanolic (3:2) extract of *B. acuminata* stem bark on anti-diabetic and anti-oxidative key enzyme activities in the targeted tissues in streptozotocin-induced diabetic male albino rats, to provide information for the development of

an herbal-based product to manage diabetes and its oxidative stress complications.

Materials and Methods

Chemicals and reagents

Methanol (99%)(Thermo Fisher Scientific India Pvt. Ltd, India); Glucose-6-phosphate dehydrogenase (>98%) (Tokyo Chemical Industry Pvt. Ltd, India), Nicotinamide adenine diphosphate (95%), Adenosine triphosphate (99%), Alpha-ketoglutarate (99%), Alanine (99%) (Sisco Research Laboratories Pvt. Ltd, India), Pyrogallol (98%), Thiobarbituric acid (98%) (LOBA Chemie Pvt. Ltd, India); Trichloro acetic acid (99%), Aspartate (99%), 4-nitrophenyl- β -D-glucopyranosiduronic acid (98%) (Merck Ltd, Germany); hydrogen peroxide (30%) (SD fine chem Ltd, India) were used in this study. All these chemicals were of analytical grades.

Plant materials

Bauhinia acuminata (L) or Shet Kanchan or Dudh Kanchan stem bark was collected from May to June 2023, in the summer season, from the naturally growing matured Kanchan plant, at Vidyasagar University campus, Arts & Commerce Block, Midnapore, West Bengal 721102, located between Latitude 22.27863° and Longitude 87.37986°. It was authenticated by the taxonomist at the Botany and Forestry Department, Vidyasagar University (voucher number- *B. acuminata*/VU/BIO/08/22 VU). The fresh bark was washed and cleaned in tap water to remove dirt as much as possible. The plant part was allowed to dry up for about fourteen days in the shade and ground in an electric grinder to prepare a coarse powder.

Extraction procedure

From our pilot study, it has been noted that out of the different solvent extracts of the bark of this plant, hydro-methanol is most effective for this purpose. Hydro-methanol solvent at a ratio of 3:2 was used as the menstruum. About 75 g of the powdered plant material was macerated in 600 mL distilled water and 400 mL methanol mixture in a 5-litre airtight glass container. The material was kept for 48 hours at average room temperature, shaken, and stirred intermittently at an interval of 2 hours. The crude extract was double-filtered with cotton and Whatman No-1 filter paper. The filtrate was concentrated in a rotary evaporator (N-1200A; Shanghai EYELA. Co., Ltd; China) at 42 °C under reduced pressure at 0.08 MPa and finally lyophilised using a lyophilizer (EYELA FDU- 1200; Factory of Rikakikai Co., Ltd; Japan) to obtain the extract in dry powder form.¹¹

Animal care

Twelve Wistar strain male albino inbred rats were purchased from Saha Enterprise, Kolkata-700051 (license no-1828PO/BT/S/15/CPCSEA). The age of each rat was about 1.5 months, with an average body weight of 100±10 g. All the rats were normoglycemic, having fasting blood glucose levels of 75±5 mg/ dl. Rats were acclimated for ten days to adapt to the new environment before starting the experiment. Clean and dry polypropylene-made broad square mesh cages were used to prevent coprophagy nature. Each cage contained a maximum of six animals. During the study phase, rats were housed in a well-ventilated room, with a relative humidity of 50-60 %, temp: 25 °C, and 12 hr:12hr dark-light cycles. All animals were allowed free access to water and food.

Ethical statement

All the conditions related to rats were maintained per Committee for Control and Supervision of Experiments on Animals (CCSEA) guidelines, Govt. of India. The experimental design was approved by our Institutional Animal Ethics Committee (IAEC), with an ethical approval number -VU/IAEC/CPCSEA/9/7/2022, dated 22.11.2022.

Induction of diabetes mellitus

After the acclimatisation of the experimental rats having a body weight of 120±10 g, six rats were kept overnight (8-12 hours) in fasting condition, and those rats were given a single intramuscular injection of streptozotocin (Sigma Aldrich, Germany) at a concentration of 4 mg/0.1 mL in 0.1 (M) citrate buffer (pH-4.5)/100 g body weight for the

induction of diabetes.¹² Streptozotocin (STZ) is a glucosamine nitrosourea compound synthesised by *Streptomyces achromogenes* that selectively damages the DNA of pancreatic beta cells.¹³ On the seventh day of intramuscular injection of STZ, blood was collected by pricking the tail vein in a fasting condition to confirm the diabetic state of experimental rats. Rats with blood glucose levels ≥ 250 mg/dl in fasting conditions were considered diabetic model animals for this investigation. The experiment was conducted for the next 28 days, and fasting blood glucose (FBG) was taken at 7-day intervals, using On Call Plus (OCP) 10s Glucometer (ACON Biotech Hangzhou Co., Ltd., China).

Experimental design

On the 29th day after diabetes development with STZ injection, the animals were sacrificed by Euthanasia. Vital metabolic tissues, i.e., liver, renal, musculoskeletal, cardiac muscle, and intestine, were dissected from each control and diabetic rat. The isolated tissues were washed in 0.9 % saline, sliced into tiny pieces, and placed in different test tubes containing 10 mL Krebs Ringer Bicarbonate (KRB) buffer solution (pH-7.6). The KRB solution (1000 mL) consists of 8 g NaCl (0.125 M), 0.2 g KCl (0.0025 M), 50 mg Na₂HPO₄ (0.0012 M), 0.2 g CaCl₂ (0.002 M), 100 mg MgCl₂ (0.001 M), 1 g NaHCO₃ (0.025 M), and 1 g glucose (0.025 M). The test tubes were grouped as control, untreated diabetic, and extract-treated diabetic. The hydro-methanolic extract of *B. acuminata* bark was dosed into test tubes marked as extract-treated diabetic groups in concentrations of 10, 20, and 40 mg/10 mL *in-vitro* media. Every test tube was placed in an incubator at 37 °C with a continuous supply of 95% O₂ and 5% CO₂ mixture at a rate of 30 bubbles/ minute for two hours. In each test tube, 7-8 tissue slices were placed at the size of 5×4×3 mm and were exposed as per control, untreated diabetic, and diabetes with extract treatment.¹⁴

Parameters investigation

All tissue samples, i.e., hepatic, kidney, small intestine, skeletal, and cardiac muscles, were washed in normal saline. Homogenates of these tissues were prepared separately using method-specific buffer [phosphate buffer saline (pH-7.4) or tris-HCL buffer (pH-7.0) or maleate buffer (pH-6.5)] in homogeniser (B-1244, Bio-Lab, India). The supernatant was collected through centrifugation (Z216MK, HERMLE, Germany) at 10000 g for 15 minutes at 4 °C.

Glucose-6-phosphatase (G-6-phosphatase)

Glucose-6-phosphatase activity in the liver, skeletal muscle, and cardiac muscle was measured spectrophotometrically (Evolution 201, Thermo Fisher, China), and the absorbance was noted at 340 nm using the standard protocol. Glucose-6-phosphate (0.1 M) as substrate solution, maleate buffer (0.5 M), target tissue homogenate (50 mg/mL phosphate-buffered saline or PBS), and 10 % TCA was used to stop the reaction in this method. This enzymatic activity was expressed in the unit of mg of inorganic phosphate liberated/ g of tissue.¹⁵

Hexokinase

Liver, skeletal, and cardiac muscles were used to measure hexokinase activity. Following the standard protocol, 60 μ L target tissue homogenate (50 mg/mL PBS) was mixed with 900 μ L assay mixture [glucose (3.7 mM), MgCl₂ (7.5 mM), thioglycerol (11 mM), HEPES (45 mM) (pH-7.5)], 10 μ L 10% (w/v) glucose-6-phosphate dehydrogenase, 10 μ L 2.5% (w/v) nicotinamide adenine dinucleotide phosphate (NADP), and 30 μ L 0.022 M adenosine triphosphate (ATP), were used for spectrophotometric analysis to record the O.D at 340 nm. The enzymatic activity was presented in the unit of μ g/ mg of tissue.¹⁶

Alpha-glucosidase

The percentage of inhibition in α -glucosidase enzyme activity was assessed by the standard method with some modification. In this method, 200 μ L assay mixture containing 100 mM maleate buffer (pH-6.0) and 2% (w/v), para-nitrophenyl glucopyranoside (pNPG) mixed thoroughly with 50 μ L enzymatic solution of α -glucosidase [1 g intestinal tissue/ mL maleate buffer (pH-6.0)], prepared from rat's small intestinal part (below the duodenum and above the cecum). The percentage of inhibition was calculated by the formula [(Abs Control – Abs Sample)/ Abs Control] \times 100.¹⁷

Catalase (CAT)

Catalase activity was assessed using the specific target tissues (liver and kidney tissues) as per the standard method.¹⁸ Homogenised tissue samples [50 mg/ mL 0.05 (M) tris-HCL, pH-7.0] were centrifuged at 10000 g at 4°C for 10 minutes. The tissue supernatants were collected, mixed with 30% H₂O₂ and used for spectrophotometric analysis at 240 nm wavelength for three minutes at 30-second intervals. The result was expressed as mM of H₂O₂ consumption/ mg of tissue/ min.

Super-Oxide Dismutase (SOD)

The activity of superoxide dismutase was measured using 20 µL targeted tissue (liver and kidney tissues) supernatant [50 mg/ml PBS (pH-7.4)], mixed with 2 mL PBS and 20 µL pyrogallol (10 mM) in a 3.5 mL cuvette. The absorbance was noted in the spectrophotometer at 420 nm for 180 seconds at every 30-second interval. The activity of SOD was stated as unit/ mg of tissue. One unit = reaction rate of SOD that inhibits pyrogallol auto-oxidation.¹⁹

Quantification of Thiobarbituric Acid Reactive Substances (TBARS)

Ice cold PBS was used to homogenise hepatic and kidney tissues at 50 mg/mL concentration, and 0.5 mL homogenate was mixed with 2 mL thiobarbituric acid-trichloroacetic acid (TBA-TCA) mixture and 0.5 mL of 0.9% NaCl. The mixture was centrifuged at 10000 g for 5 minutes at 4°C. The prepared supernatant was collected and used in a spectrophotometer to quantify TBARS at 540 nm. The outcome was stated as nM/ mg of tissue.²⁰

Measurement of Glutamate Oxaloacetate Transaminase (GOT) and Glutamate Pyruvate Transaminase (GPT) activities

In this assay, 1 mL substrate solution [(1.33 g aspartate + 15 mg α-ketoglutarate)/ 20.5 ml PBS (pH-7.5) for GOT, and (1.78 g alanine + 30 mg α-ketoglutarate)/ 20 mL PBS (pH-7.5) for GPT activity assessment] was mixed with 0.2 mL hepatic or renal tissue homogenate (50 mg/mL PBS) separately. Then 1 mL of 1 mM DNPH (2,4-dinitrophenylhydrazine), reaction arresting reagent was added, and the O.D was determined at 520 nm wavelength in the spectrophotometer. The enzymatic activities of GOT and GPT were expressed as Unit/ mg of tissue.²¹⁻²²

Qualitative Phytochemical analysis of hydro-methanolic *B. acuminata* bark extract

Qualitative phytochemical screening of crude extract of *B. acuminata* was conducted using the standard protocol.²³ The presence of anthraquinone, tannin, phenol, alkaloid, terpenoid, steroid, saponin, and glycoside in the extract were determined. Colour formation, layer development, and precipitation were the critical determinants for analytical testing.

Thin-layer Chromatographic analysis

Silica gel TLC plates (F₂₅₄) were used as the stationary phase, while ethyl acetate, methanol, and distilled water mixture in the ratio of 1.61:

0.21: 0.16 was used as the mobile phase. The gel plate was dried and observed under a UV analyser (Eastern Instruments, Wilmington) at 254 nm.²⁴

Screening of hydro-methanolic *B. acuminata* bark extract by Liquid Chromatography- Mass Spectrophotometry (LC-MS) study

Screening of phytochemicals present in the hydro-methanol extract of *B. acuminata* bark was conducted using a Quattro Micro™ API (Waters, Milford, Massachusetts, USA) mass spectrophotometer. This spectrophotometer consists of a column of Silica gel coat as the stationary phase, temperature 25°C; quaternary pump for solvent moving through the column (mobile phase: water, acetonitrile and methanol=94 : 2: 4, flow rate-0.3 ml/min); vacuum interface for ionisation of macromolecules with solvent vaporisation (ES⁺, ES⁻); auto-sampler for sample analysis (sampling rate-10 points/sec), and photodiode array (PDA) detector, transferring data to Waters MASS LYNX 4.1 software at the wavelength range 190-690 nm. This technique requires a total of 30 minutes to complete the process. Data was obtained using MS scanning with a 100-1900 (m/z) scan range.²⁵

Statistical analysis

Values were stated as Mean ± SEM, n = 6. ANOVA followed by "Multiple Comparison Student's Two tail 't'-test" was adopted.²⁶

Results and Discussion

One of the major causes of post-prandial hyperglycemia is increased α-glucosidase activity, a brush border enzyme of the intestine that catalyses the hydrolysis of starch.²⁷ The methanol extract of *B. acuminata* bark exhibited a dose-dependent inhibition of α-glucosidase activity. The extract's inhibitory activity of α-glucosidase was assessed by determining IC₅₀ values compared to acarbose, having 46.15 mg/ mL and 23.09 mg/ mL, respectively, as shown in Figure 2. The secondary metabolites present in the extract may bind competitively or noncompetitively with the enzyme or enzyme-substrate complex or inhibit the enzyme noncompetitively by attaching to its allosteric site.²⁸

After 28 days of STZ injection, glucose-6-phosphatase activity was increased, and hexokinase activity was decreased in the liver, skeletal, and cardiac muscle tissues of the untreated diabetic group compared to the control. This may be explained by inadequate insulin, activation of cyclic AMP, and a greater ratio of ATP to Mg²⁺, which increased glucose-6-phosphatase and glucose-6-phosphate translocase activities.²⁹ Each of these factors markedly raised the activity of glucose-6-phosphatase and inhibited the activity of hexokinase.³⁰⁻³¹ These findings were compatible with our present study. Dosing hydro-methanolic *B. acuminata* stem bark extract at three different doses to the tissues caused a significant reduction ($p < 0.05$) in the glucose-6-phosphatase activity in the extract-treated diabetic groups. However, no significant changes were noted in hexokinase activity with respect to the untreated diabetic group (Figure 1B).

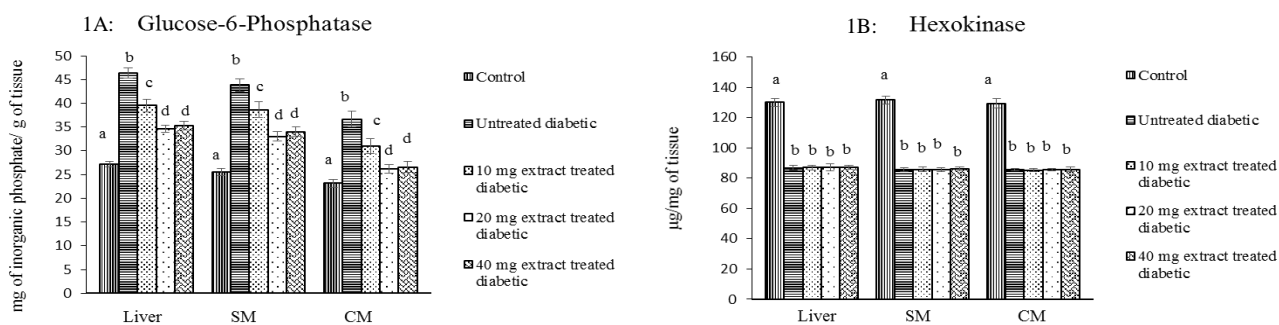


Figure 1: Direct effects of hydro-methanol (3:2) extract at different doses of *B. acuminata* bark on the activities of glucose-6-phosphatase (1A) and hexokinase (1B) in the liver, skeletal muscle (SM), and cardiac muscle (CM) in the male albino rat. Values were expressed as Mean ± SEM, n = 6. ANOVA followed by "Multiple Comparison Student's Two tail 't'-test". Bars with different superscripts (a,b,c & d) for glucose-6-phosphatase differ from each other significantly, $p < 0.05$. In the case of hexokinase, no significant difference has been noted among the groups, $p > 0.05$.

The inhibition in glucose-6-phosphatase activity was most significant in the 20 mg extract-treated diabetic group, and no significant difference ($p > 0.05$) was observed between 20 and 40 mg doses of the extract, presented in Figure 1A. This ameliorative effect of the extract on glucose-6-phosphatase activity may be linked with the competitive or non-competitive inhibition in the enzyme activity directly or inhibition in enzyme-linked substrate transporter protein T1 and T2, as reported earlier with other plants.³²⁻³³ The reason behind the unchanged activity of hexokinase in extract-charged groups may be because the phytoconstituents in the bark extract have no direct ability to modulate hexokinase activity or are relatively less specific to this particular enzyme activation in such laboratory conditions.

Activities of CAT and SOD were reduced significantly ($p < 0.05$) in STZ-induced untreated diabetic tissues, which may be due to the excessive ROS generation, and micro-nutrient deficiencies resulting from increased fluid loss and altered protein metabolism,³⁴⁻³⁵ which served as co-factors for such enzymes. Two-hour exposure of hepatic and renal tissues with 10, 20, and 40 mg of the extract significantly increased both enzyme activities ($p < 0.05$). Maximum attenuation of CAT and SOD activities was noted in the 20 mg extract-treated group compared to the 10 mg extract-treated group (Figure 3A and Figure 3B). The recovery in enzyme activities may be associated with the binding of the phytochemicals as a full agonist to their particular allosteric site. It may act as a positive allosteric modulator. This mechanism is compatible with previously reported in-silico studies on phytochemicals.³⁶

TBARS, an important metabolomic marker of oxidative stress, was raised in the hepatic and renal tissues of the diabetic group, as diabetes is linked with increased lipid peroxidation.³⁷ TBARS was significantly ($p < 0.05$) elevated in the tissues of the untreated diabetic group

compared to the control. After the addition of the extract to the tissues under study, a significant reduction ($p < 0.05$) was noticed in TBARS level compared to the untreated diabetic group; possibly, the phytochemicals either directly quench these lipid peroxidation products or scavenge highly reactive hydroxyl radicals and reduced the formation of TBA-MDA adduct.³⁸⁻³⁹ Among all the extract doses, the maximum inhibition was noted in 20 mg of hydro-methanolic *B. acuminata* bark extract. No further significant reduction was observed after dose increment (Figure 4). This may be explained by the saturation-dependent effect achieved at a 20 mg dose.

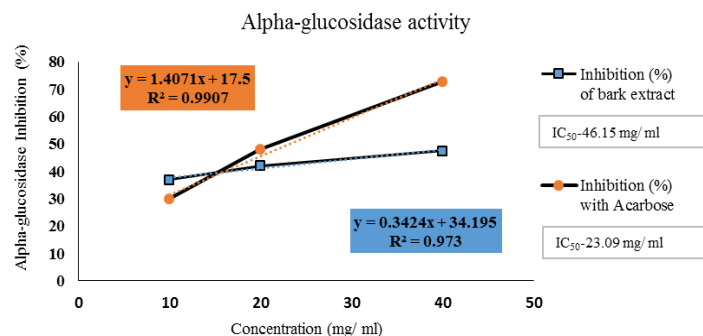


Figure 2: Effect of different doses of hydro-methanol (3:2) extract of *B. acuminata* bark on inhibiting alpha-glucosidase activity in male albino rats. IC₅₀ value for extract- 46.15 mg/ml and standard drug acarbose- 23.09 mg/ml.

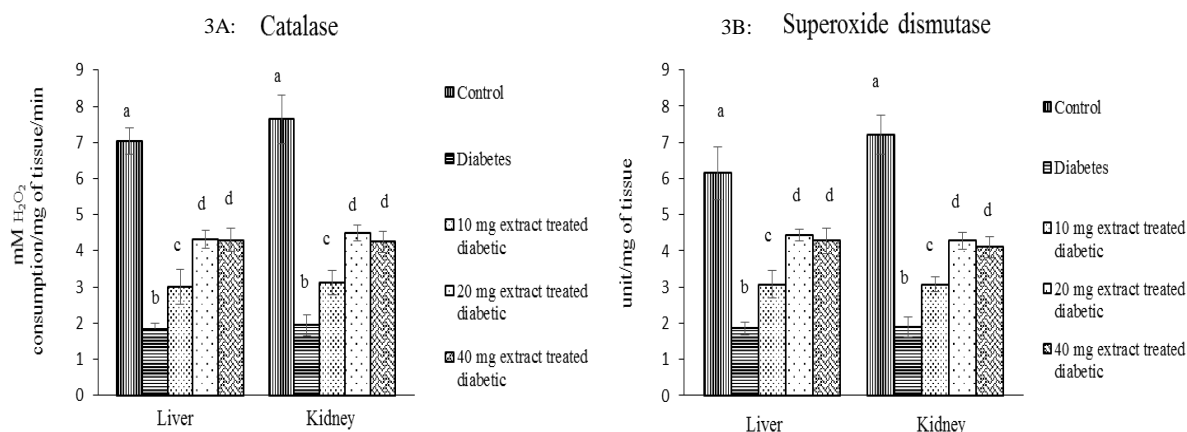


Figure 3: Dose dependent anti-oxidative effect of different doses of hydro-methanol (3:2) extract of *B. acuminata* bark on the activities of catalase (3A) and superoxide dismutase (3B) in hepatic and renal tissues. Values were expressed as Mean \pm SEM, n = 6. ANOVA followed by "Multiple Comparison Student's Two tail 't'-test". Bars with different superscripts (a, b, c & d) differ from each other significantly, $p < 0.05$.

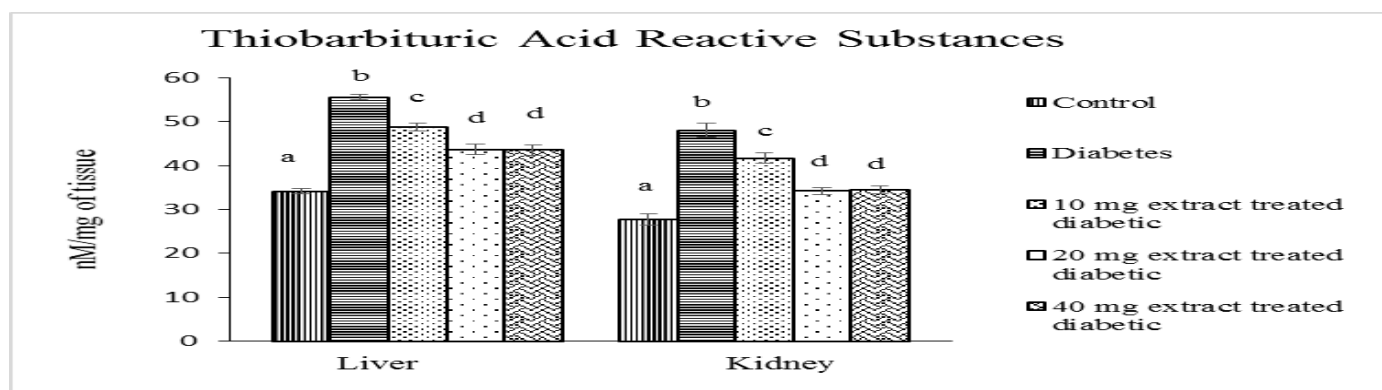


Figure 4: Levels of TBARS in the liver and kidney after charging at different doses of hydro-methanol (3:2) bark extract of *B. acuminata*. Bars indicate Mean \pm SEM (n = 6), ANOVA followed by "Multiple Comparison Student's Two tail 't'-test". Bars with a, b, c & d superscripts differ significantly from one another, $p < 0.05$.

Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) are the marker enzymes of hepatic and renal toxicity.⁴⁰ These enzyme activities in liver and kidney tissues were elevated significantly ($p < 0.05$) in the untreated diabetic group compared to the control. Both enzyme activities were increased in diabetic conditions due to the oxidative-stress-induced inflammation that raises the secretion of pro-inflammatory cytokines and reduces anti-inflammatory adiponectin. These factors are directly linked with increased GOT and GPT activities.⁴¹⁻⁴² Dosing of hydro-methanolic bark extract of *B. acuminata* resulted in a significant reduction ($p < 0.05$) in the said enzyme activities (Figure 5A & 5B) in diabetic extract-treated groups against the untreated diabetic group. The inhibition of these enzymes with the extract may be through blocking pyridoxal phosphate or stabilising the plasma and endosomal membrane that prevents their release from the intracellular part to the extracellular region. This mechanism is consistent with previous findings.⁴³⁻⁴⁴

Qualitative analysis of phytochemicals showed the presence of flavonoids, alkaloids, terpenoids, tannins, and phenols (Table 1). Five bands or spots were separated in TLC with R_f values 0.5, 0.6, 0.64, 0.7, and 0.8 (Figure 6). LC-MS analysis of hydro-methanolic bark extract of *B. acuminata* revealed the presence of 14 phyto-compounds, as shown in Table 2. The peaks of mass to charge ratio (m/z) were 199.92, 218.47, 269.38, 278.17, 292.37, 306.16, 336.51, 392.19, 438.20, 499.78, 631.98 [in (+) Electrospray Ionization (ESI)] and 174.53, 490.01, 625.78 [in (-) ESI]. The obtained mass spectrum of the respective phytochemicals is presented in Figure 7.

Regarding the possible mode of nongenomic actions, phytochemicals worked on the target tissues quickly under *in-vitro* conditions. Three hypotheses may be postulated. The first hypothesis is enzymatic inhibition (competitive, non-competitive, un-competitive, allosteric binding), the second is membrane stabilisation, and the third alternative

includes the prediction of target receptor saturation cum spare receptor concept. All these hypotheses are crucial steps for any phytomolecule(s) to improve drug efficacy.⁴⁵ The direct exposure of targeted tissues with hydro-methanolic *B. acuminata* bark extract in three doses, 10, 20, and 40 mg, revealed that 20 mg is the most potent dose with the maximum target enzyme or receptor occupancy.⁴⁶ The number of unbound or spare receptors may remain at the same level at 40 mg of the herbal extract.⁴⁷ This investigation supports the extract's potential for anti-oxidative and anti-diabetic properties. There is a need to determine the exact mechanism of the activity of the phytoconstituents through extensive *in-vivo* studies.

Conclusion

The experimental results of this study concluded that 20 mg hydro-methanolic (3:2) bark extract of *B. acuminata* showed maximal efficacy for the recovery in different selected parameters and to correct toxicity in general. The use of the relevant diabetic biomarkers highlighted the promising role of the extract for diabetes and diabetes-linked oxidative stress management.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 1: Qualitative analysis of secondary metabolites present in hydro-methanol (3:2) extract of *B. acuminata* bark

Serial no	Name of Phytochemical	Determinants	Test name
1.	Flavonoid (+)	Blackish red	Ferric chloride test
2.	Alkaloid (+)	Precipitation	Dragendroff test
3.	Terpenoid (+)	Reddish brown	Salkowski test
4.	Tannin (+)	Brownish green	Ferric chloride test
5.	Phenol (+)	Bluish black	Ferric chloride test
6.	Steroid (-)	Greenish-red layer	Libermann-Burchard reaction
7.	Saponin (-)	Soapy appearance	Foam test
8.	Glycosides (-)	Browning junction	Kellar killan test
9.	Anthraquinone (-)	Pink	Brontagers test

The results were represented as (+) for presence and (-) for absence.

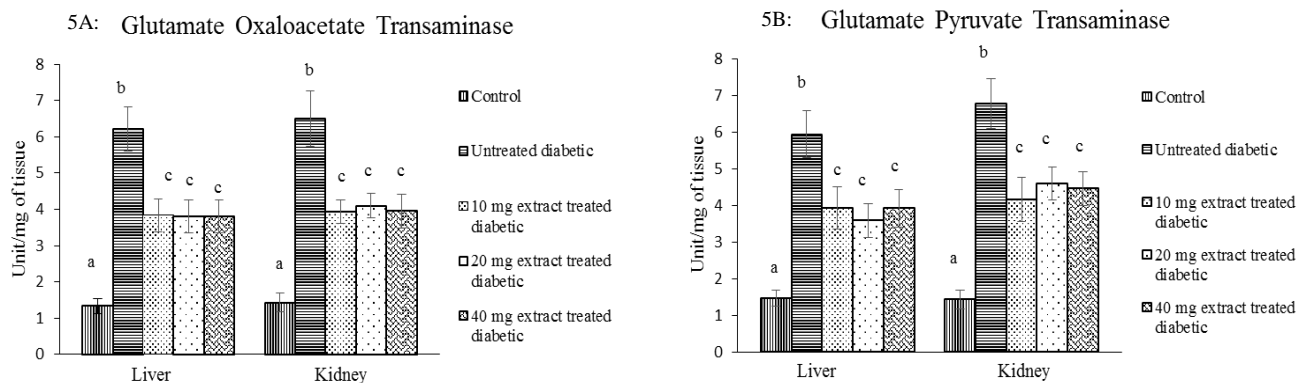
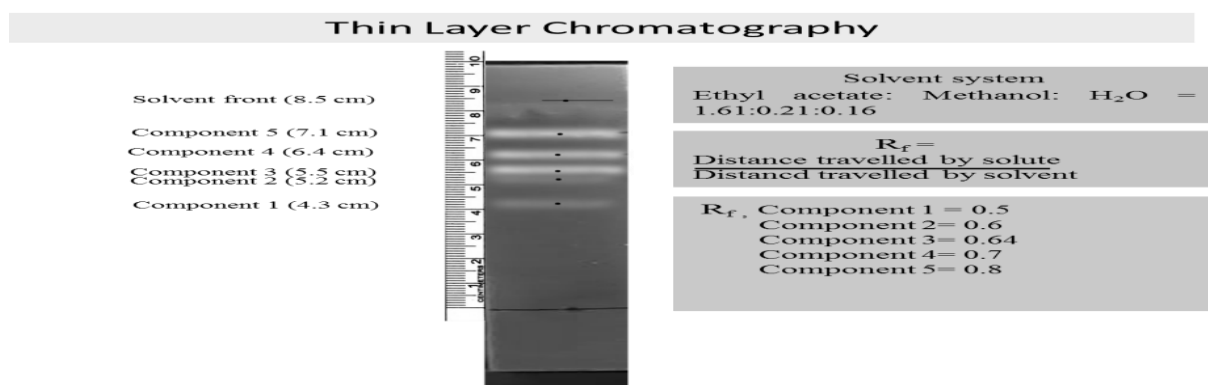
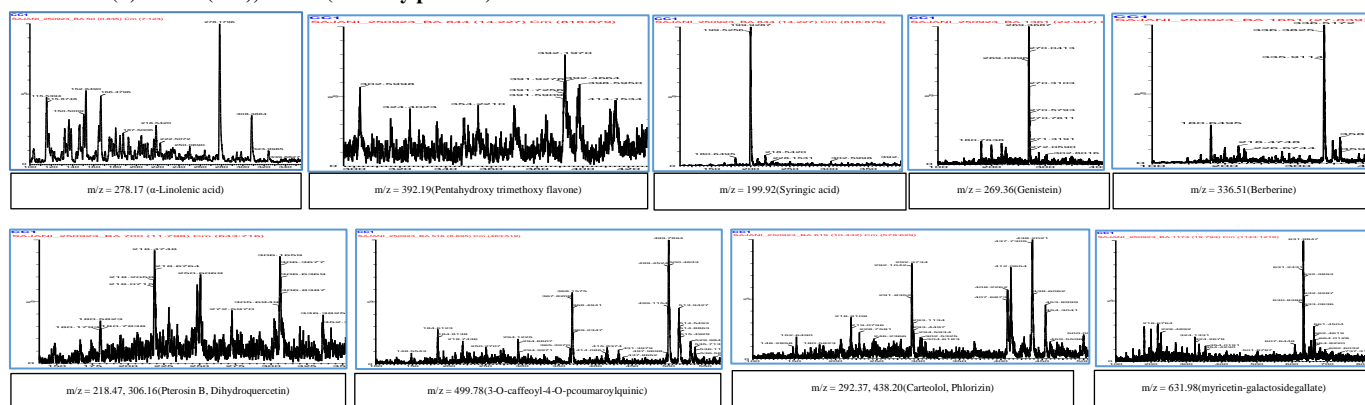
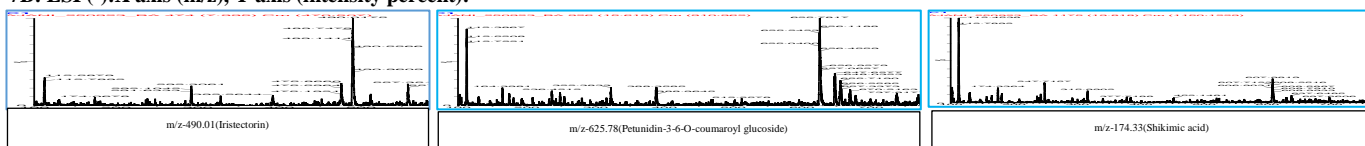


Figure 5: Assessment of GOT (5A) & GPT (5B) activities in liver and kidney tissue after treatment with different doses of hydro-methanol (3:2) extract of *B. acuminata* bark. Values were mentioned as mean \pm SEM, $n=6$. ANOVA followed by "Multiple Comparison Student's Two tail 't'-test". Bars with different superscripts, i.e. a, b & c differ from each other significantly, $p < 0.05$.

Table 2: Quantitative screening for secondary metabolites in hydro-methanol (3:2) extract of *B. acuminata* bark by LCMS analysis

SL No	ESI mode	Observed m/z	Reference m/z	RT	Proposed compound	Nature
1.	[M-H] ⁺	199.92	199.00	14.227	Syringic acid (benzoic a, cedar acid)	Phenolic
2.	[M-H] ⁺	218.40	218.29	12.644	Pterosin B	Sesquiterpenes
3.	[M-H] ⁺	269.36	269.00	22.947	Apigenin	Isoflavone
4.	[M-H] ⁺	278.17	278.00	0.835	α -Linolenic acid/ 9,12,15-Octadecatrienoic acid	Fatty acid
5.	[M-H] ⁺	292.37	292.37	10.432	Carteolol	Secondary alcohol
6.	[M-H] ⁺	306.16	305.00	11.798	Dihydroquercetin	Flavonoids
7.	[M-H] ⁺	336.38	336.36	27.839	Berberine	Alkaloid
8.	[M-H] ⁺	392.19	392.31	14.227	Pentahydroxy trimethoxy flavone	Flavonoid
9.	[M-H] ⁺	438.20	437.00	10.432	Phlorizin	Glucoside
10.	[M-H] ⁺	499.78	500.00	8.695	3-O-caffeoyl-4-O-pcoumaroylquinic	Phenolic
11.	[M-H] ⁺	631.98	631.00	19.793	Myricetin-galactosidegallate	Flavonoids
12.	[M-H] ⁻	174.53	174.15	19.818	Shikimic acid	Tannin
13.	[M-H] ⁻	490.01	491.00	7.995	Iristectorin A/B (3,5,7-trihydroxy4,6 dimethoxy isoflavone 7-Ohexoside	Isoflavone
14.	[M-H] ⁻	625.78	625.55	15.619	Petunidin-3-6-O-coumaroyl glucoside	Flavonoids

Here, m/z= mass to charge ratio, RT= retention time.

**Figure 6:** Thin Layer Chromatography of hydro-methanol (3:2) extract of *B. acuminata* bark. Here, R_f means retention factor.**7A: ESI (+): X axis (m/z), Y axis (intensity percent):****7B: ESI (-): X axis (m/z), Y axis (intensity percent):****Figure 7:** Phytomolecular screening and analysis with Liquid Chromatography-Mass Spectrophotometric study of *B. acuminata* hydro-methanol (3:2) bark extract. Mass spectrum peaks in positive ESI (7A) and negative ESI (7B) are evaluated.

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