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ORIGINAL ARTICLE

Extract of *Bauhinia vahlii* Shows Antihyperglycemic Activity, Reverses Oxidative Stress, and Protects against Liver Damage in Streptozotocin-induced Diabetic Rats

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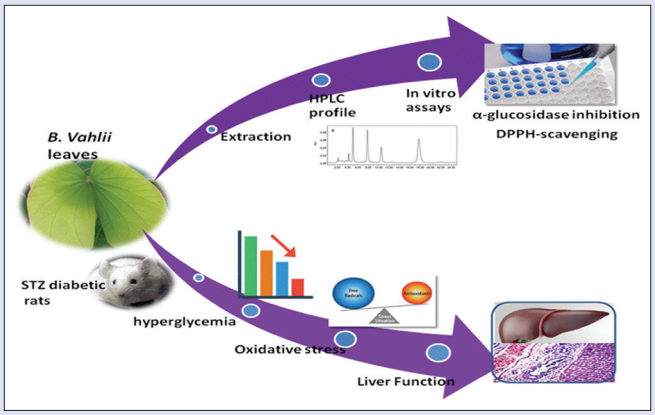
ABSTRACT

Background: Several studies have affirmed the effectiveness of some *Bauhinia* plants as antihyperglycemic agents. **Objective:** We investigated the possible effect of *Bauhinia vahlii* leaves extract in reducing hyperglycemia and reversing signs of organ damage associated with diabetes in streptozotocin (STZ) rat model. **Materials and Methods:** Both polar fraction of the *B. vahlii* leaves (defatted ethanolic extract [DEE]) and nonpolar fraction (*n*-hexane extract) were evaluated *in vitro* for α-glucosidase inhibition and 2,2-diphenyl-1-picrylhydrazyl radical scavenging potential. DEE was selected for further *in vivo* studies and was administered at two doses, i.e., 150 or 300 mg/kg to STZ-diabetic rats for 4 weeks. **Results:** Only DEE exhibited *in vitro* antioxidant and antihyperglycemic activities and its oral administration at both dose levels resulted in significant reduction in fasting blood glucose and glycated hemoglobin. Furthermore, signs of oxidative stress as indicated by hepatic reduced glutathione, nitric oxide, and malondialdehyde levels were completely reversed. In addition, histopathological examination and measurement of serum aspartate transaminase and alanine transaminase levels showed that DEE protected the liver from signs of liver pathogenesis when compared to diabetic untreated animals and those treated with metformin. Phytochemical analysis of DEE showed high flavonoids content with quercitrin as the major constituent along with other quercetin glycosides. **Conclusion:** This study strongly highlights the possible beneficial effect of *B. vahlii* leaves extract in relieving hyperglycemia and liver damage in STZ-diabetic rats and recommends further investigation of the value of quercetin derivatives in controlling diabetes and ameliorating liver damage associated with it.

Key words: Antihyperglycemic, antioxidant, *Bauhinia vahlii*, quercitrin, α-glucosidase inhibitor

SUMMARY

- The polar fraction of the *Bauhinia vahlii* leaves (defatted ethanolic extract [DEE]) exhibited both *in vitro* antioxidant activity in 2,2-diphenyl-1-picrylhydrazyl scavenging assay and strong α-glucosidase inhibition while the nonpolar fraction (*n*-hexane extract) failed to show any activity in both assays. DEE was further investigated in streptozotocin-induced diabetic rat model where oral administration of DEE at 2 doses (150 and 300 mg/kg) for 4 weeks resulted in significant reduction in fasting blood glucose and glycated hemoglobin and reversal of oxidative stress signs as indicated by measurement of hepatic reduced glutathione, nitric oxide, and malondialdehyde levels. In addition, histopathological examination and measurement of serum aspartate transaminase and alanine transaminase levels showed that DEE protected the liver from signs of pathogenesis observed in diabetic untreated rats. Phytochemical analysis of DEE showed high flavonoid content with quercitrin as the major constituent (62.9 ± 0.18 μg/mg).



Abbreviations used: ALT: Alanine transaminase, AST: Aspartate transaminase, DEE: Defatted ethanol extract, DPPH: 2,2-diphenyl-1-picrylhydrazyl, FBG: Fasting blood glucose, GAE: Gallic acid equivalent, GSH: Reduced glutathione, Hb1Ac: Glycated hemoglobin, HE: Hexane extract MDA: Malondialdehyde, QE: Quercetin equivalent, STZ: Streptozotocin, TAC: Total antioxidant capacity.

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INTRODUCTION

Diabetes mellitus, a metabolic disorder characterized by hyperglycemia, is associated with a shift in the balance between oxidants and antioxidants in favor of the former creating a metabolic status known as "oxidative stress."[1] Increased oxidative stress is not only involved in the development and progression of diabetes but also causes tissue damage to vital organs such as liver and kidney hence many complications of diabetes.[1,2] An ideal treatment of diabetes should not only control the hyperglycemia but also preferentially work to eliminate the oxidative stress associated with the disease to prevent other organs damage.

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Many plants are used in traditional medicine for the treatment of diabetes mellitus. Among these plants of known antihyperglycemic activities are different species of the genus *Bauhinia* family Fabaceae.[3] Several biological studies have affirmed the antihyperglycemic effect of some *Bauhinia* species.[3-7] For example, *Bauhinia forficata* leaves decoction exhibited glucose-lowering action in both *in vivo* and *in vitro* models.[8,9] Leaves extract of *Bauhinia variegata* expressed antihyperglycemic activity in alloxan-induced diabetic mice which was partially attributed to its insulinotropic effect due to the presence of a chloroplast protein and a secondary metabolite known as roseoside.[10,11]

Bauhinia vahlii Wight and Arnott is a huge climber, native to India. The methanolic extract of its leaves was shown to have good antioxidant activity through various *in vitro* models.[12] Recent investigation of the main secondary metabolites of *B. vahlii* leaves resulted in the identification of several antioxidant compounds including quercetin, quercitrin, hyperoside, avicularin, quercetin-3-*O*-*β*-sophoroside, and gallic acid.[13] Previous biological investigations of different organs of *B. vahlii* identified potential anti-inflammatory, antimicrobial, and antidiabetic effects.[14,15] The present study was designed to evaluate possible beneficial effect of *B. vahlii* leaves extracts in deterring diabetes symptoms and complications in streptozotocin (STZ)-induced diabetic rat model. First, most of the nonpolar secondary metabolites of *B. vahlii* leaves were extracted using *n*-hexane to obtain hexane extract (HE), whereas polar metabolites were extracted from the marc by maceration in 70% ethanol to obtain defatted ethanol extract (DEE). Second, both extracts were screened for potential antihyperglycemic and antioxidant activities adopting *in vitro* *α*-glucosidase inhibition assay and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, respectively. Based on the results obtained from these *in vitro* assays, DEE was carefully analyzed by high-performance liquid chromatography (HPLC) and the standardized extract was evaluated in STZ-diabetic rat model for antihyperglycemic and antioxidant activities. Fasting blood glucose (FBG) and plasma glycated hemoglobin (HbA1c%) levels were determined as indicatives for degree of hyperglycemia. Total antioxidant capacity (TAC) in addition to hepatic nitric oxide (NO), reduced glutathione (GSH), and malondialdehyde (MDA) levels were measured as indicators of the degree of oxidative stress. Furthermore, liver functions were assessed by measuring serum level of hepatic enzymes (alanine transaminase [ALT] and aspartate transaminase [AST]) and examining histopathological signs of liver damage.

MATERIALS AND METHODS

Plant material

Leaves of *B. vahlii* were collected during the flowering stage in May 2013 from Mohammed Ali Museum's Garden, Kasr Al-Aini, El-Manial, Egypt. The plant material was identified and authenticated by Dr. Mohammed El-Gebaly, senior botanist at National Research Center, Giza, Egypt. A voucher specimen (No. 11-6-2013s-1) was kept at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

Preparation of plant extractives

Air-dried and powdered *B. vahlii* leaves (2.9 kg) were extracted exhaustively with *n*-hexane (6 × 1.5 L) and resulted extracts were combined and evaporated under reduced pressure yielding the *n*-HE, 25 g. The defatted marc was then extracted with 70% ethanol till exhaustion (8 × 1.5 L), and the combined extracts were evaporated under vacuum yielding the DEE, 255 g. Compounds used for standardization of DEE were isolated from the ethyl acetate fraction obtained after suspending DEE residue in distilled water and performing successive liquid–liquid extraction with dichloromethane followed by EtOAc. The residue obtained after evaporation of EtOAc under vacuum was then chromatographed to isolate quercitrin, quercetin, hyperoside, and avicularin as described previously.[13]

Chemicals

Ascorbic and gallic acids were obtained from Misr Company for Pharmaceutical Industry (Cairo, Egypt). DPPH, *α*-glucosidase (Brewer's yeast, EC 3.2.1.20), *p*-NPG, acarbose, and STZ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Metformin was obtained from Chemical Industries Development Co., Giza, Egypt. All other chemicals used in the *in vivo* study were of the highest purity and analytical grade. Acetonitrile and methanol were of HPLC grade and were purchased from Sigma-Aldrich, Steinheim, Germany.

Apparatus and equipment

Tecan Infinite F50 microplate reader was used for spectrophotometric measurement for the DPPH scavenging assay. Absorbance in total phenolics and flavonoids contents and *α*-glucosidase inhibition assays were determined using Spectramax 340 microplate reader. Agilent Technologies 1260 series HPLC system equipped with a quaternary pump, degasser G1322A. and a diode array detector (DAD) was used for HPLC analysis. Separation was achieved using an Eclipse XDB-C18 column (150 mm × 4.6 mm, particle size 5 µm) and Agilent Chemstation software (Hewlett-Packard, Germany) was utilized for data acquisition and processing.

Total phenolics and flavonoids contents

The total phenolic content of HE and DEE was assessed using Folin–Ciocalteu colorimetric method described previously.[14] Results were expressed as gallic acid equivalents (GAE) per milligram of extract through calibration curve plotted using serial dilutions of gallic acid (5–50 µg/ml). The flavonoids content of *B. vahlii* leaves was assessed spectrophotometrically through complexing reaction with AlC_{l3} (0.1 M).[14] A standard calibration curve of quercetin was established at a concentration range of 5–50 µg/ml and results were expressed as quercetin equivalents per milligram of the extract.

High-performance liquid chromatography fingerprinting of active extractives

DEE (5 mg) was dissolved in 2 ml methanol and filtered through a 0.2 µm membrane filter. Samples were analyzed through injection of 20 µl and separation was achieved applying a 32 min gradient of acetonitrile (A) and 0.1% acetic acid (B) at a flow rate of 1 ml/min as follows: 10%–15% A (0–5 min), 15%–30% A (5–28 min), and 30%–95% A (28–32 min). Elution of different metabolites was detected using DAD set at 280, 310, and 340 nm. For identification of different peaks in HPLC chromatogram, 20 µl each of methanolic solution of quercetin, avicularin, quercitrin, and hyperoside was injected and their corresponding retention time (RT) and area under the curve (AUC) were determined. AUCs of serial dilutions of quercitrin in methanol (50–500 µg/ml) obtained under the same HPLC conditions were used to establish a standard calibration curve of quercitrin.

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The assay was carried out in a 96-wells plate as described previously.[16,17] Both HE and DEE of *B. vahlii* leaves were dissolved in methanol yielding a set of serial dilutions that were tested in triplicate for quenching DPPH radical. Concentration of the sample scavenging 50% of DPPH radicals (EC_{50}) was calculated by plotting the absorbance of remaining nonquenched DPPH radicals at 492 nm.

α-glucosidase inhibitory assay

The experiment was carried out in a 96-wells plate using serial dilutions of both HE and DEE in dimethyl sulfoxide.[18] Absorbance of the final reaction mixture was recorded at 405 nm and percentage of enzyme inhibition was calculated and expressed in terms of EC_{50} (concentration of the sample inhibiting 50% of the *α*-glucosidase).

In vivo study in streptozotocin-induced diabetic rats

Fifty male Wistar rats were used in this study, and all procedures were approved by the Ethical Committee at Faculty of Pharmacy Cairo University. Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ, 50 mg/kg body weight (b.wt.), freshly prepared in 0.1 M citrate buffer (pH 4.5). A normal control group (Group 1: $n = 10$) was injected with the appropriate volume of the citrate buffer. During the first 24 h of diabetes induction, STZ-treated rats ($n = 40$) were given 5% glucose solution to avoid hypoglycemia resulting from massive destruction of β-cells. Three days later, a blood sample was collected from the tail bleeding, and hyperglycemia was confirmed by a blood glucose level ≥300 mg/dl. Blood glucose level was determined using a commercial glucometer (Roche Diagnostic ACCU-CHECK Active Test Strips, Germany). Diabetic rats were then randomly divided into four groups (ten rats, each) which received different treatments for 4 weeks as follows: group 2, remained untreated during the whole study period; Group 3 was supplied orally with 150 mg/kg of DEE suspension in distilled water, Group 4 received 300 mg/kg of DEE suspension, and Group 5 received 300 mg/kg b.wt. metformin hydrochloride dissolved in distilled water orally (standard antihyperglycemic). At the end of the experimental period, animals were fasted overnight and blood samples were collected from the retro-orbital sinus, under mild ether anesthesia. Blood samples were divided into two portions; one was processed for serum preparations and used for assaying FBG, TAC,[19] ALT, and AST levels. The other portion was collected in heparinized tubes and used for the estimation of plasma HbA1c%.[20]

After blood collection, animals were immediately decapitated under mild ether anesthesia. The liver was removed, immersed in ice-cold physiological saline, and blot-dried. Specimen of each liver was used for histopathological examination, and the remaining tissues were stored at −20°C until the use for assessment of oxidative stress parameters: GSH level,[21] MDA level,[22] and NO content quantified indirectly as nitrite.[23]

Sections of the liver, collected immediately after decapitation, were stored in 10% formal saline for 24 h, dehydrated in ethanol, embedded in paraffin, cut at 4 µm thicknesses, mounted on slides, and stained with hematoxylin and eosin.[24] All histopathologic processing and assessment of the specimens were performed by an experienced histologist blinded to the identity of the samples being examined to avoid any bias.

The results were expressed as the mean ± standard error of the mean and statistical comparisons were carried out using one-way analysis of variance followed by Tukey's multiple comparisons test. The minimal level of significance was identified at $P < 0.05$.

RESULTS

In vitro evaluation of *Bauhinia vahlii* extracts

DEE and HE were evaluated for total phenolics and flavonoids contents, DPPH radical scavenging activity, and *α*-glucosidase inhibitory activity. DEE showed high total phenolics content of 40.23 ± 0.94 µg GAE/mg compared to 5.3 ± 0.01 µg GAE/mg for HE. Moreover, *in vitro* biological investigation of *B. vahlii* leaves extracts indicated that DEE exhibited excellent *α*-glucosidase inhibition activity compared to a reference *α*-glucosidase inhibitor, acarbose, whereas HE extract showed no activity [Table 1]. DEE also scavenged DPPH radicals with EC_{50} of 55.4 ± 2.25 µg/ml, whereas HE extract was inactive [Table 1]. The antioxidant activity and the *α*-glucosidase inhibitory activity observed for the DEE strongly supported further chemical investigation and biological evaluation of DEE in an *in vivo* diabetic model.

Chemical characterization of defatted ethanolic extract

Based on $AlCl_3$ colorimetric assay, total flavonoids content of DEE was estimated at 108.95 ± 2.89 µg/mg calculated as quercetin. HPLC chromatograms of DEE, with UV detector at 340 nm, revealed four major peaks observed at RT 11.7, 13.5, 14.1, and 22.8 min. After comparing these RTs with those of compounds previously isolated from DEE and analyzed under the same HPLC conditions, these major peaks were identified as hyperoside, avicularin, quercitrin, and quercetin, respectively.

The single major metabolite in DEE as revealed from HPLC chromatogram was identified as quercitrin (RT = 14.1 min). A standard calibration curve of quercitrin was established and used to quantify other metabolites. Results are shown in Table 2.

Activity of defatted ethanolic extract in streptozotocin diabetic model

Injection of STZ in male Wistar rats resulted in a successful induction of diabetes as indicated by high FBG level (FBG ≥300 mg/dl). DEE at the specified doses, 150 and 300 mg/kg, for 4 weeks reduced biochemical signs of hyperglycemia as shown in Figure 1. DEE reduced FBG levels by 21% and 43% in Groups 3 and 4, respectively, when compared to diabetic untreated rats. Meanwhile, metformin (500 mg), a standard treatment for diabetes, caused a reduction of FBG by 62%. To further confirm the antihyperglycemic effect of DEE, levels of HbA1c were measured, and oral administration of DEE resulted in a reduction of HbA1c by 25% and 37% in Group 3 and 4, respectively, compared to Group 2. Animals treated with metformin showed 53% reduction in HbA1c% when compared to untreated animals.

As diabetes conditions persisted for 4 weeks, signs of oxidative stress became apparent; however, treatment with DEE was able to reverse most

Table 1: Chemical characterization of defatted ethanolic extract

Compounds	Concentration
Total phenolic content	40.23±0.94[a]
Total flavonoid content	108.95±2.89[b]
Quercitrin	62.9±0.18[c]
Avicularin	15.1±0.02[c]
Hyperoside	2.5±0.005[c]
Quercetin	1.4±0.0001[c]

[a]Calculated as gallic acid equivalent µg/mg DEE, [b]Calculated as quercetin equivalent µg/mg DEE, [c]Calculatedas quercitrin mg/100 mg DEE. DEE: Defatted ethanolic extract

Table 2: 2,2-diphenyl-1-picrylhydrazyl radical scavenging and *α*-glucosidase inhibitory activities of hexane extract and defatted ethanolic extract expressed as EC_{50} (µg/mL)

Compounds	DPPH radical scavenging (µg/mL)	*α*-glucosidase inhibition (µg/mL)
DEE	55.4±2.25	30.7±1.3
HE	NA	NA
Gallic acid	18.85±0.59	ND
Ascorbic acid	19.75±0.77	ND
Acarbose	ND	224±0.77

NA: Not active; ND: Not determined; DEE: Defatted ethanolic extract; HE: Hexane extract; DPPH: 2,2-diphenyl-1-picrylhydrazyl

of these signs [Figure 1]. TAC was lowered by 28% in Group 2 when compared to nondiabetic animals (Group 1), whereas animals in Groups 3 and 5 showed TAC levels which were measured at 85% and 92% of the base level observed in Group 1. On the other hand, administration of 300 mg/kg of DEE caused a 6% increase in the serum TAC. In addition, other signs of oxidative stress were evaluated in the liver tissue, namely GSH, MDA, and NO. Hepatic levels of GSH in Group 2 were found to be 40% lower than those observed in normal control group indicating a state of oxidative stress. Treatment with 300 mg/kg of DEE was able to completely restore hepatic GSH level to its normal level, whereas administration of both metformin and DEE at 150 mg/kg dose restored hepatic GSH level to about 80% of the normal levels of the nondiabetic group [Figure 2].

Levels of MDA, an indicator of lipid peroxidation, showed a 57% increase in the liver tissues of animals in Group 2, whereas those in Groups 3 and 4 showed only moderate-to-slight increase of MDA level at 39% and 10%, respectively. Treatment with metformin showed an increase of hepatic MDA level by approximately 24% [Figure 1]. Similarly, levels of hepatic NO were higher in Group 2 when compared to normal control (57% increase). Oral administration of either 300 mg/kg of DEE or metformin was able to restore hepatic NO level to its base value, whereas treatment with 150 mg/kg of DEE caused 82% reduction in the surge of NO levels caused by diabetes [Figure 1].

The extent of liver damage caused by diabetes and/or oxidative stress was examined by evaluating histopathological and biochemical signs of liver injury in animals of different treatment groups. Normal architecture

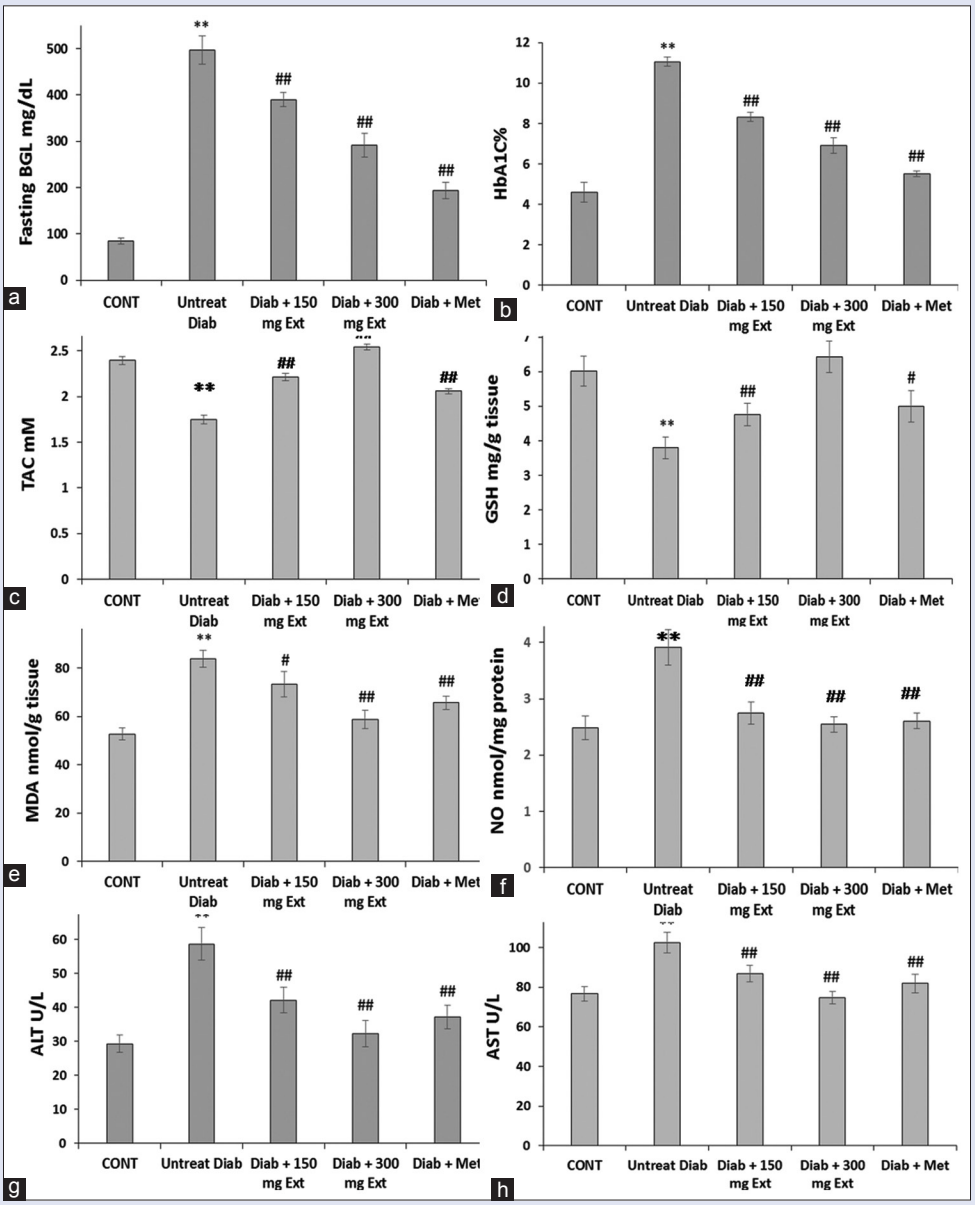

Figure 1: Biochemical parameters of hyperglycemia, oxidative stress, and liver functions as measured in five animals groups. (a) Serum levels of fasting blood glucose; (b) percentage of glycated hemoglobin, (c) serum total antioxidant capacity, (d) hepatic reduced glutathione, (e) hepatic malondialdehyde, (f) hepatic nitric oxide, (g) serum alanine transaminase and (h) serum aspartate transaminase. Biochemical parameters were measured in serum and hepatic tissue of streptozotocin-induced diabetic animal after 4 weeks of treatments. **Significantly different from the control group at $P < 0.01$, ##Significantly different from the untreated diabetic group at $P < 0.01$, #Significantly different from the untreated diabetic group at $P < 0.05$

of central vein and surrounding hepatocytes was observed in the liver sections from normal healthy rats [Figure 2 and Table 3]. However, when diabetic conditions were untreated, severe hyperplasia in bile ducts with severe congestion in portal vein, moderate inflammatory cells infiltration, and moderate fibrosis were evident [Figure 2 and Table 3]. Although all treatments used in the study improved histopathological features in the hepatic tissue [Figure 2 and Table 3], animals receiving 300 mg/kg DEE showed fewer pathological signs including mild hyperplasia in bile ducts and mild congestion in portal vein with no fibrosis or signs of inflammation.

For biochemical evaluation of liver functions, serum levels of hepatic enzymes; AST and ALT were measured in all animal groups [Figure 1]. In the diabetic untreated group, levels of AST and ALT were higher by 34% and 93%, respectively, when compared to the normal control group. Results showed that animals receiving 300 mg/kg of DEE had serum AST and ALT levels that were comparable to those observed in normal healthy animals. Meanwhile, treatment with the extract at a dose of 150 mg/kg or metformin showed the slight elevation of serum AST and ALT when compared to healthy nondiabetic animals.

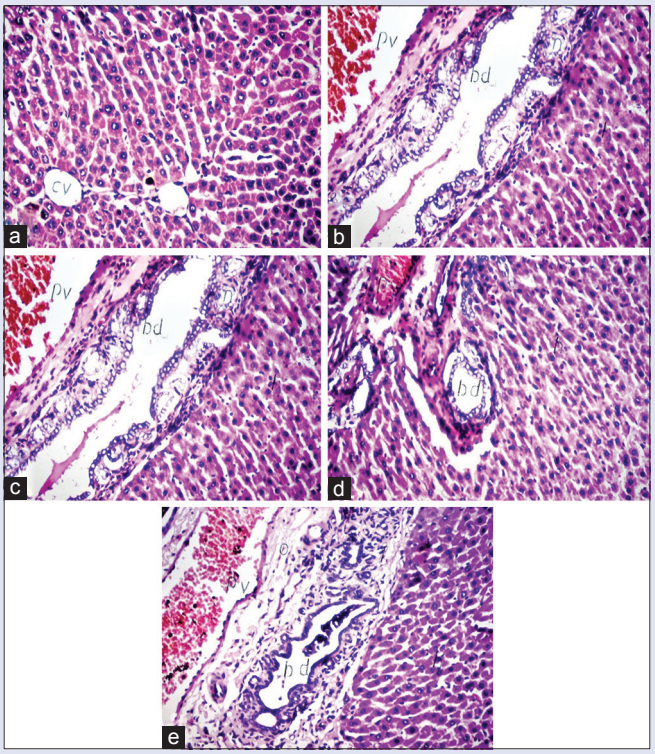

Figure 2: Photomicrographs of liver tissue from (a) normal group, (b) diabetic untreated, (c) diabetic + 150 mg DEE/kg, (d) diabetic + 300 DEE mg/kg, (e) diabetic + 300 mg metformin/kg. Photomicrographs of the effect of DEE of *Bauhinia vahlii* leaves on liver tissues in streptozotocin-induced diabetic rats *cv*: Central vein, *h*: Hepatocytes, *bd*: Bile ducts, *n*: Newly formed ductules, *pv*: Portal vein, *m*: Inflammatory cell infiltration, *o*: edema. DEE: Defatted ethanolic extract

DISCUSSION

Antihyperglycemic activity has been previously reported for extracts prepared from different plant organs (mainly leaves and stems) belonging to genus *Bauhinia*. Among these, *B. vahlii* root bark extracts displayed acute antihyperglycemic activities in STZ-induced hyperglycemic rats.[14] Beside possible antihyperglycemic activity, extracts from different organs of *B. vahlii* showed anti-inflammatory, antimicrobial, and strong antioxidant activities.[12-14] Flavonoids and triterpenes were identified as the major secondary metabolite in *B. vahlii* leaves extract.[13] The polar extract DEE was found to be enriched in antioxidant compounds and exhibited excellent α-glucosidase inhibitory activity compared to the standard drug acarbose. HPLC analysis of DEE identified several quercetin glycosides with quercitrin identified as the major glycoside present at 62.9 ± 0.18 mg/g of DEE. This may explain the α-glucosidase inhibitory activity of DEE since flavonol glycosides have been previously reported as good inhibitors of the enzyme.[25]

In STZ-diabetic rats model, DEE showed moderate antihyperglycemic activity when compared to metformin. Upon examining oxidants/antioxidants balance, administration of DEE was more effective in relieving the oxidative stress status associated with diabetes than the antidiabetic drug. In fact, DEE at a dose of 300 mg/kg ameliorated all signs of oxidative stress and restored levels of serum TAC, hepatic GSH, NO, and MDA to the levels reported for normal healthy rats. In addition, hepatic damage observed in diabetic animals that remained untreated for the length of the trial was generally not observed in animals treated with 300 mg/kg of DEE, whereas certain tissue damage and elevated levels of serum AST and ALT were observed in all other treatment groups of diabetic rats.

CONCLUSION

This is the first report of the potential of *B. vahlii* leaves extract in relieving hyperglycemia and oxidative stress in STZ-diabetic rats model. These results point to the beneficial effects of *B. vahlii* and quercetin glycosides in general in controlling diabetic conditions and complications. Further investigation may be required to assess how other vital organs affected by diabetes might respond to treatment with DEE. In conclusion, our study highly suggests that other plants enriched in quercetin glycosides are very good candidates for investigation of their antidiabetic activity.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

Table 3: Histopathological manifestations of the effect of defatted ethanolic extract of *Buxus vahlii* leaves on liver tissues in streptozotocin-induced diabetic rats

Signs of toxicity	Control	Untreated	150 mg DEE	300 mg DEE	Metformin
Hyperplasia of bile duct	-	+++	++	+	+
Newly formed bile ductules	-	+++	-	-	-
Inflammatory cell infiltration in portal area	-	++	++	-	-
Fibrosis in portal area	-	++	+	-	+
Collagen in portal area	-	+	++	-	++
Congestion in portal vein	-	+++	++	+	++

DEE: Defatted ethanolic extract

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