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Flavonoid chemical composition and antidiabetic potential of *Brachychiton acerifolius* leaves extract

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ABSTRACT

Objective: To evaluate *Brachychiton acerifolius* leaf extracts as antidiabetic potential agent and to identify the main active constituents using bioactivity guided fractionation.
Methods: *In vitro* antioxidant activity was evaluated for *B. acerifolius* different extracts using DPPH assay and vitamin C as control. Antidiabetic activity was then determined using STZ-induced rats treated daily with ethyl acetate and 70% ethanol leaf extracts for 4 weeks at a dose of 200 g/kg body weight against gliclazide reference drug. Blood glucose, α-amylase, lipid profile, liver function enzymes and oxidative stress markers were assessed along with histopathological study for liver and pancreatic tissues. Isolation and structural elucidation of active compounds were made using Diaion and Sephadex followed by spectral analyses.
Results: The results indicated that ethyl acetate and ethanol leaf extracts exhibited the strongest antioxidant activity compared to that of vitamin C (IC_{50} 0.05, 0.03 and 12 mg/mL, respectively). Both extracts showed potent anti-hyperglycemic activity evidenced by a significant decrease in serum glucose levels by 82.5% and 80.9% and α-amylase by 45.2% and 53.6%, as compared with gliclazide 68% and 59.4%, respectively. Fractionation of ethanol extract resulted in the isolation of 9 flavonoids including apigenin-7-O-α-rhamnosyl(1→2)-β-D-glucuronide, apigenin-7-O-β-D-glucuronide, apigenin-7-O-β-D-glucoside and luteolin-7-O-β-D-glucuronide.
Conclusions: This study highlights the potential use of *B. acerifolius* leaf extract enriched in flavones for the treatment of diabetes that would warrant further clinical trials investigation.

1. Introduction

Diabetes mellitus is a heterogeneous group of disorders in protein, fat and carbohydrate metabolism, portrayed by chronic hyperglycemia [1]. As indicated by the International Diabetes Federation (2015), 415 million adult people are diabetic; by 2040 this will ascend to 642 million. Diabetes brought on 4.9 million deaths in 2014 and this is cited in the IDF report as being the fourth or fifth driving reason of death in most high income countries and an epidemic disease in economically developing ones. The expanded morbidity associated with

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diabetes is secondary to a number of pathological conditions, embracing dyslipidemia, neuropathy, cerebrovascular disorders, renal insufficiency and coronary heart disease [2]. Free radicals height and hepatic failure were additionally associated with diabetes [3].

In response to the increasing demands for discovering new antidiabetic drugs of natural origin, this study assessed the antidiabetic activity of *Brachychiton acerifolius* (*B. acerifolius*) (A. Cunn. ex G. Don) Macarthur (Malvaceae) defatted leaf extract using bioactivity guided fractionation. *B. acerifolius*, an Australian tree cultivated worldwide, belongs to a genus and family of known antidiabetic herbal drugs among several other activities [4–8]. The genus is reported for a myriad of chemical compounds, including flavonoids, alkaloids, sterols, terpenes and coumarins [9,10]. However, no previous biological activities have been reported for *B. acerifolius* except for the strong antioxidant activity of its leaf crude extract [11]. This study included successive extraction of *B. acerifolius* leaf powder with solvents of increasing polarity, *in vitro* antioxidant activity assay for the successive extracts as a rapid screening tool for extract activity [12], most potent of which were further assessed for their antidiabetic activity. Finally, isolation attempt was made to isolate and identify the major chemical forms present in the leaf alcohol extract and are likely to mediate for its antidiabetic effect.

2. Materials and methods

2.1. Plant material and animals

B. acerifolius leaf was collected during April–May 2012 from Agricultural Museum, Giza Governorate, Egypt. The plant material was kindly authenticated by Therese Labib, Agricultural engineer, specialist of plant scientific classification at the Ministry of Agriculture and ex-director of the Orman botanical garden, Giza, Egypt. Plant samples were deposited at Faculty of Pharmacy-Cairo University (voucher No.: 16-6-2014). In the current study, male Wistar albino rats (150:180 g) were provided from the Animal House, National Research Centre, Egypt. All experimental procedures involving animals were conducted in accordance with the moral rules of the Medical Ethical Committee of National Research Centre in Egypt (Approval No: 12035).

2.2. Chemicals, reagents and materials

Petroleum ether 60–80 °C, ethanol and acetic acid were purchased from Adwic (Egypt). Chloroform, ethyl acetate, methanol, formaldehyde, streptozotocin, quercetin, kaempferol, NaOMe, $AlCl_3$, HCl, NaOAc and H_3BO_3 were obtained from Sigma Aldrich (USA). Shift reagents and chemicals for UV spectroscopic analysis of flavonoids were prepared as mentioned in detail elsewhere [13]. Sephadex LH-20 (25–100 μm) and 1,1-diphenyl-2-picryl-hydrazil (DPPH) were purchased from Sigma–Aldrich Chemie GmbH (Germany). Diaion HP-20 was obtained from SUPELCO (USA), vitamin C from Cid Co., (Egypt), gliclazide (diamicron®) from Servier (France) and n-butanol from alpha chemicals (Egypt). Kits for the assessment of: blood glucose [14], α-amylase [15], lipid profile [16], liver oxidative stress markers: GSH, MDA and SOD and serum liver function enzymes [17] were provided from Biodiagnostics (Spectrum, Egypt).

2.3. Extraction procedure for B. acerifolius leaves

One kilogram of *B. acerifolius* air dried leaves powder was macerated on cold with petroleum ether 60–80 °C for defatting. The marc was then successively macerated on cold with chloroform, ethyl acetate and 70% ethanol to yield 27 g, 7.5 g and 100 g, respectively.

2.4. In vitro antioxidant activity

Serial dilutions of chloroform, ethyl acetate and 70% ethanol leaf extracts spanning from 10 till 100 mg/mL were assessed for their antioxidant effect by the method of Awad *et al.* [16], where DPPH solution prepared at a concentration of 50 μmol/L react with plant antioxidants and the decrease in absorbance (A) of $DPPH^-$ (at 517 nm) was calculated in relation to absorbance of control (blank) as follows: Percentage inhibition = $(A_{control} - A_{sample})/A_{control} \times 100$. Vitamin C was used as a standard.

Thirty male Wistar strain albino rats (150–180 g) were divided to estimate the acute toxicity of ethyl acetate and 70% ethanol leaf extracts at different concentrations (100, 200 and 500 g/kg body weight) with 5 rats/group. Animals were observed for 15 consecutive days and accordingly, safety dose was monitored and recorded.

2.5. In vivo antidiabetic activity determination

Thirty-five male Wistar strain albino rats (150–180 g) were divided into 7 groups (5 rats/group) in which Group 1: normal healthy control rats, Groups 2–3: normal healthy rats, orally administrated daily with ethyl acetate and 70% ethanol leaf extracts (200 g/kg, as determined by LD_{50} experiment) for 4 weeks to evaluate their adverse effects over liver and pancreas, Group 4: injected with a single dose of streptozotocin *i.p.* (STZ, 35 mg/kg) to induce diabetes [18], Groups 5–6: *i.p.* injected with STZ and orally treated, after five days of STZ injection, with ethyl acetate and 70% ethanol leaf extracts (200 g/kg) daily for 4 weeks, Group 7: *i.p.* injected with STZ and treated with gliclazide (30 mg/kg body weight) after five days of STZ injection, for 4 weeks and serve as a reference group. Diabetic rats with a glucose level >300 mg/dL were used for this experiment. Blood samples were collected in clean dry test tubes, left for 10 min to clot and then centrifuged at 3000 r/min for serum separation. The separated serum was stored at −80 °C for determination of glucose, α-amylase, lipid profile and liver function enzymes level. Liver tissue was homogenized in cold 0.9 mol/L NaCl (1:10 w/v), centrifuged at 3000 r/min for 10 min, the supernatant was separated and stored at −80 °C for the assay of oxidative stress markers and total protein content.

2.6. Histopathological study

Representative slices of liver and pancreatic tissues were fixed in 10% formalin. Paraffin-embedded sections (4 μm thick) were stained with haematoxylin and eosin (H&E) [19]. Slides were seen under a light microscope with magnification power 200×.

2.7. Statistics

All data were expressed as mean ± SD of rats' number in each group. Statistical analysis was carried out by One-way

ANOVA, Costat Software Computer Program. A significant value between groups was set at $P < 0.05$.

$$\%\ \text{of change} = \frac{\text{Control mean} - \text{Treated mean}}{\text{Control mean}}$$

$$\%\ \text{of improvement} = \frac{\text{Treated mean} - \text{Diabetic mean}}{\text{Control mean}}$$

2.8. Isolation and purification of chemical constituents from 70% ethanol leaf extract

70% ethanol leaf dry extract (80 g) was fractionated twice sequentially using diaion (5:1), ion exchange resin for column chromatography, and eluting system of decreasing polarity; 100% H_2O, 25% MeOH, 50% MeOH, 75% MeOH and 100% MeOH. 50% MeOH fraction (14 g) was selected for further fractionation on diaion using the same eluting system. Eventually, upon spotting on PC Whatmann No. 1 in solvent systems BAW (S1) and 15% acetic acid (S2), the 25% methanol fraction (1.5 g) was selected for separation and purification of compounds on Sephadex column (10:1) with gradient eluting system: 100% H_2O, 5% MeOH, 10% MeOH, 20% MeOH and 50% MeOH. Similar sub-fractions were pooled together and further purified on Sephadex (10:1) using 50% MeOH to yield 5 flavonoid glycosides and 4 aglycones.

2.9. Spectral analyses

UV spectroscopy used for detecting λ_{max} in MeOH and shift reagents (UV–VIS double beam UVD-3500 spectrophotometer, Labomed, Inc.). Mass spectral data were obtained by UPLC–PDA–high resolution (HR) qTOF–ESI/MS (Bruker Daltonics) with the mechanism and conditions explained in Farag *et al.* [14], and UPLC/ITMS (Thermo Electron, San Jose, USA) equipped with an ESI source (electrospray voltage 4.0 kV, sheath gas: nitrogen; capillary temperature: 275 °C) in negative ionization mode with ion-trap mass detector for performing tandem MS/MS and confirming MS fragment entities. Acquired NMR data include ^1H, ^{13}C, Correlation spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear multiple-bond correlation spectroscopy (HMBC). All ^1H and ^{13}C spectra were recorded on an Agilent (Varian) VNMRS 600 NMR spectrometer operating at a proton and carbon NMR frequencies of 599.83 and 150.83 (MHz), respectively. 2D NMR spectra were recorded using standard CHEMPACK 4.1 pulse sequences (DQCOSY, HSQCAD, HMBCAD) implemented in Varian VNMRJ 2.2C spectrometer software. The conditions for 1D and 2D NMR analyses were adjusted as mentioned in Farag *et al.* [20].

3. Results

3.1. In vitro antioxidant activity determination for successive leaf extract using DPPH assay

Results of scavenging DPPH free radicals are expressed as IC_{50} values (mg/mL): 0.03, 0.05, 0.3 and 12; for 70% ethanol, ethyl acetate, chloroform successive leaf extracts and vitamin C, respectively.

3.2. Acute toxicity determination (LD_{50})

Ethyl acetate and 70% ethanol leaf extracts were orally administered at three concentrations (100, 200 and 500 g/kg body weight). Absence of death was revealed for both extracts at different dose levels after 15 days of treatment, accordingly the median dose (200 g/kg body weight) was selected for further *in vivo* experiments.

3.3. In vivo antidiabetic activity determination

Leaf extracts [*i.e.* ethyl acetate (EA) and 70% ethanol (E)] were evaluated as antidiabetic agents *in vivo* by monitoring changes in rat serum glucose, α-amylase, lipid profile, liver function enzymes and oxidative stress parameter levels to provide a complete overview on the biological impact of *B. acerifolius* extracts in healthy and diabetic rats. Change and improvement percentages for each parameter are presented along with the histopathological examination of liver and pancreatic tissues (Tables 1–3 and Figures 1–2).

3.4. Chemical investigation of E extract

Both *B. acerifolius* leaf extracts (EA and E) were found active against diabetes, as evidenced by a significant decrease in glucose and α-amylase levels in diabetic rats. Nevertheless, E showed an overall more favourable biochemical and histopathological effects in both healthy and diabetic rats and was thus subjected to further isolation attempts. UPLC–MS profiling of *B. acerifolius* crude leaf ethanol extract revealed the enrichment of flavone type glycosides suggesting that these forms amount for secondary metabolite composition in *B. acerifolius* leaf. To further clarify *B. acerifolius* flavonoids repertoire, additional purification and optimised chromatographic conditions were attempted. Moreover, further NMR analyses were made for the isolates, that is, the absolute stereochemistry and attachment position of sugars to flavonoid aglycones, not easily determined using UPLC–MS analysis. Fractionation and isolation attempts for most active 70% ethanol leaf extract led to the isolation of 9 flavonoids.

3.5. Spectral data of isolated compounds

Compound 1; apigenin-7-O-α-rhamnosyl (1→2) β-D-glucuronide (25 mg): R_f values in S1: 0.22 and S2: 0.37. UVλ_{max}, nm: MeOH: 331, 270; +NaOMe: 380, 273; +$AlCl_3$: 385, 325, 300, 280; +$AlCl_3$/HCl: 380, 325, 300, 280; +NaOAc: 333, 269; +NaOAc/H_3BO_3: 332, 269. MS data: $[M-H]^-$ 591.1373, $C_{27}H_{27}O_{15}^-$; fragments at *m/z* 445.0774, $C_{21}H_{17}O_{11}^-$ and *m/z* 269.0468, $C_{15}H_9O_5^-$. NMR data: Table 4.

Compound 2; quercetin-3-O-α-rhamnosyl (1→6)-β-D-glucoside or rutin (20 mg): R_f values in S1: 0.36 and S2: 0.4. UVλ_{max}, nm: MeOH: 355, 290, 260; +NaOMe: 405, 275; +$AlCl_3$: 425, 305, 275; +$AlCl_3$/HCl: 400, 355, 270; +NaOAc: 390, 270; +NaOAc/H_3BO_3: 380, 265. MS data: $[M-H]^-$ 609.1468, $C_{27}H_{29}O_{16}^-$; fragments at *m/z* 463.0835, $C_{21}H_{19}O_{12}^-$ and *m/z* 301. NMR data: Table 4.

Compound 3; luteolin-7-O-β-D-glucuronide (15 mg): R_f values in S1: 0.3 and S2: 0.1. UVλ_{max}, nm: MeOH: 347, 268, 256sh; +NaOMe: 397, 268; +$AlCl_3$: 420, 346sh, 300, 277; +$AlCl_3$/HCl: 390, 356, 298, 273, 256; +NaOAc: 406, 328, 269; +NaOAc/H_3BO_3: 400, 269. MS data: $[M-H]^-$ 461.0726,

Table 1

Effect of ethyl acetate and 70% ethanol *B. acerifolius* successive leaf extracts on the different biochemical parameters in normal healthy rats.

Parameter	Control	Healthy rats + Leaf extracts		*P*
		Ethyl acetate (EA)	70% ethanol (E)	
Glucose (mg/dL)	$74.60^{a} \pm 3.30$	$69.20^{a} \pm 7.70$	$72.90^{a} \pm 4.00$	0.019
α-Amylase (IU/L)	$684.10^{a} \pm 125.00$	$431.50^{b} \pm 115.30$	$375.50^{b} \pm 153.00$	0.005
Lipid profile (mg/dL)				
Total cholesterol	$69.50^{a} \pm 2.70$	$66.50^{ab} \pm 13.10$	$54.80^{b} \pm 2.20$	0.100
HDL-C	$64.40^{a} \pm 2.20$	$46.08^{b} \pm 5.30$	$48.70^{b} \pm 2.20$	0.0001
LDL-C	$25.80^{a} \pm 1.10$	$5.84^{c} \pm 2.10$	$17.50^{b} \pm 3.60$	0.001
Triglycerides	$103.20^{a} \pm 10.50$	$75.20^{b} \pm 10.90$	$102.13^{a} \pm 21.00$	0.008
Total lipids	$3500.00^{a} \pm 20.40$	$3250.00^{a} \pm 204.10$	$3000.00^{b} \pm 204.10$	0.170
Liver function enzymes (IU/L)				
AST	$2.10^{a} \pm 0.05$	$2.20^{a} \pm 0.18$	$2.50^{a} \pm 0.58$	0.289
ALT	$1.44^{a} \pm 0.06$	$1.60^{b} \pm 0.02$	$1.48^{a} \pm 0.02$	0.289
ALP	$91.60^{a} \pm 6.70$	$84.80^{a} \pm 4.90$	$81.90^{b} \pm 5.20$	0.275
Oxidative stress markers (μg/mg protein)				
GSH	$10.80^{a} \pm 1.06$	$10.70^{a} \pm 1.10$	$11.47^{a} \pm 2.40$	0.300
MDA	$0.47^{ab} \pm 0.03$	$0.46^{ab} \pm 0.08$	$0.41^{ab} \pm 0.05$	0.033
SOD	$294.60^{a} \pm 45.30$	$95.03^{b} \pm 31.20$	$80.60^{b} \pm 14.50$	0.001

Values are expressed as mean ± SD of five rats in each group. Statistical analysis is carried out by One-way ANOVA accompanied by Costat computer program using least significant difference (LSD) at $P < 0.05$. Unshared superscript letters indicate values of significant difference between groups.

Table 2

Effect of ethyl acetate (EA) and 70% ethanol (E) of *B. acerifolius* successive leaf extracts on the different biochemical parameters in diabetic rats.

Parameter	Control	Diabetic (D.)	D. + EA	D. + E	D. + Gliclazide
Glucose (mg/dL)	$74.63^{c} \pm 3.30$	$397.1^{a} \pm 13.8$	$69.3^{c} \pm 5.5$	$75.6^{c} \pm 4.4$	$127^{b} \pm 2.5$
α-amylase (IU/L)	$684.1^{d} \pm 125.7$	$2925.8^{a} \pm 524$	$1603.1^{b} \pm 115.4$	$1356.5^{bc} \pm 87.2$	$1187.5^{c} \pm 100$
Lipid profile (mg/dL)					
Total cholesterol	$69.6^{b} \pm 2.7$	$245^{a} \pm 13.3$	$56.1^{c} \pm 4.9$	$63.1^{bc} \pm 8.6$	$57.6^{bc} \pm 6$
HDL-C	$64.5^{a} \pm 2.2$	$42.5^{c} \pm 4.5$	$36.1^{d} \pm 5.6$	$53.4^{b} \pm 1.3$	$60.5^{a} \pm 3.3$
LDL-C	$25.8^{b} \pm 1.1$	$152.5^{a} \pm 14$	$8.6^{c} \pm 1.1$	$14.6^{c} \pm 1.8$	$32.1^{b} \pm 1.3$
Triglycerides	$103.2^{b} \pm 10.5$	$263.4^{a} \pm 32.2$	$109.4^{b} \pm 13.7$	$63.1^{c} \pm 1.9$	$127^{b} \pm 1.6$
Total lipids	$3500^{d} \pm 204$	$9166.5^{a} \pm 311.8$	$6166.5^{b} \pm 425$	$5000^{c} \pm 408.2$	$5125^{c} \pm 478.7$
Liver function enzymes (IU/L)					
AST	$2.1^{b} \pm 0.06$	$2.6^{a} \pm 0.02$	$2.5^{a} \pm 0.06$	$2.56^{a} \pm 0.04$	$2.2^{b} \pm 0.2$
ALT	$1.4^{b} \pm 0.05$	$1.76^{a} \pm 0.12$	$1.65^{a} \pm 0.06$	$1.71^{a} \pm 0.13$	$1.65^{a} \pm 0.05$
ALP	$91.6^{d} \pm 6.80$	$173.5^{a} \pm 5.19$	$119.4^{b} \pm 8.5$	$108.3^{c} \pm 6.8$	$123^{b} \pm 2.5$
Oxidative stress markers (μg/mg protein)					
GSH	$10.8^{a} \pm 1.1$	$5.6^{d} \pm 0.26$	$9.21^{b} \pm 0.56$	$9.07^{bc} \pm 0.58$	$8.16^{c} \pm 0.52$
MDA	$0.47^{d} \pm 0.03$	$2.02^{a} \pm 0.17$	$0.82^{c} \pm 0.07$	$0.55^{d} \pm 0.04$	$1.09^{b} \pm 0.09$
SOD	$294.6^{b} \pm 45.2$	$466.7^{a} \pm 122.7$	$288.8^{b} \pm 41.7$	$292.6^{b} \pm 39.5$	$272.1^{b} \pm 87.9$

Values are expressed as mean ± SD of five rats in each group. Statistical analysis is carried out by One-way ANOVA accompanied by Costat computer program using least significant difference (LSD) at $P < 0.05$. Unshared superscript letters indicate values of significant difference between groups.

Table 3

Percentage of improvement in different parameters after treatment of diabetic rats with *B. acerifolius* leaf extracts and gliclazide.

Parameters	Leaf extracts		
	Ethyl acetate (EA)	70% Ethanol (E)	Gliclazide
Glucose	439.2	430.8	361.9
α-Amylase	193.3	229.4	254.1
TC	271.4	261.3	269.2
HDL-C	9.9	16.9	27.9
LDL-C	557.7	534.5	466.7
TG	149.2	194.1	132.2
Total lipids	85.7	119.1	115.5
AST	4.7	1.9	18.9
ALT	7.6	3.5	7.6
ALP	59.1	71.2	55.1
GSH	33.4	32.1	23.7
MDA	252.7	310.1	196.2
SOD	60.4	59.1	66.1

$C_{21}H_{17}O_{12}^{-}$; fragment at *m/z* 285.0394, $C_{15}H_9O_6^{-}$. NMR data: Table 4.

Compound 4; apigenin-7-O-β-D-glucoside (12 mg): R_f values in S1: 0.28 and S2: 0.15. UVλ_{max}, nm: MeOH: 331, 270; +NaOMe: 390, 270; +$AlCl_3$: 360, 320, 300, 270, 239; +$AlCl_3$/HCl: 361, 325, 300, 273, 240; +NaOAc: 334, 271; +NaOAc/H_3BO_3: 332, 272. MS data: $[M-H]^{-}$ 431.2189, $C_{21}H_{19}O_{10}^{-}$; fragment at *m/z* 269.0454, $C_{15}H_9O_5^{-}$. NMR data: Table 4.

Compound 5; apigenin-7-O-β-D-glucuronide(10 mg): R_f values in S1: 0.35 and S2: 0.15. UVλ_{max}, nm: MeOH: 335, 270; +NaOMe: 395sh, 350, 300, 275; +AlCl3: 355, 300, 275; +$AlCl_3$/HCl: 345, 300, 275; +NaOAc: 340, 270; +NaOAc/H_3BO_3: 340, 270. MS data: $[M-H]^{-}$ 445.0781, $C_{21}H_{17}O_{11}^{-}$; fragment at *m/z* 269.0447, $C_{15}H_9O_5^{-}$. NMR data: ^{1}H NMR (400 MHz, CD_3OD):δ7.9 (H-2′ & 6′, d, $J = 8.3$ Hz), 6.9 (H-3′ & 5′, d, $J = 8.3$ Hz), 6.2 (H-6, d, $J = 2.1$ Hz), 6.5 (H-3, s), 6.75 (H-8, d, $J = 2.1$ Hz), 5.2 (H-1″, complex multiplet), 3.5–3.8 (sugar protons, m).

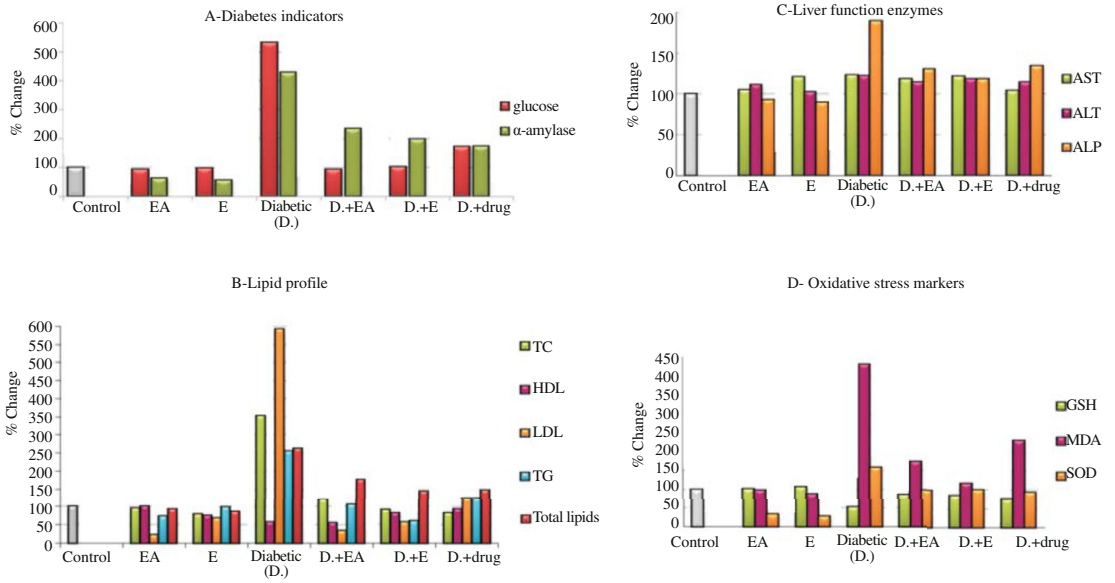

Figure 1. Percentage of change in different biochemical parameters compared to normal control group upon the administration of *B. acerifolius* leaf extracts to healthy and diabetic rats.
EA: Ethyl acetate leaf extract; E: 70% ethanol leaf extract; D.: Diabetic rats; drug: Gliclazide. (A) Diabetes indicators: glucose and α-amylase. (B) Lipid profile: total cholesterol, HDL, LDL, triglycerides and total lipids. (C) Liver function enzymes: AST, ALT and ALP. (D) Oxidative stress markers: GSH, MDA and SOD.

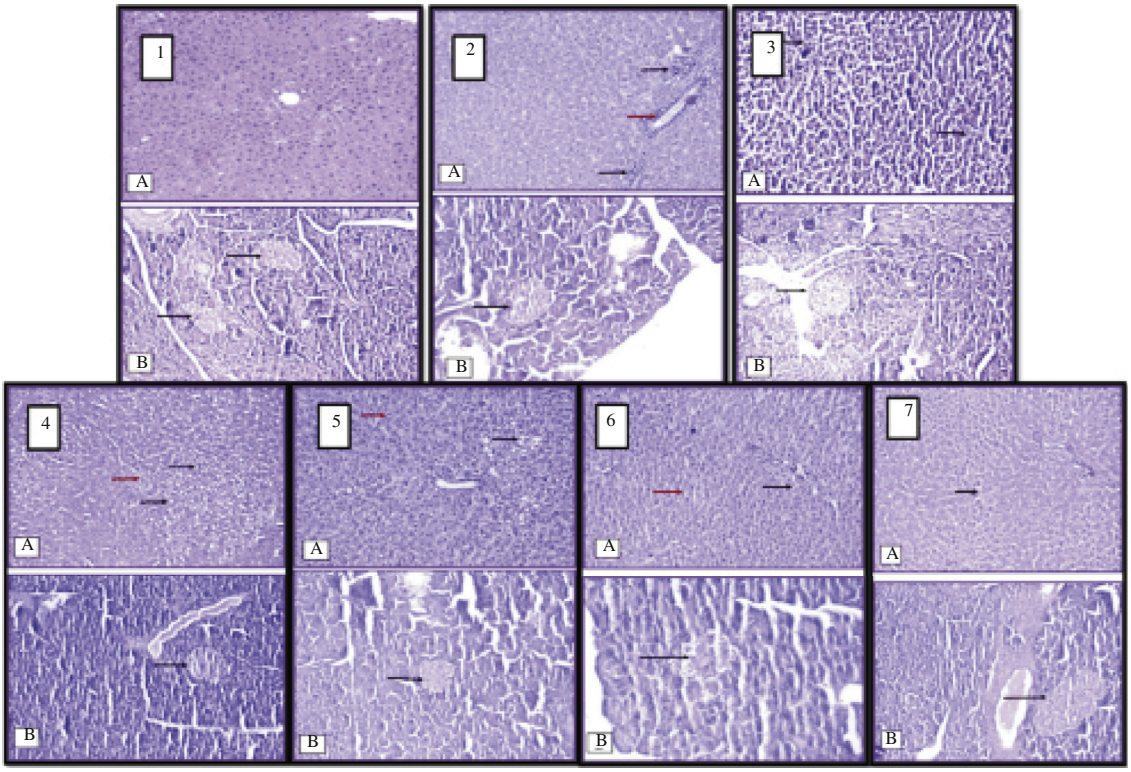

Figure 2. H&E stained histopathological sections (200×) of liver (A) and pancreatic (B) tissues from healthy (1–3) and diabetic (4–7) rats after 4 weeks of experiment duration.
1: normal control; (1A) Liver section showed normal morphological appearance. (1B) Pancreatic section: showed regular appearance of pancreatic islets (arrows) 2: healthy rats treated with (EA) extract; (2A) Liver section: intact lobular hepatic architecture with mild lobular inflammation (black arrows) and mild infiltration (red arrow). (2B)Pancreatic section: pancreatic islets with regular shape and arrangement (black arrow). 3: healthy rats treated with (E) extract; (3A) Liver section: preserved lobular hepatic architecture and normal morphological appearance with mild intralobular inflammation (arrow). (3B) Pancreatic section: pancreatic islets with mild capillary congestion (arrow). 4: diabetic rats; (4A) Liver section: moderate to severe micro and macro vesicular steatosis, hepatocyte ballooning (black arrow) and moderate lobular inflammation (red arrow). (4B) Pancreatic section: atrophic pancreatic islets. 5: diabetic rats treated with (EA) extract; (5A) Liver section: intact lobular hepatic architecture, moderate micro and macro vesicular steatosis and hepatocyte ballooning (black arrow) with mild interlobular inflammation (red arrow). (5B) Pancreatic section: moderate pancreatic islets size (black arrow). 6: diabetic rats treated with successive (E) extract; (6A) Liver section: scattered few micro steatotic changes (black arrows) and mild intralobular inflammation (red arrows). (6B) Pancreatic: showed pancreatic islets with normal size and mild disorganization (black arrow). 7: diabetic rats treated with gliclazide reference drug; (7A) Liver section: mild vacuolization of hepatocytes (arrow). (7B) Pancreatic section: large pancreatic islets, capillary congestion and focal necrosis (arrow).

Table 4
NMR spectral data of selected flavonoid glycosides (1–4) isolated from 70% ethanol *B. acerifolius* leaves extract.

Cpd no.:	1			2		3	4
C/H-aglycone	δC	δH (m, *J* value-Hz)	Key HMBC correlations	δC	δH (m, *J* value-Hz)	δH (m, *J* value-Hz)	
	Confirmed by HSQC			Confirmed by HSQC			
2	163	–	–	158.5	–	–	–
3	102	6.6 (s)	C-2	135	–	6.60 (s)	6.65 (s)
4	180.9	–	–	179.4	–	–	–
5	162	–	–	162.9	–	–	–
6	99.8	6.4 (s)	C-7, C-8	99.9	6.2 (d, 1.7)	6.50 (d, 2)	6.50 (d, 1.7)
7	162.4	–	–	166	–	–	–
8	96	6.8 (s)	C-7, C-9	94.8	6.4 (d, 1.7)	6.85 (d, 2)	6.87 (d, 1.7)
9	157	–	–	159.3	–	–	–
10	103	–	–	105.6	–	–	–
1′	123	–	–	123.1	–	–	–
2′	129	7.89 (d, 8.3)	C-4′, C-2	117.6	7.65 (d, 1.8)	7.40*	7.9 (d, 8.7)
3′	117	6.92 (d, 8.3)	C-4′, C-1′	145.8	–	–	6.9 (d, 8.7)
4′	159	–	–	149.8	–	–	–
5′	117	6.92 (d, 8.3)	C-4′, C-1′	116	6.9 (d, 8.3)	6.89 (d, 8.7)	6.9 (d, 8.7)
6′	129	7.89 (d, 8.3)	C-4′, C-2	123.5	7.6 (dd, 1.8, 8.3)	7.40*	7.9 (d, 8.7)
Glucuronic acid							
1″	100.7	5.25 (d, 7.4)	C-7	–	–	–	5.1 (d,7)
2″	79	3.7	H-1‴	–	–	–	3.1–3.5 (m)
3″	77.6	3.3–3.9 (m)	–	–	–	–	
4″	72.4		–	–	–	–	
5″	75		–	–	–	–	3.8 (d, 9)
6″	172		–	–	–	–	–
Glucose							
1″	–	–	–	104.7	5.1 (d, 7.4)	5.1 (complex m)	–
2″	–	–	–	75.7	3.2–3.8 (m)	3.5–3.8 (m)	–
3″	–	–	–	77.2			–
4″	–	–	–	71.4			–
5″	–	–	–	104.7			–
6″	–	–	–	75.7			–
Rhamnose							
1‴	102.2	5.31 (s)	C-2″	102.4	4.5 (d, 1.7)	–	–
2‴	72	3.3–3.9 (m)	–	72	3.2–3.8 (m)	–	–
3‴	72.2		–	72.2		–	–
4‴	73.9		–	73.9		–	–
5‴	70		–	69.7		–	–
6‴	18.2	1.29 (d, 6.1)	–	17.8	1.1 (d, 6.1)	–	–

All spectra were obtained in CD_3OD; *: Overlapped peaks with unassigned multiplicity.

Compound 6; quercetin (8 mg): R_f values in S1: 0.53 and S2: 0.03. UVλ_{max}, nm: MeOH: 375, 255; +NaOMe: 445, 340, 280; +$AlCl_3$: 450, 360, 325, 275; +$AlCl_3$/HCl: 425, 305, 260; +NaOAc: 435, 325sh, 275; +NaOAc/H_3BO_3: 450, 325sh, 275.

Compound 7; kaempferol (8 mg): R_f values in S1: 0.69 and S2: 0.04. UVλ_{max}, nm: MeOH: 360, 290, 270; +NaOMe: 435, 330, 285; +$AlCl_3$: 420, 350, 300, 295; +$AlCl_3$/HCl: 415, 350, 298, 295; +NaOAc: 425, 280; +NaOAc/H_3BO_3: 420, 360, 290, 270.

Compound 8; luteolin (15 mg): R_f values in S1: 0.5 and S2: 0.08. UVλ_{max}, nm: MeOH: 346, 269sh, 250; +NaOMe: 405, 276; +$AlCl_3$: 419, 302, 273; +$AlCl_3$/HCl: 387, 356, 298, 275, 261; +NaOAc: 403, 269; +NaOAc/H_3BO_3: 400, 301, 269. MS data: $[M-H]^-$ 285.0402, $C_{15}H_9O_6$.

Compound 9; apigenin (7 mg): *Rf* values in S1: 0.65 and S2: 0.1. UVλ_{max} in MeOH: 333 nm, 267 nm. MS data: Molecular ion $[M-H]^-$ 269.0461; $C_{15}H_9O_5$.

4. Discussion

4.1. In vitro antioxidant activity of successive leaf extracts

The correlation between antioxidants and treatment of diabetes (type I & II) is well established in terms of ameliorating the diabetic status [21,22]. Antioxidant assay of successive *B. acerifolius* leaf extracts showed promising antioxidant effects, albeit relatively less able to scavenge DPPH if compared to that of crude extract (IC_{50} 0.015 mg/mL) [11], suggesting for a synergistic effect among *B. acerifolius* leaf constituents. Considering that both (E) and (EA) leaf extracts showed much lower IC_{50} (0.03 and 0.05 mg/mL, respectively) than that of chloroform (0.3 mg/mL), both extracts were selected for further *in vivo* biological examination.

4.2. In vivo antidiabetic activity determination

Healthy rats treated with (EA) and (E) leaf extracts showed insignificant changes ($P < 0.019$) in glucose levels (−7.3% and −2.3%, respectively) compared to control group and thus exhibiting no hypoglycemic activity on normal blood glucose levels (Figure 1A). With regard to lipid profile, LDL-C was significantly reduced upon (EA) administration by 77.4% and (E) by 32.2%. Whereas, HDL-C showed a significant decrease in both extracts by 28.4% and 24.4%, respectively (Table 1). Generally, the effect of extracts on healthy rats' lipid profile after 4 weeks was generally favourable except for HDL (Figure 1B). Kent *et al.* [23] reported reduction in HDL with a concurrent improvement in other cardiovascular risk indicators in healthy human subjects who follow plant-based diets. Such observation

uncertain relying on HDL levels solely and suggests that non-HDL cholesterol levels ought to be measured for predicting the risk of cardiovascular diseases [24].

Concerning liver function enzymes, insignificant changes in AST, ALT and ALP enzymes activity were recorded (Figure 1C) compared to control group, revealing the extracts' safety on liver functions. Similarly, oxidative stress markers, malondialdehyde (MDA) and glutathione (GSH), showed insignificant changes (Table 1). In contrast, superoxide dismutase (SOD) recorded a significant decrease in healthy rats treated with (EA) and (E) by 67.7% and 72.6%, respectively (Figure 1D) suggesting for a decrease in free radical formation as SOD transcription is known to increase following an increase in free radicals to dismutate superoxide radicals into water and oxygen [25].

On the other hand, upon *i.p.* STZ injection, the diabetic rats group recorded significant increase ($P < 0.0001$) in glucose and α-amylase levels by 432.1% and 327.6%, respectively, compared with healthy rats. After 4 weeks of treatment, significant decrease ($P < 0.0001$) in glucose was recorded by 82.5% (EA) and 80.9% (E) compared to the diabetic group, α-amylase level was also decreased by 45.2% and 53.6%, respectively (Table 2, Figure 1A). Similar results were observed in the reference drug (gliclazide) on glucose levels (reduced by 68%) and α-amylase (reduced by 59.4%). Consequently, both leaf extracts were considered to have remarkable anti-hyperglycemic activity with a comparable improvement % in glucose (EA: 439.2%, E: 430.8%) and α-amylase levels (EA: 193.3%, E: 229.4%) post treatment (Table 3).

The lipid profile (Figure 1B), showed significant reduction in TC, LDL, TG and total lipids upon treatment with both extracts to variable extents. Contradictory, HDL showed significant increase post treatment with (E) and gliclazide by 25.6% and 42.3%, respectively (Table 2).

Liver function enzymes showed the highest improvement % in ALP (E: 71.2%) whereas other enzymes were not significantly altered in both extracts (Table 3). The oxidative stress markers MDA and SOD that recorded a notable increase in diabetic rats by 329.7% and 58.4%, respectively, were significantly decreased upon treatment with (EA) by 59.3% and 38.1%, and (E) by 72.7% and 37.3%, respectively (Table 2). In contrast, GSH was significantly increased upon treatment with (EA) and (E) by 64.5%, 61.9%, respectively, compared to the diabetic group (Figure 1D).

Histopathological examination of liver tissue showed no signs of hepatotoxicity or damage in healthy rats treated with both extracts compared to the control group. Additionally, pancreatic tissue showed a remarkable improvement compared to the diabetic group as revealed by the size and arrangement of pancreatic islets (Figure 2).

From the above results, the effect of (EA) and (E) leaf extracts proved to be promising anti-hyperglycemic agents compared to the reference drug gliclazide, and no signs of obvious acute or chronic toxicities.

4.3. Chemical investigation of B. acerifolius 70% ethanol leaf extract

UV spectral data of the 9 isolated flavonoids revealed that compounds 1, 3–5, 8 and 9 were flavones from their band I λ_{MeOH} (<350 nm), whereas 2,6 and 7 were flavonols (band I λ_{MeOH} > 350 nm). UV data and co-chromatography with Authentics identified compounds 6 and 7 as quercetin and kaempferol, respectively. High resolution (-) ESI/MS enabled the detection of the molecular ion and the main fragments due to sugar losses in flavonoid glycosides (1–5). The nature of the attached sugars in glycosides could be identified from the lost moieties in which 162, 146 and 176 amu corresponding to hexose, rhamnose and hexouronic acid, respectively. In detail, compound 1, exhibited fragments at *m/z* 445.0774, $C_{21}H_{17}O_{11}^-$ and *m/z* 269.0468, $C_{15}H_9O_5^-$ for the sequential loss of rhamnose (146 amu) and glucuronic acid (176 amu), from the molecular ion at *m/z* 591.1373, $C_{27}H_{27}O_{15}^-$ [26,27]. While compound 2 was assigned as a diglycoside containing glucose and rhamnose moieties attached to a quercetin aglycone (*m/z* 301) [28]. Compounds 1, 4, 5 and 9 exhibited the same aglycone mass at *m/z* 269, $C_{15}H_9O_5^-$ whereas compounds 3 [29] and 8 [30] aglycone ion mass was measured at *m/z* 285; $C_{15}H_9O_6^-$ for apigenin and luteolin, respectively, and in consistent with the UV data.

^{1}H NMR analysis further confirmed sugar type stereochemistry and attachment position. In detail, compound 1, apigenin-7-O-α-rhamnosyl (1→2)-β-glucuronide, showed two doublet peaks for protons H-3′/5′and H-2′/6′at δ6.92 and 7.89 ppm (J = 8.3 Hz) and three singlet peaks at δ6.4, 6.6 and 6.8 ppm for H-6, H-3 and H-8 respectively (Table 4). Glucuronic acid anomeric proton was observed at δ5.25 ppm as a doublet peak (J = 7.4 Hz) indicating β-sugar configuration. With regard to the rhamnose moiety; a singlet anomeric proton was observed at an unusual downfield chemical shift (δ5.31 ppm) and a doublet peak for C-6 methyl protons at δ1.29 ppm (J = 6.1 Hz). Such NMR signal features of rhamnose protons suggest for 1→2 diglycoside sugar linkage with glucuronic acid further confirmed by 2D-NMR spectra. In detail, long range HMBC detected a cross peak between the anomeric proton of rhamnose H-1‴ at δ5.3 ppm and a carbon peak at δ79 ppm (glucuronic acid, C-2″). HSQC exhibited a correlation between C-2″ and a proton at δ3.7 ppm (glucuronic acid, H-2″). The latter was traced by ^{1}H−^{1}H COSY and confirmed its coupling with the anomeric proton of glucuronic acid H-1″ at δ5.2 ppm (Table 4) thus proving the 1→2 sugar linkage [26,27]. To our knowledge, this is the first report for the presence ofapigenin-7-O-α-rhamnosyl (1→2)-β-glucuronide not only in *B. acerifolius* but rather in the whole genus.

The ^{1}H NMR spectra of compounds 4 and 5 showed the same NMR peaks pattern for apigenin aglycone with sugar anomeric proton at δ5.1/5.2 ppm as a complex multiplet signal due to the free rotation of the sugar moiety with respect to the aglycone [30], albeit could be easily distinguished from their MS data.

In contrast, ^{1}H NMR of compound 2 (rutin) showed signals for H-5′ at δ6.9 ppm (d, J = 8.3 Hz), H-2′ at δ7.65 ppm (d, J = 1.8 Hz) and H-6′ at δ7.6 ppm (dd, J = 1.8 and 8.3 Hz) consistent with the quercetin substitution pattern. The sugars anomeric protons/carbons were observed at δ_H5.1 (d, J = 7.4 Hz) and δ_C104 ppm for β-glucose and δ_H4.5 (d, J = 1.7 Hz) and δ_C102 ppm for rhamnose which exists in α-form as indicated from small J-value. The upfield chemical shift of rhamnose anomeric proton inferred 1→6 sugar linkage [27].

Compound 3, luteolin-7-O-β-glucuronide, was confirmed through a complex NMR peak signal at δ7.4 ppm for H-2′ and 6′, and the doublet at δ6.89 (J = 8.7 Hz) for H-5′. Two doublets were detected at δ6.50 and 6.85 ppm with an equal coupling constant (J = 2 Hz) representing H-6 and H-8, respectively, in addition to H-3 singlet peak at δ6.60 ppm. The anomeric proton of glucuronic acid was observed at δ5.1 ppm (d, J = 7 Hz) with another distinct doublet peak for H-5″ at δ3.8 ppm (J = 9 Hz) [29] (Table 4).

To our knowledge, all identified flavonoids in this study are reported for the first time in *B. acerifolius* leaves except for compounds 2,6,7 and 8 [31]. It is noteworthy that the majority of the identified compounds in this investigation was consistent with the results derived via the UPLC/PDA/q-TOF/ESI-MS profiling analysis of *B. acerifolius* crude leaf extract, thus posing UPLC-MS as a robust technique for flavonoids profiling [11]. Most of the identified compounds are aglycones and glycosides of apigenin and luteolin, which are well known to reduce glucose absorption remarkably by competitive inhibition of sodium-dependent glucose transporter-1. Additionally, flavones can control blood glucose level via an inhibitory effect on α-glucosidase activity [32]. Furthermore, Mukherjee *et al.* [33] recommended apigenin and luteolin as leading drugs for treatment of diabetes mellitus.

Conflict of interest statement

We declare that we have no conflict of interest.

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