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Chemical profile and beneficial effect of standardized extract of *Stevia rebaudiana* Bertoni leaves on metabolic syndrome in high fat diet streptozotocin-induced diabetic rats

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Abstract

Stevia (*Stevia rebaudiana* Bertoni) is a natural zero calorie sweetener with significant economic and medicinal values due to its high contents of steviosides (SVGs) in the leaves. The aqueous extract of *Stevia* leaves (TAqE) was standardized to contain 8.5% w/w of SVGs (HPLC), total phenolics (164.63 ± 1.39 µg Gallic acid/mg extract) and total flavonoids of 100.5 ± 0.79 µg QE/mg extract. Twenty-one compounds were tentatively identified in the leaves via UPLC-Orbitrap HRMS and stevioside, rebaudioside A, and quercetrin were isolated from TAqE by repeated column chromatography. Stevioside showed significant inhibition of pancreatic lipase, α-amylase, and α-glucosidase enzymes. The effect of a standardized TAqE on high fat diet (HFD)-streptozotocin (STZ)-induced diabetic rats was investigated. Thirty-six animals were divided into 6 groups (each of 6). Rats in group I (control) and group II (control/HFD-STZ) received distilled water, and rats in groups III and IV received TAqE for 4 weeks in two doses; 300 mg/kg b.wt., and 500 mg/kg b.wt., respectively. Rats in group V received metformin (200 mg/kg), while those in group VI received statin (1 mg/kg). Body weight, fasting blood glucose, lipid profile (total cholesterol and triglycerides), liver enzymes (alanine transaminase and aspartic transaminase), and serum kidney parameters (urea and creatinine) were decreased in rats treated with TAqE (300 mg/kg b.wt.), while insulin sensitivity was enhanced, when compared to that in group II. These findings could justify the use of *Stevia* as a complementary medicine for the prevention and treatment of metabolic changes associated with diabetes mellitus type 2.

Keywords: *Stevia rebaudiana* Bertoni, Fat-fed/STZ rats, Metabolic symptoms, Antihyperglycemic, Antihyperlipidemic, HPLC standardization

Introduction

Metabolic Syndrome (MS) is a constellation of risk factors that increases a person's risk of developing cardiovascular disease. These factors include abdominal obesity, atherogenic dyslipidemia (elevated triglycerides, small LDL particles, low HDL cholesterol), raised blood pressure, insulin resistance (with or without glucose intolerance), pro inflammatory state and prothrombotic state [1, 2].It is reported that MS is a major public and clinical problem worldwide; over a billion people in the world are now affected with MS and the prevalence of obesity and its consequent metabolic abnormalities was found to be 32.2% [2]. MS usually starts with insulin resistance leading finally to metabolic disturbances such as hyperglycemia, hyperlipidemia, kidney, and liver impairments. Clinically, treatment of each is prescribed according to the

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patient's state. However, acetylcholinesterase inhibitors used for hypertension such as enalapril and captopril, may increase serum creatinine level, or cause cough, headache, and skin rash [3]. Also, metformin, a drug used for type 2 diabetes, can induce gastrointestinal symptoms and lactic acidosis [4].Therefore, traditionally used herbal drugs such as *Camellia sinensis*, *Hibiscus sabdariffa*, *Citrus limon*, and *Punica granatum* can be considered as a complementary or alternative medicine for metabolic diseases [5].

Stevia rebaudiana Bertoni (Fam. *Asteraceae*) popularly known as stevia or sweet leaf, is native to Paraguay, where it has a long history of use as a non-calorie sweetener in beverages and foods [6]. Recently, the leaves have gained increased industrial and scientific interests as a perfect alternative to sucrose and artificial sweeteners that have many health hazards [7]. Sweetness of the leaves is imparted by the presence of a complex mixture of zero caloric sweeteners (steviol glycosides, SVGs); mainly stevioside and rebaudioside A (250–300 sweeter than sucrose) [8]. The leaves also contain other important phytochemicals, such as vitamin C and polyphenols (flavonoids and phenolics), which are mostly responsible for the antioxidant activities of its extracts [9]. Accordingly, Stevia has great economical and health values in food industry as non-alcoholic beverages, as food additive. Also, as a natural control for diabetes and to help control weight in obese persons [10]. In number of animal experiments, stevia extracts showed anti-hyperglycemic [10], antioxidant [11], antihypertensive [12], anti-inflammatory [13] and anti-obesity [14] activities. Also, inhibited α-amylase [15] and decreased fasting blood glucose, glycosylated hemoglobin, and improved insulin and glycogen levels in STZ-induced diabetic rats [16]. However, nothing was reported concerning the effect of the extract of stevia cultivated in Egypt or steviosides on MS. Therefore, it deemed of interest to assess the effect of a standardized *Stevia* extract on hyperglycemia, hyperlipidemia and relevant metabolic parameters associated with MS in a high fat diet (HFD)-streptozotocin (STZ)-induced diabetic rats [17]. This combination of high-fat diet with STZ generates rats with hyperglycemia associated with hypertriglyceridemia and introduces many other metabolic alterations present in human diabetes type 2 (DM2) [17, 18]. Additionally, the antioxidant effect and effect on key digestive enzymes in the hydrolysis of carbohydrates and fats such as pancreatic lipase, α-amylase, and α-glucosidase were investigated [19]. Besides, the chemical profile of the extract was identified by UPLC/MS/MS and major SVGs and flavonoids were isolated and quantified in the leaves of the Egyptian stevia cultivar using HPLC.

Experimental

Materials

Plant material Samples of the leaves of *S. rebaudiana* (Bertoni) Egyptian cultivar (through crossing between Chinese and Spanish varieties) were collected from the Sugar Crops Research Institute (SCRI), Agricultural Research Centre (ARC), Giza, Egypt and identified by Dr. Ahmed Attia; Senior Researcher, SCRI, ARC (Breeding and Genetic Department). A voucher specimen (No. 2.9.2019.I) is kept at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University. The leaves were collected, air-dried in shade, powdered (with mesh size of 0.2–0.636 mm) and kept in tightly closed glass containers till use.

Chemicals Porcine pancreatic lipase enzyme, *p*-nitrophenylbutyrate (PNPB), α-amylase, α-glucosidase, acarbose, *p*-nitrophenyl-α-D-maltopentoside (PNPM), *p*-nitrophenyl-α-D-glucopyranoside (PNPG), Streptozotocin (STZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and gallic acid were all purchased from (Sigma, St Louis, MO, USA). Metformin and Orlistat were kindly supplied from Eva Pharma, Egypt. Quercetin (Misr Company for Pharmaceutical Industry, Cairo, Egypt). Statin was obtained from EIPICO (Cairo, Egypt). Stevioside and rebaudioside A were isolated in our lab and chemical structures were identified (see Additional file 1).

Solvents The solvents used in this work viz.; petroleum ether (60–80 °C), *n*-hexane, methylene chloride, ethyl acetate and methanol were of analytical grade and purchased from the local market. Acetonitrile and methanol used for HPLC and spectrophotometric analyses were from Sigma- Aldrich (Steinheim, Germany).

Extraction, fractionation, and isolation One kg of stevia leaves powder was extracted with boiling distilled water (4 × 2 L) then filtrated. The filtrates were combined, concentrated, and freeze dried to give 280 g of dry residue (TAqE). Part of the residue (100 g) was suspended in water and repeatedly applied onto a column of Diaion HP-20 (40 cm L × 4 cm i.d.) from (Pharmacia, Fine Chemicals AB, Uppsala, Sweden). Gradient elution started with water (4 L) and decreasing the polarity by 25% increments of methanol till 100% methanol (4 L each) to give 5 fractions. Steviosides rich fraction (SRF, 3.46 g) was obtained from fractions eluted with 25–50% aqueous methanol, and flavonoid-rich fraction (FRF, 5 g) was obtained from fractions eluted with 75%-100% methanol. Stevioside and rebaudioside A were isolated by crystallization from SRF. Also, quercetrin was isolated from FRF. Chemical structures of the isolated compounds were identified using different spectroscopic methods (see Additional file 1).

Total phenolic content (TPC) The total phenolic content of TAqE was determined using the Folin-Ciocalteu method described by [20], and expressed as µg gallic acid equivalents (GAE) per mg of the extract. All samples were analyzed in triplicate.

Total flavonoid content (TFC) Total flavonoidal content of TAqE was determined by the aluminium chloride colorimetric assay described by [20], and expressed as µg quercetin equivalents (QE) per mg of the extract. All samples were analyzed in triplicates.

Determination of DPPH radical scavenging activity The DPPH radical scavenging activity was determined using the method reported by [21]. Absorbance was measured at 492 nm and ascorbic acid concentrations (0.1575–1 mg/ml) were used as standard. A blank was set up in parallel as a control. Each sample was tested twice with triplicate measurements in each experiment. The DPPH radical scavenging activity (%) was calculated as follows:

$$EC_{50} = [(Ac - As)/Ac] \times 100$$

where, Ac was the absorbance of control (DPPH solution without test sample) and As was the absorbance of sample [DPPH solution + sample (extract/standard)]. The EC_{50} is defined as the concentration of substrate that causes 50% reduction of the DPPH color. Results are displayed in Fig. 1.

UPLC-Orbitrap HRESI-MS analysis The chemical profile of the methanolic extract (5 mg) of *S. rebaudiana* leaves (Egyptian cultivar) was identified using UPLC coupled to a photodiode array detector (PAD) and an Orbitrap Elite mass spectrophotometer equipped with heated electrospray ionization (ESI) source. Analysis was performed using water (A) and acetonitrile (B) with 0.1% formic acid as mobile phases. The following binary gradient was applied: 0–1 min (isocratic 5% B/A), 1–11 min (linear gradient of B/A from 5 to 100%), 11–19 min (isocratic 100% B) and 19–30 min (isocratic 5% B). The flow rate was 150 µL/min, and the injection volume was 2 µL. The CID mass spectra (buffer gas; helium) were recorded using normalized collision energy (NCE) of 35%. The instrument was equipped with a heated electrospray ion source (negative spray voltage at 3 kV, capillary temperature of 300 °C, source heater temperature of 250 °C, FTMS resolution of 30.000) and RP-18 column (particle size 1.8 µm, pore size 100 Å, 150 × 1 mm i.d., Acquity HSS T3, Waters; column temperature of 40 °C). It was externally calibrated by the Pierce ESI negative ion calibration solution (product No. 88324) from Thermo Fisher Scientific. The data were evaluated using the software Xcalibur 2.2 SP1. Metabolites were also characterized by their UV–VIS spectra (220–600 nm) [22].

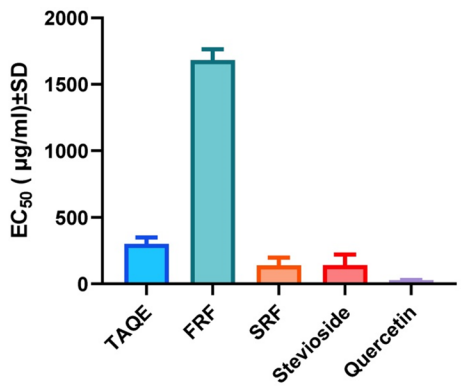


Fig. 1 DPPH antioxidant activity of TAqE, fractions and isolated compounds

HPLC quantitation of steviosides The chromatographic analysis was performed on Agilent Technologies 1100 series HPLC system Agilent Technologies, Palo Alto, CA), equipped with a quaternary pump, degasser G1322A and UV detector. Agilent Chemstation software was used for data acquisition and processing. Lichrospher RP-C18 column (250 mm L × 4.6 mm ID, 5 µm, Merck, Germany), preceded by a C18 guard column (10 mm L × 4 mm ID, 5 µm) was used. The mobile phase was composed of acetonitrile "solvent A" and 0.3% H_3PO_4 in H_2O "solvent B" applying gradient elution: 20% A/B to 33.7% A/B in 7 min, then to 34% A/B in another 7 min and to 50% A/B in 1 min then to 100% A in 2 min, then to 20% A/B in 3 min. The flow rate was 1 ml/min, injection volume was 20 µL, and detection (UV) was performed at 210 nm.

Sample preparation Sample (500 mg) of powdered *S. rebaudiana* leaves (mesh size of 0.2–0.636 mm) was extracted with distilled water (10 × 10 mL) by frequent sonication (for 3 min) and heating on water bath (80°C for 2 min). The extract was filtered using Whatmann filter paper, and the volume was adjusted to the mark (100 mL) with water. An aliquot (20 µL) of the extract was used for HPLC analysis.

Construction of standard curves for stevioside and rebaudioside A A standard stock solution of stevioside in water (4 mg/5 mL) was prepared and diluted with water to yield 4 concentrations (25, 64, 96 and 128 µg/mL). An aliquot (20 µL) of each dilution was injected in triplicates and corresponding peak area recorded. The standard calibration curve of stevioside was constructed ($r^2 = 0.998$) by

plotting mean peak areas versus corresponding concentrations.

Similarly, a stock solution of rebaudioside A in water (2 mg/5 ml) was prepared and diluted to yield 4 concentrations (32, 64,120 and 160 µg/ml). As mentioned above, standard calibration curve of rebaudioside A was constructed ($r^2 = 0.9889$).

Pancreatic lipase inhibitory assay The lipase inhibition activity was determined by a method in [23]. In this method, the enzyme was dissolved in phosphate buffer (pH 6.8) at a concentration of (100 µg/mL) and then centrifuged at 2000 rpm for 5 min to remove insoluble matter. The PNPB solution (substrate) was dissolved in acetonitrile (20 mM) and the stevia extract (TAqE) and fractions (SRF and FRF) were prepared in DMSO at different concentration (1000–7.81 µg/mL). The enzyme (20 µL) was incubated first with 20 µL of the sample solution and 20 µL of the phosphate buffer at 30 °C for 5 min in a 96 well plate. Subsequently, 20 µL of PNPB solution was added and the mixture was incubated at 37 °C for 60 min. The absorbance was measured at 405 nm. Orlistat was used at the same concentrations as a standard. Enzyme inhibitory activity was calculated as follows:

$$\text{Inhibitory activity } (I\%) = \left(\text{Abs. 100\% enzyme activity} - \text{Abs. extract}\right) / \left(\text{Abs. 100\% enzyme}\right) \times (100)$$

Results are displayed in Fig. 5.

alpha-Amylase inhibitory assay According to the method described in [24] the enzyme α-glucosidase (from *Saccharomyces cervisiae*) was dissolved in phosphate buffer (pH 6.8) in concentration of 4 U/mL and then centrifuged at 2000 rpm for 5 min to remove insoluble matter. PNPM solution (substrate) was dissolved in buffer at concentration of 1.25 mM and the stevia extract (TAqE) and fractions (SRF and FRF) were prepared in methanol in varying concentrations from 1000 to 7.81 µg/mL. Then, 20 µL phosphate buffer (50 mM, pH = 6.8) with 20 µL of enzyme, 20 µL of the sample solution and 20 µL PNPG were incubated at 37 °C for 10 min in a 96 well plate. The absorbance of the released *p*-nitrophenol was measured at 405 nm using multiplate reader. Acarbose (Sigma-Aldrich, Bangalore) was used as a standard at concentrations of 0.1575–1 mg/ml. Blank was set up in parallel as a control. Enzyme inhibitory activity was calculated as follows:

$$\text{Inhibitory activity } (I\%) = \left(\text{Abs. 100\% enzyme activity} - \text{Abs. extract}\right) / \left(\text{Abs. 100\% enzyme}\right) \times (100)$$

Results are displayed in Fig. 6.

alpha-Glucosidase inhibitory assay According to [25] the enzyme (*α*-glucosidase from *Saccharomyces cerevisiae*) was dissolved in phosphate buffer (pH 6.8) in concentration of 1 U/ml and then centrifuged at 2000 rpm for 5 min to remove insoluble matter. The *p*-nitro-phenyl-α–D-glucopyranoside (*p*-NPG) substrate (Hi-media) PNP solution (substrate) was prepared by dissolving it in phosphate buffer in concentration of 5 mM. The stevia extract (TAqE) and fractions (SRF and FRF) were prepared in varying concentrations from 1000 to 7.81 µg/mL in DMSO. Then, 20 µL phosphate buffer (50 mM, pH = 6.8) was incubated with 20 µL of enzyme, 20 µL of the sample solution and 20 µL PNPG at 37 °C for 20 min, in a 96 well plate. The absorbance of the released *p*-nitrophenol was measured at 405 nm using multiplate reader. Acarbose at various concentrations (0.1575–1 mg/ml) was used as a standard. A blank was set up in parallel as a control. Enzyme inhibitory activity was calculated as follows:

$$\text{Inhibitory activity } (I\%) = \left(\text{Abs. 100\% enzyme activity} - \text{Abs. extract}\right) / \left(\text{Abs. 100\% enzyme}\right) \times (100)$$

Results are displayed in Fig. 7.

Acute oral toxicity test Median lethal dose (LD_{50}) was determined for evaluating the safety of TAqE of *S. rebaudiana* leaves as described in [26]. Forty-eight male Westar rats (200 g) were divided into eight groups (6 animals each). They were orally administered single doses of the extract (ranging from 1 to 5 g/kg b.wt., the maximum soluble dose).

In vivo antihyperlipidemic and antihyperglycemic activities According to [17] all animals except normal control were fed a high-fat diet HFD (total energy 25.07 kJ/g including fat 60%, protein 20% and carbohydrate 20%) for 4 successive weeks. After overnight fasting, STZ (40 mg/kg) was freshly prepared in a 0.05 M citrate buffer (pH 4.5) and injected i.p. Blood glucose level was monitored after 2 days using an Accu-check blood glucose meter (Roche Diagnostics, Basel, Switzerland). Animals having blood

glucose levels ≥ 200 mg/dl were included in the experiment.

Group I: Normal-control group received saline and normal diet orally.
Group II: HFD/STZ was kept as positive control.
Group III: HFD/STZ induced diabetic group was administered an oral dose of TAqE (300 mg/kg).
Group IV: HFD/STZ induced diabetic group was administered TAqE with an oral dose of (500 mg/kg).
Group V: HFD/STZ induced diabetic group was administered an oral dose of metformin (200 mg/kg) [27] as a standard anti-hyperglycemic drug.
Group VI: HFD/STZ induced diabetic group was administered an oral dose of statin (1 mg/kg) [28] as anti-hyperlipidemic standard.

Body weight of the rats was measured weekly and fasting blood glucose was determined every 2 weeks. At the end of the experiment, the animals were fasted overnight, and blood samples were then collected by cardiac puncture. After standing for at least 30 min, the blood samples were centrifuged in centrifuge machine (Labcent 5000, Biosan England) at 3000 rpm for 15 min and the sera were stored at − 20 °C until use. Blood insulin level was determined by Rat Insulin ELISA kit (Alpco, UK). Serum lipid profile including total cholesterol and triglycerides was measured using Biodiagnostic colorimetric kits (Biodiagnostic, Cairo, Egypt). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by kits provided by Randox Laboratories Co. (Crumlin, Antrim, UK).

Statistical analysis Data are presented as the mean ± SD and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparison using SPSS Software (Chicago, USA) and a trial version of Graph Pad Prism. Differences were significant at $p^{<} 0.05$.

Results and discussion

Extraction and isolation of compounds

The leaves of stevia were extracted with boiling distilled water to give TAqE. Phytochemical fractionation of the extract afforded 3 compounds: stevioside, rebaudioside A and quercetrin (see Additional file 1).

Total phenolic content (TPC) and total flavonoid content (TFC)

Egyptian cultivar of stevia leaves showed total phenolics of 164.63 ± 1.39 µg Gallic acid/mg extract) and total flavonoids of (100.5 ± 0.79 µg QE/mg extract).

The antioxidant activity against DPPH radical

The antioxidant activity of TAqE, fractions and isolated compounds was evaluated (Fig. 1). Stevioside showed EC_{50} of 139.14 ± 58.70 (µg/mL), TAqE (560.71 ± 52.50), FRF (301 ± 48.00), SRF (1681 ± 48). The results indicate that stevioside has the highest activity to quench the DPPH radical, which reflects its high antioxidant activity.

Tentative identification of metabolites in *Stevia* leaves

Chemical profile of the methanolic extract of *S. rebaudiana* leaves was performed in the negative ESI mode. It revealed the presence of 29 peaks belonging to phenolic acids, flavonoids, diterpene glycosides (steviosides) and labdane diterpenes (Fig. 2). The tentatively identified compounds were classified and the main parameters that support their identification are compiled in Table 1.

Phenolic acids

Conjugates formed from the reaction of hydroxycinnamic acids with quinic acid are of common occurrence in *Stevia* species [29–32]. Several of the conjugates such as di-*O*-caffeoylquinic acid and its conjugates, *p*-sinapoylquinic acid and feruloylquinic acid were tentatively identified in this study (peaks #2, 3, 4, 5, 11, 16, 17, and 19. The predominant fragment of *m/z* 191 amu for the quinic acid moiety in the MS spectrum and the characteristic UV λ_{max} at 325–330 nm are diagnostic for hydroxycinnamic acid derivatives. Also, fragment ions at m/z 179 (for the loss of caffeoyl moiety). In case of caffeoylquinic acid dimers and trimers, the fragment ion at *m/z* 353 $[M\text{-}H\text{-}353.08]^-$ indicates the loss of caffeoylquinic acid moiety.

Flavonoids

Generally, occur as sugar conjugates, principally as *O*-glycosides, The loss of mass units 162, 146 and 132 amu are indicative for *O*-hexosides, *O*-deoxyhexosides and *O*-pentosides; respectively. *O*-glycosides are of common occurrence in *Stevia* species [30, 33, 34]. Peak #14 a flavonol glycoside (quercetin-3-*O*-hexosyl-7-*O*-deoxyhexoside or rutin)] at *m/z* 609.1450($C_{27}H_{29}O_{16}^-$) and fragment ions at 447 $[M–H–162]^-$, 463 $[M–H–146]^-$ and 301 characteristic to quercetin. Peak#15 Flavone glycoside (luteolin 7-*O*-hexoside) with an $[M–H]^-$ at *m/z* 447.0925 ($C_{21}H_{19}O_{11}^-$) and a fragment ion at 285 $[M–H–162]^-$.

Diterpene glycosides (Steviosides)

All the stevia glycosides, as well as steviol and isosteviol, yielded abundant $[M–H]^-$ ions in preliminary

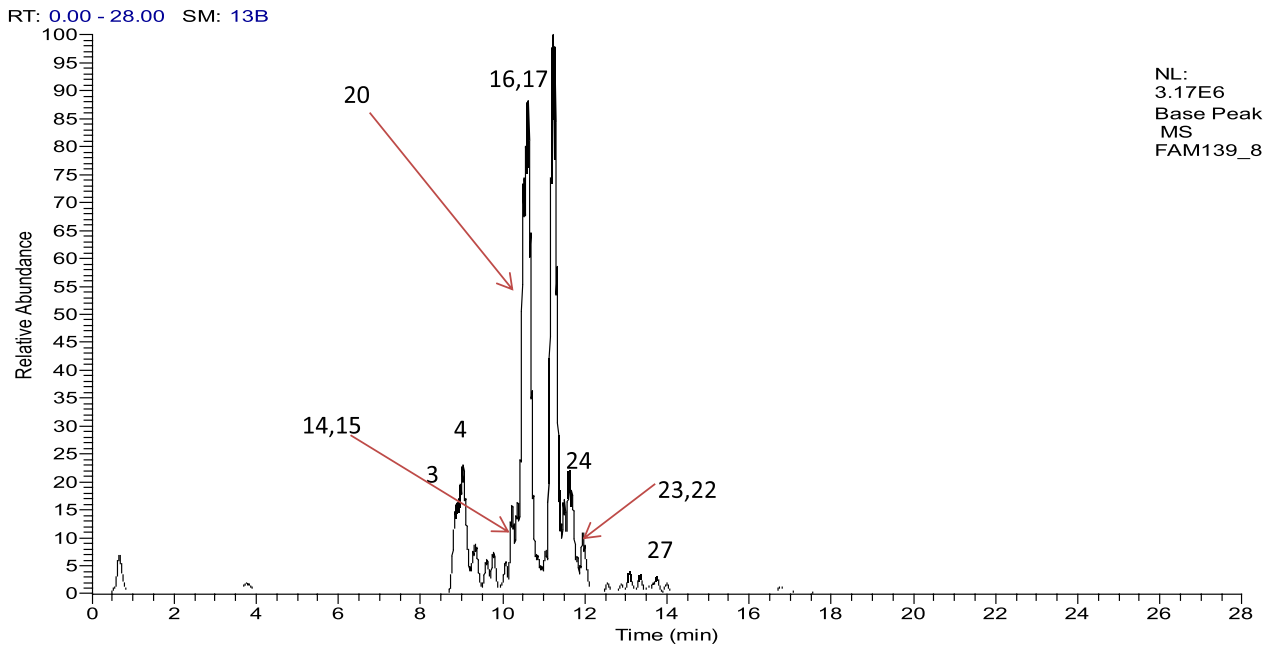


Fig. 2 UPLC-Orbitrap HRESI-MS base peak chromatogram of the methanolic extract of *Stevia rebaudiana* Bert. leaves in negative ion mode

investigations of their ionization behavior. Such behavior was expected for the aglycones steviol and isosteviol as well as Reb B and steviolbioside, as these substances have carboxylic acid groups that readily undergo deprotonation [35]. Eight diterpene glycosides were tentatively identified in *S. rebaudiana* leaves (peaks# 20, 21, 22, 23, 24, 25, 27, 28) [6, 9, 15, 33, 36, 37]. Identification was based on their $[M–H]^-$ ions and the facile sequential cleavage of sugar units (loss of 162 amu for hexose and loss of 146 for deoxyhexose) in the ion source, even at low cone voltages [35, 36, 38].

HPLC standardization

From the established standard calibration curves, the results showed each 100 g of the dried Stevia leaves contain 7.98 g stevioside and 1.03 g rebaudioside A. (Structures shown in Fig. 3). HPLC chromatograms (Fig. 4i and ii) were developed at 210 nm (for detecting steviosides) and at 325 nm (for detecting flavonoids and phenolics), respectively. The detected compounds were stevioside (St), rebaudioside A (Reb A), quercetin-3-*O*-α-rhamnopyranoside (C4), apigenin–7-*O*-α-rhamnopyranoside (C3), as well as caffeic acid (C2) and chlorogenic acid (C1) by comparing their retention times with those of standard samples.

In vitro pancreatic lipase inhibitory assay

Results showed that TAqE has no activity at tested concentration, while SRF and FRF fractions demonstrated weak inhibitory effects. However, stevioside exerted the most significant inhibition of lipase enzyme (60.5 ± 1.5 µg/mL), comparable to that of orlistat (Fig. 5).

In-vitro α-glucosidase and α-amylase inhibitory assays

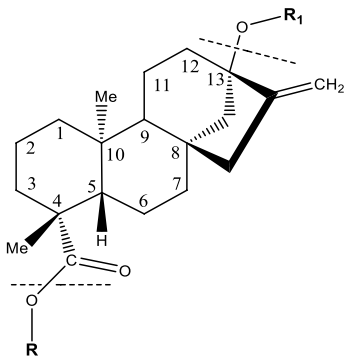
Favorable α-amylase and α-glucosidase inhibitory effects were observed by TAqE (Figs. 6 and 7). On the other hand, stevioside exhibited the highest ***α***-amylase inhibition activity compared to acarbose standard and showed high *α*-glucosidase inhibitory effect relative to acarbose (Figs. 6 and 7).

Acute oral toxicity test

No signs of toxicity or mortality were observed in any group during 24 h after oral administration of TAqE ranging from 1 to 5 g/kg. The extract was considered safe up to 5 g/kg b.wt. Thus, therapeutic doses would be 1/10, 1/20 and 1/40 of the maximum soluble dose. Accordingly, 300 and 500 mg/kg were chosen as therapeutic doses of TAqE.

Table 1 Metabolites tentatively identified in the methanolic extract of *S. rebaudiana* Bert. leaves using UPLC-Orbitrap HRESI-MS in the negative ion mode

Peak no.	R_t (min.)	M^-	M. Formula	Error (ppm)	MS/MS^-	UV (nm)	Identification
1	0.66	377.0901	$C_{12}H_{21}O^-$	3.5	341.11, 215	–	Sugar alcohol
2	8.71	179.0351	$C_9H_7O_4^-$	0.5	135	333	Caffeic acid
3	8.71	353.0871	$C_{16}H_{17}O_9^-$	1.87	191, 173, 179, 135	327	3-*O*-Caffeoylquinic acid (neochlorogenic acid)
4	9.03	707.1814	$C_{32}H_{36}O_{18}^-$	0.127	353	326	Dicaffeoylquinic acid dimer
5	9.23	353.0856	$C_{16}H_{17}O_9^-$	1.8	173, 179, 191	327	5-*O*-Caffeoylquinic acid (Chlorogenic acid)
6	9.27	431.1522	$C_{19}H_{27}O_{11}^-$	0.283	299.11, 131.04 149.05	328	Hexosylpentosyl 2-(*p*-hydroxyphenyl)ethanol
7	9.54	381.0820	$C_{18}H_{21}O_9^-$	1.5	161, 179, 135	328	Ethyl chlorogenate isomer
8	9.63	447.1499	$C_{18}H_{25}O_{10}^-$	2.5	401	328	Benzylhexosylpentoside
9	9.72	431.1913	$C_{19}H_{29}O_8^-$	2.5	385, 223, 205, 153	332	Roseoside
10	9.87	397.1131	$C_{18}H_{21}O_{10}$	O.44	191,179, 173	332	*p*-Sinapoylquinic acid
11	9.93	367.1027	$C_{17}H_{19}O_9^-$	1.14	191, 173, 147	326	5-*O*-Feruloylquinic acid
12	10.00	537.2020	$C_{26}H_{33}O_{12}^-$	− 0.56	489.12, 375.14, 327.12	325	Hydroxylariciresinol hexoside
13	10.01	611.2481	$C_{26}H_{43}O_{16}^-$	0.22	431, 251	325	Unknown
14	10.15	609.1450	$C_{27}H_{29}O_{16}^-$	0.05	463, 447, 301, 300	335–369	Quercetin hexosyl deoxyhexoside Quercetin -3-*O*-rutinoside (Rutin)
15	10.31	447.0925	$C_{21}H_{19}O_{11}^-$	1.369	285, 244	344	Luteolin-7-*O*-glucoside (Cynaroside)
16	10.41	515.1184	$C_{25}H_{23}O_{12}^-$	0.849	353, 191, 173	329	3,4-di-*O*-Caffeoylquinic acid
17	10.61	515.1189	$C_{25}H_{23}O_{12}^-$	3.061	353, 299, 173	328	4,5-di-*O*-Caffeoylquinic acid
18	10.92	705.3327	$C_{26}H_{41}O_9^-$	2.63	659, 497	308–328	Unknown
19	11.00	677.1502	$C_{34}H_{29}O_{15}^-$	− 0.91	515, 353, 191	329	3,4,5-tri-*O*-Caffeoylquinic acid
20	11.15	965.4564	$C_{44}H_{69}O_{23}^-$	0.92	803-	nd	Rebaudioside A
21	11.24	849.3761	$C_{39}H_{61}O_{20}^-$	0.42	641, 803	nd	Steviol + 2 Hexose + 4-Methylglucuronide
22	11.41	787.3744	$C_{38}H_{59}O_{17}^-$	1.65	787, 625, 641	nd	Dulcoside A
23	11.54	687.3244	$C_{33}H_{51}O_{15}^-$	0.732	641, 623, 479	nd	Steviol + hexose + 4-methylglucoronid
24	11.55	803.3698	$C_{38}H_{59}O_{18}^-$	0.272	641, 479, 317	nd	Stevioside/Rebaudioside B
25	11.58	641.3172	$C_{32}H_{49}O_{13}^-$	0.767	479, 317	nd	Steviolbioside/rubusoside
26	11.78	359.0766	$C_{18}H_{15}O_8^-$	2.18	344, 329, 286	nd	Centaureidin
27	11.89	335.2229	$C_{20}H_{30}O_3^-$	3.93	317	289	Steviol
28	12.15	479.2653	$C_{26}H_{39}O_8^-$	2.55	317.12	nd	Kaurenoic acid hexoside
29	13.16	295.2273	$C_{18}H_{31}O_3^-$	2.70	277, 195, 171	nd	Unknown



Compound	R	R_1
Stevioside	β-Glc	β-Glc-β-Glc (2→1)
Steviolbioside	H	β-Glc-β-Glc (2→1)
Rebaudioside A	β-Glc	β-Glc-β-Glc (2→1) | β-Glc (3→1)
Dulcoside A	β-Glc	β-Glc-α-Rha (2→1)

Fig. 3 Structures of major diterpene glycosides detected by UPLC—Orbitrap HRMS analysis in the extract of *Stevia rebaudiana*

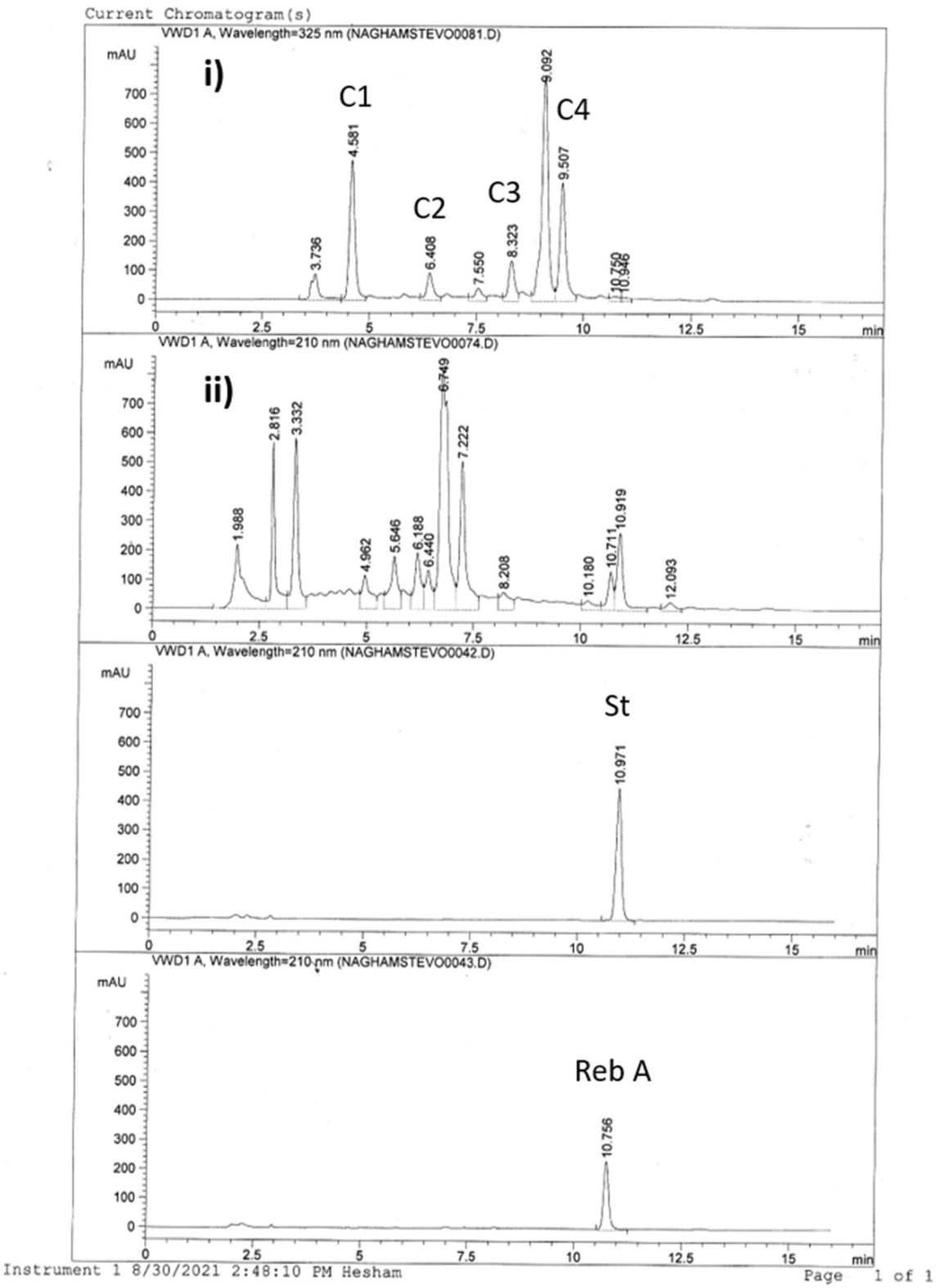


Fig. 4 HPLCchromatograms (i: at325 and ii: at 210 nm) of total aqueous extract of *S. rebaudiana* leaves. C1 = chlorogenic acid, C2 = caffeic acid, C3 = apigenin-7-O rhamnopyranoside, C4 = quercetin-3-*O*-α-rhamnopyranoside, *St* stevioside, *Reb* A rebaudioside A

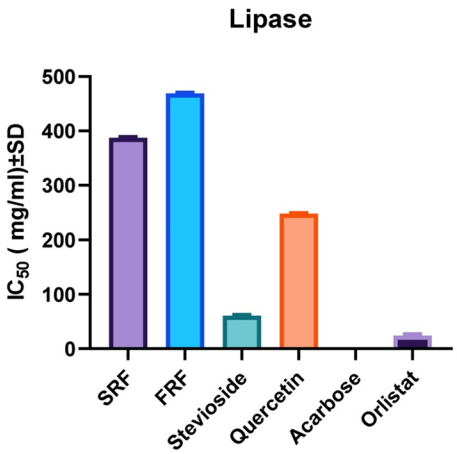


Fig. 5 In-vitro lipase inhibitory activities of TAqE, fractions, and isolated compounds of *S. rebaudiana* leaves compared to orlistat

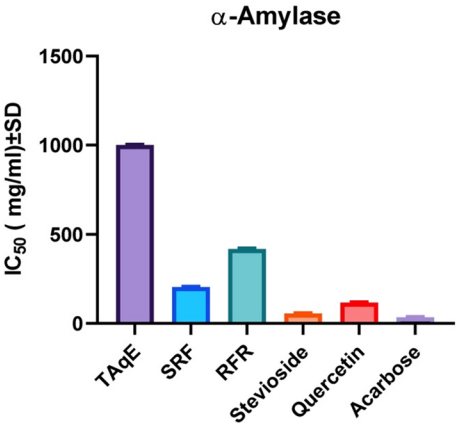


Fig. 6 In-vitro α-amylase inhibitory activities of TAqE, fractions and isolated compounds of *S. rebaudiana* leaves compared to acarbose

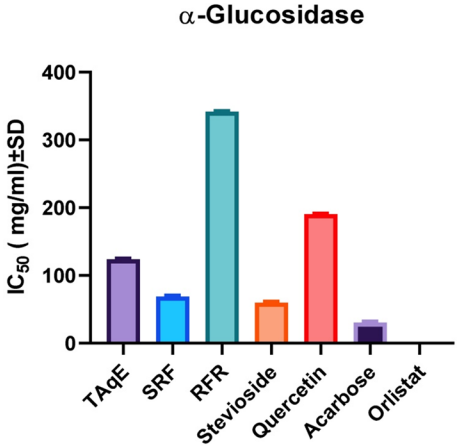


Fig.7 In-vitro α-glucosidase inhibitory activities of TAqE, fractions and isolated compounds of *S. rebaudiana* leaves compared to acarbose

In-vivo biological potential of *S. rebaudiana* extract in hyperglycemic and hyperlipidemic rats

High fat diet altogether with STZ triggered various metabolic changes in the rats (HFD-STZ group). Significant increase in body weight, hyperglycemia, hyperlipidemia, liver and kidney dysfunctions compared to normal control group. Body weight was increased by 26.4% (Fig. 8A), fasting blood glucose and insulin were elevated by 168.83% and 32.27%, respectively in HFD-STZ group compared to normal rats (Fig. 8B and C). Lipid profile was up regulated significantly as well; total glycerides, total cholesterol were elevated by 66.23, 130.35%, respectively (Fig. 8D and E). The level of liver enzymes; ALT and AST was increased by 105% and 15.19%, respectively in HFD-STZ group compared to normal group (Fig. 8 F and G). Also, kidney functions were impaired; serum urea and creatinine levels were elevated by 76.02% and 59.57%, respectively (Fig. 8H and I). The oral administration of TAqE (300 and 500 mg/kg) for 4 weeks was found to be effective in ameliorating the induced metabolic changes as follows: the body weight was significantly decreased by 12% and 18%, respectively in a manner comparable to standard statin (1 mg/kg) and metformin (200 mg/kg) that decreased the body weight gain by 13 and 18%, respectively (Fig. 8A). As presented in (Fig. 8B), fasting blood glucose level of the diabetic control group rats was significantly ($P < 0.05$) restored toward normal after treatment with TAqE (300 and 500 mg/kg) from the 1st week to the fourth week by 43.50–56%. Also, TAqE (500 mg/kg) showed significant decrease in blood insulin by 10.35% (Fig. 8C).

Treatment of the HFD-STZ rats with TAqE (300 and 500 mg/kg) significantly ($P^{<}$ 0.05) decreased triglycerides by 17% and 27.24%, respectively (Fig. 8D). Similarly, total cholesterol was significantly decreased by 22.12% and 34%, respectively after treatment in a manner comparable to that shown by standard metformin (41.15%) and statin (25.32%) (Fig. 8E). Also, the liver enzymes; ALT & AST were significantly ($P^{<}$ 0.05) down regulated by 35.25% and 36.68%, respectively, and in a manner comparable to that of demonstrated by metformin and statin -treated groups (45.11% and 34.46%, respectively) (Fig. 8F and G). On the other hand, administration of TAqE (300 and 500 mg/kg) decreased serum urea by 18.55% and 32.39%, respectively, and a decrease in serum creatinine level of 1.04 and 1.02 mg/dL, was obtained (Fig. 8H and I).

Discussion

This study showed that HFD along with high carbohydrates administration led to physiological and metabolic changes similar to that of MS with elevated levels of serum glucose, insulin, lipid profile parameters, liver enzymes, and kidney function parameters [38]. It also

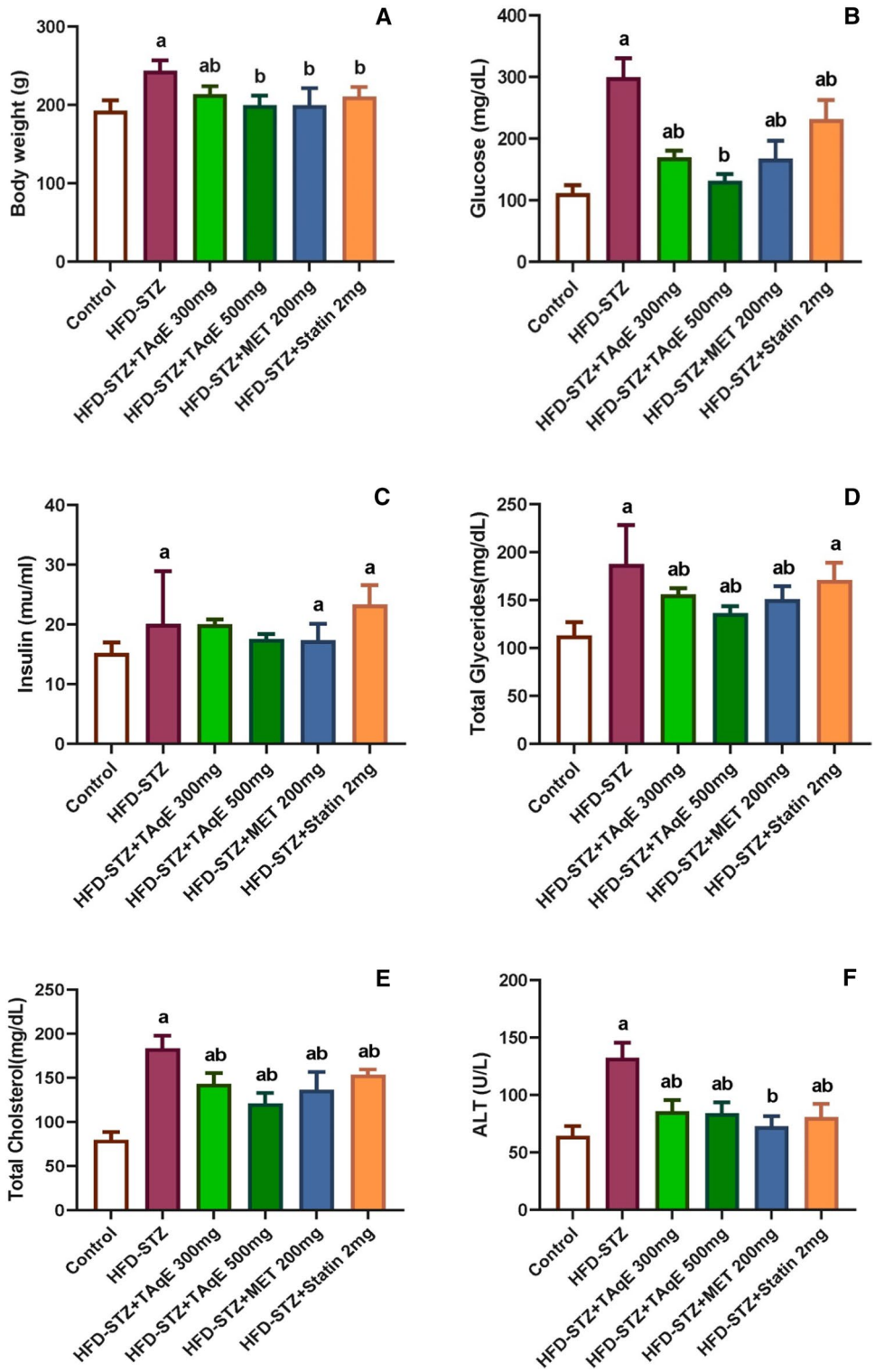


Fig. 8 Effect of *S. rebaudiana* extract on different metabolic parameters of hyperlipidemic and hyperglycemic rats. **A** Average body weight, **B** glucose level, **C** insulin level, **D** Total glycerides, **E** Total cholesterol, **F** ALT level, **G** AST level, **H** Urea and **I** Creatinine. Data are calculated as mean ± SD (n = 6) (one-way ANOVA followed by Tukey's multiple comparison test). [a]Significant difference from control group at $p < 0.05$. [b]Significant difference from HFD-inducted group at $p < 0.05$. [ab]Significant difference from control group and HFD-inducted group at $p < 0.05$

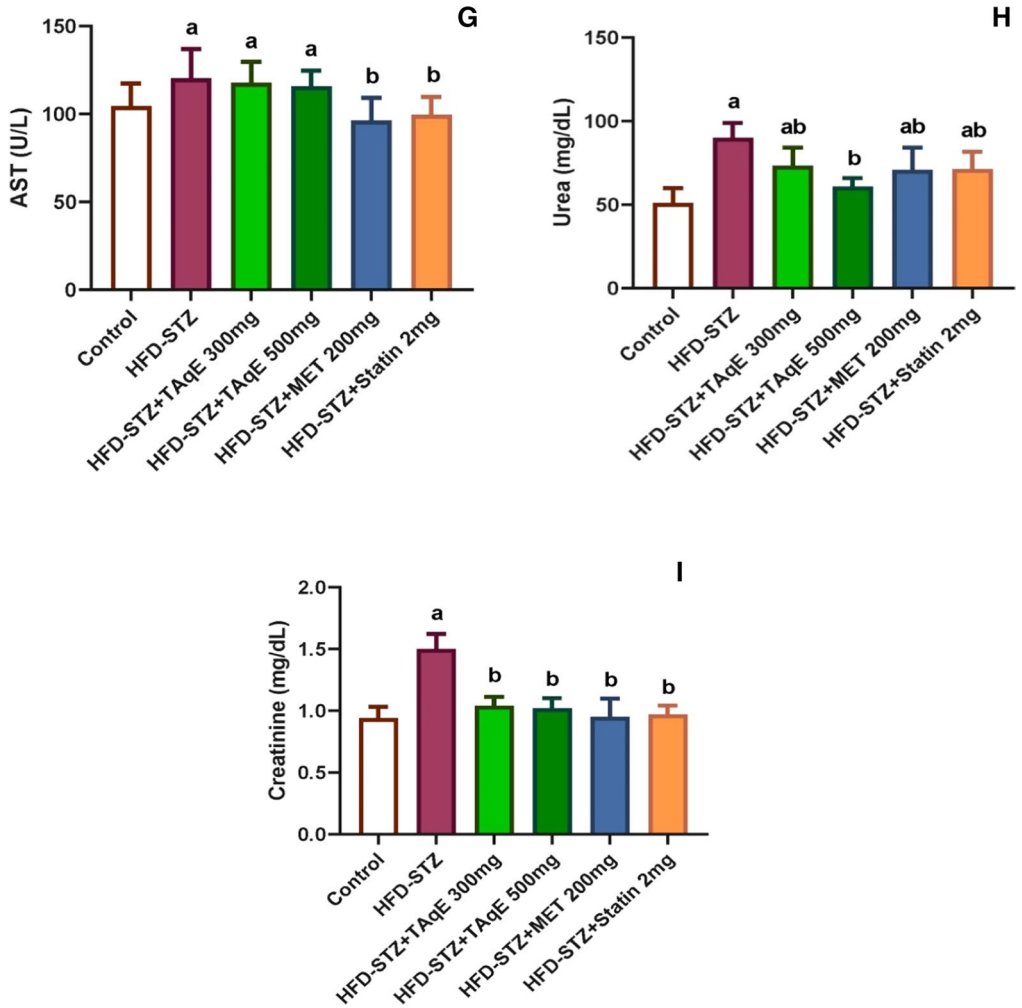


Fig. 8 continued

increased body weight (visceral adiposity in particular), lead to CVS disorders and defects in antioxidant stability [38–40]. Insulin resistance is a major underlying mechanism for the MS; insulin and its signaling cascade normally control cell growth and metabolism. Therefore, alleviation of oxidative stress and enzymes controlling carbohydrates and lipid metabolism suppression support MS management.

Concerning MS animal model, diet induced hyperglycemia and hyperlipidemia is one of the most popular and reliable models due to its similarity in modeling the common route of MS in humans [2]. The present work aimed to evaluate the harmful effect of HFD on metabolic profile and beneficial effect of *S. rebaudiana* aqueous extract (TAqE) on MS. In this context, products' quality to control obesity associated with metabolic syndrome is determined by its ability to induce weight loss [41]. In this study, administration of the TAqE (300 and 500 mg/kg) was found to significantly decrease body weight and the hyperglycemic parameters. Also, at a dose of 500 mg/kg it showed the highest significant decrease in the abruptly increased serum lipid profile parameters. Moreover, liver and kidney inflammatory markers were significantly down regulated upon treatment with TAqE. These findings prove to high extent the effective actions exerted by Stevia leaves on metabolic disorders associated with diabetes.

Enhanced anti-hyperlipidemic and anti-hyperglycemic effects of TAqE could be attributed to its inhibitory effects on carbohydrates and lipid metabolizing enzymes. As was shown by the pronounced antioxidant, α -amylase and glucosidase enzymes inhibitory activities of TAqE as well as the highest inhibitory activities shown by SRF. It is also worth to mention that oxidative stress was remarkably ameliorated and FRF showed the highest antioxidant activity (when compared to standard ascorbic acid).

Phenolics and flavonoids are of interest because of their apparent health-promoting effects as antioxidants, antidiabetic and anti-CVS disorders [42]. Accordingly, antioxidant activity and its role in managing oxidative stress and enzymes inhibitory activities were the suggested mechanisms in correlation with the in vivo study. Our findings are consistent with those of previously published studies. Assi et al. [43] reported that Stevia extract at a dose of (300 mg/kg) has a significant anti-hyperglycemic action in diabetic rats. Also, Stevia crystal reduced body weight and BGL [44].

Biological potential of TAqE could be attributed to its predominating compounds; steviol glycosides, phenolic acids and flavonoids as was shown in the results of in vitro assessment. Stevioside is a potent free radicle scavenger when compared to standard ascorbic acid. Its inhibitory activities on lipase, α-amylase and α-glucosidase enzymes are the proposed mechanisms for controlling hyperglycemia and hyperlipidemia accompanying MS.

Chemical profiling of *S. rebaudiana* leaves was tentatively clarified using UPLC-Orbitrap HRMS analysis. It was characterized by its enrichment of steviol glycosides, phenolic acids and flavonoids, where 21 compounds were tentatively identified including stevioside, rebaudioside A, steviol, chlorogenic acid and quercetrin, as previously detected [15, 31–33]. However, it is the first time to use advanced technique like UPLC-Orbitrap HRMS analysis in the metabolic profiling of the leaves of the Egyptian cultivar of *S. rebaudiana*.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-022-00724-8.

Additional file 1.

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Author contributions
NHK performed the investigation, resources, visualization and writing the original draft; AE: methodology, investigation, and formal analysis of biological experiments; HIEA, methodology, investigation, formal analysis of HPLC experiment; HMEH: conceptualization, supervision, visualization, writing review and editing of the manuscript; SMAW: conceptualization and supervision; MRM conceptualization, supervision, visualization, reviewing and editing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Competing interests
The authors declare that they have no competing interests.

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