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Reno Protective Effect of Methanolic *Stevia Rebaudiana Bertoni* Leaves Extract and Its Phenolic Compounds in Type-1- Diabetes

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THE AIM of this study is to evaluate the reno-protective effect of the defatted methanolic extract of Stevia rebaudiana which showed the highest improvement of renal parameters, as it reduced the protein urea, blood urea nitrogen and blood creatinine and nephroprotective properties with reduced hyperglycemia complications through protection of renal tissues in type-1 diabetes. Also, identification of bioactive phenolic compounds of defatted methanolic extract using HPLC analysis, demonstrate the presence of diterpene glycosides, stevioside, rebaudioside A and chlorogenic acid.

Keywords: *Stevia rebaudiana*, Diterpene glycosides, Chlorogenic acid, Phenolic, HPLC, Diabetic nephropathy

Introduction

Stevia rebaudiana Bertoni is an ancient perennial shrub, a member among the 950 genera of the Asteraceae (Compositae) family, commonly known as candy leaf, sweet leaf, or sugar leaf, and native to the Amambay region in the north east of Paraguay. It also grows in neighboring areas of Brazil and Argentina, favors humid, wet environments, although the root does not tolerate standing water [1,2]. *Stevia* end-products have been used for various purposes during the past [3]. This shrub has gained an important economic value and scientific interest, due to the presence of a high concentration of natural, dietetically valuable sweeteners in the leaves. Japan was the first country in Asia to commercialize stevioside as a sweetener in the food and drug industry [4]. Crops of *Stevia* have been planted in several other countries in Asia, including China, Malaysia, Singapore, South Korea, Taiwan, and Thailand [5]. Hyperglycemia is considered to be the dominant pathological characteristic of DM, as it causes the majority of the symptoms associated with DM. Chronic hyperglycemia results in the accumulation of advanced glycation end products (AGEs) in the body, which further increase the risk for vascular complications in patients with DM as damaged kidney tissues, including reduced glomeruli, dilation of the renal capsule and kidney tubules [6,7].Diabetic nephropathy (DN) induced by streptozotocin (STZ) is widely used in studies investigating DM. High-dose STZ induced direct nephrotoxicity, making it unable to distinguish between the direct toxic effect of STZ and the lesions that resulted from STZ-induced hyperglycemia [8,9].

Diabetic nephropathy is the chronic loss of kidney function occurring in those with diabetes mellitus and causes widespread damage to small and large blood vessels, which is a marker for cardiovascular disease, a common cause of death in these patients. Chronicity of hyperglycemia is associated with long-term damage and failure of various organ systems mainly affecting the eyes, nerves, kidneys, and the heart [10]. Approximately 200 manufacturers including all of the major food manufacturers showed interest in using stevia in the manufacturing of their products.

Stevia leaves used in the studies met all current specifications set by the Joint FAO/WHO Expert Committee on Food additives [11]. Due to the high content of various phytoconstituents, *Stevia* extracts have shown significant antimicrobial, hypoglycemic, anti-hypertensive, antitumour, anti-inflammatory, hepatoprotective and

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immunomodulatory activities both in vitro and in animal studies [12].

The aim of the present work, in continuation of our research [13] on methanolic extract of *Stevia rebaudiana* plant is to characterize the content of the main phytochemical component and biological activity of extracts prepared from dried *Stevia rebaudiana* leaves on diabetic nephropathy induced by streptozotocin(STZ).

Materials and Methods

Plant material

Stevia leaves plant (*S. rebaudiana*) (1.5 kg), were collected from International Company from Agro-industry Product (SICAP), Cairo, Egypt in March 2016. The voucher specimen of plant was stored in Botany Department, Faculty of Science, Zagazig University.

Extraction and fractionation

Powdered air-dried leaves of *Stevia rebaudiana bertoni* (300 gm) were defatted with petroleum ether (60-80°C) and extracted with methanol (80%) to yield a dry extract (75 gm).

Chemicals and instruments

All solvents and HPLC grade was acquired from Sigma-Aldrich (Bellfonte, PA), standards kit containing stevia glycosides, dulcoside A, stevioside, rebaudioside A rubusoside, steviolbioside, rebaudioside C, rebaudioside B, obtained from Chromadex (P/N KIT-00019568-010, Irvine CA).

Chromatography Analysis

Paper chromatographic analysis was carried out on Whatman paper No. 1, using solvent systems: (1) H_2O; (2) 6 % HOAc; (3) BAW (n-BuOH: HOAc: H_2O, 4:1:5, upper layer). BAW solvent system was used for preparative paper chromatography (Prep. PC) using Whatman paper No. 3.

The purification of stevioside and chlorogenic acid through a column silica gel 60, using a gradient mobile phase solvent system of acetonitrile: water (20:80) was achieved [16]. The fractions were collected and dried under reduced pressure to give 8.9 gm pure compound of stevioside. While Chlorogenic acid was isolated from methanolic *Stevia* extract through a silica gel column using toluene/ethyl acetate (90: 10, v/v) as an eluant, 300 mg of pure powder was produced [17]

HPLC Analysis

Separation and determination of phenolics were performed by reverse phase HPLC (Waters M6000A, Milford, USA) using HPLC grade acetonitrile and deionised water. The detector was set-up at UV 210 nm. The column used for HPLC analysis was a reversed-phase RP- C_{18}, (Length: 150 mm. inner diameter 4.6 mm. particle size: 5 μm) with a guard column. The HPLC method employed was an isocratic binary solvent mobile phase system with a 30:70 mixture of acetonitrile and 10 mmol/L sodium phosphate buffer (pH: 2.63), very similar to the method reported in JECFA [14,15]

Nuclear Magnetic Resonance Spectroscopic Analysis

NMR spectra were measured by a Bruker 400-MHz NMR spectrometer. ^{1}H chemical shifts (δ) were measured in *ppm*, relative to TMS and ^{13}C NMR chemical shifts relative to DMSO-d_6 and converted to TMS scale by adding 39.5 ppm.

Experimental Animals

Forty eight healthy male Sprague Dawely rats, weighting 170-200 g were purchased and housed at the Medical Experimental Research Centre (MERC), faculty of medicine, Mansoura University. All animals were given food and distilled water and housed at a temperature maintained under a 12 hour light/dark cycle and a temperature of 25°C (±3ºC). Rats were randomly divided into six groups (n = 8/group): the first group was accounted as normal control rats (NC), they received 0.75 ml normal saline subcutaneous (S.C) once daily for 4 weeks, the second group (DM) diabetic rats were induced by a single dose STZ (50 mg/kg in 0.1 M citrate buffer, pH 4.0). Seventy-two hours after STZ injection, diabetes was diagnosed (blood glucose levels were over 300 mg/dl). The third group (DM+I) diabetic rats were treated with mixtard insulin 30 in at a dose of 1.0 IU/ 100g dissolved in 0.75 ml normal saline subcutaneous (S.C) [18], the fourth group (DM+MSE) diabetic rats were treated with 200 mg/kg of methanolic *Stevia* extract, while the fifth group diabetic rats were treated with 2 mg/kg of pure natural stevioside (DM+S) extracted from *Stevia*, and the last group diabetic rats were treated with 10 mg/kg of pure natural chlorogenic acid (DM+CGA) extracted from *Stevia*. Treated groups (4, 5, 6) fed with aqueous solution of (dried methanolic extract, pure stevioside and pure chlorogenic acid) by gavages once daily for 4 weeks.

Biochemical assays

At the end of experiment, the rats were anaesthetized by high dose of Na thiopental (120 mg/Kg), then blood samples were collected by heart puncture and centrifuged at 1000 rpm and the sera were kept at -20°C till the time of analysis. Quantitative determination of urea (BIOMED / BU210217), uric acid (SPINREACT/LIQ625) and creatinine (HUMAN/16011) in serum were performed according to the manufacturer's protocols.

Statistical Analysis

Data obtained from experiment have been analyzed using SPSS 20; they were expressed as the mean ± SD., with one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test. $P < 0.05$ was considered statistically significant.

Results and Discussion

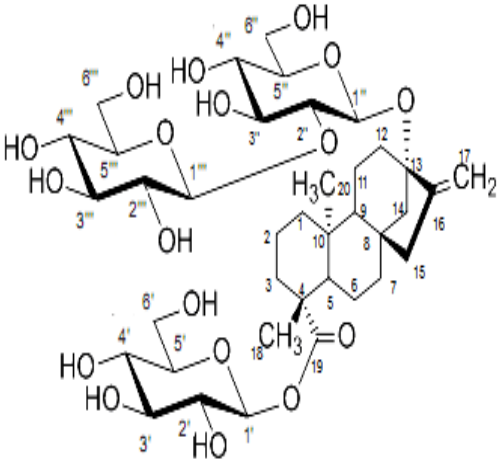
High Performance Liquid Chromatography (HPLC) Analysis

High Performance Liquid Chromatography (HPLC) analysis have been performed on methanolic *Stevia* extract. all the six naturally occurring steviol glycosides Dulcoside A, Stevioside, Rebaudioside A, Steviolbioside, Rebaudioside C, Rebaudioside B were identified by comparative study with the retention times of those standards and are given in Table 1, Fig 1. A close observation of their retention times indicated that the two major steviol glycosides of *S. rebaudiana* are rebaudioside A (3) and stevioside (2) and the three minor compounds rebaudioside C (6), and dulcoside A (1) based on the RP-HPLC method using UV detection [19].

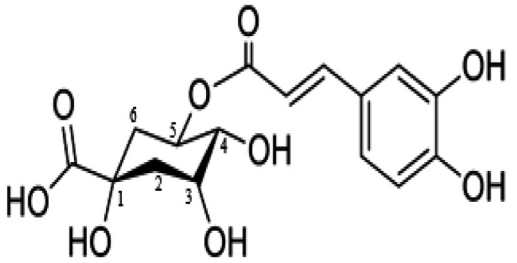
Identification of Stevioside

White powder; mp 225-227°C; With molecular formula $C_{38}H_{60}O_{18}$ and was supported by ^{1}H- NMR spectral data (DMSO-d6) at δ (ppm) :0.8,1.78 (m,H-1) ,1.34, 1.99 (m,H-2), 1.03,2.13(m,H-3), 1.06(m,H-5), 1.76,2.05 (m,H-6) , 1.37,1.49 (m,H-7), 0.92(m,H-9), 1.69 , 1.72(m,H-11) , 1.47, 2.02(m,H-12) , 1.39, 2.16(d,j =10 H-14), 2.05(m,H-15) , two olefinic protons at δppm = 4.7, 5.02 (s,H-17), two CH3 singlets at 1.14 (s, H-18) , 0.87 (s, H-20) ,and three doublet anomeric glycosyl signals at δppm = 5.2(d, J=8.5 Hz, H-1`), 4.64 (d, J= 8.3 Hz H-1``), 4.63 (d,J= 7.8 Hz,H-1```) beside glycosyl protons at δppm = 3.3 – 3.9 (m). ^{13}C-NMR Spectral Data (DMSO-d6) at δppm = 40.3 (C-1), 19.1 (C-2), 37.85 (C-3), 42.9 (C-4), 56.9 (C-5), 21.6 (C-6), 41.4 (C-7), 40.3(C-8), 53.5 (C-9), 39.3(C-10), 20.4 (C-11), 36.05 (C-12), 85.1 (C-13),43.9 (C-14), 47.36(C-15), 78.8 (C-16),22.2 (C-17), 178.5(C-18),28.3(C-19),15.59(C-20) . Three glucopyranose moieties in this compound appeared at : 94.6(C-1'), 73.01(C-2') , 77.42(C-3') , 69.98(C-4') , 76.71(C-5') , 60.97(C-6') , 96.8(C-1"), 83.08(C-2") , 77.42(C-3") , 70.8(C-4") , 78.1(C-5") , 61.49(C-6") and 104.7 (C-1"') , 75.75(C-2"') ,76.49(C-3"') , 70.12(C-4"') , 76.67(C-5"') , 61.15(C-6"').Based on the above spectral and chemical results, this compound was assigned as13-[(2-O- β -D -glucopyranosyl- β -D glucopyranosyl) oxy]-16β-hydroxy-ent-kauran-19- oic acid β -D-glucopyranosyl ester , (stevioside).

Identification of Chlorogenic acid:



Stevioside



Chlorogenic Acid

TABLE 1. Retention (*tR*) times of steviol glycosides 1-7.

Peak No.	Compound Name	Retention Times (*tR*, min)
	Dulcoside A	13.99
	Stevioside	10.05
	Rebaudioside A	9.74
	rubusoside	17.88
	Steviolbioside	26.00
	Rebaudioside C	12.531
	Rebaudioside B	24.71

Qualitative HPLC method for the identification of steviol glycosides, Dulcoside A (1), s Stevioside (2), Rebaudioside A (3), rubusoside (4), Steviolbioside (5), Rebaudioside C (6), Rebaudioside B (7), based on the RP-HPLC method using UV detection system.

Figure 1A

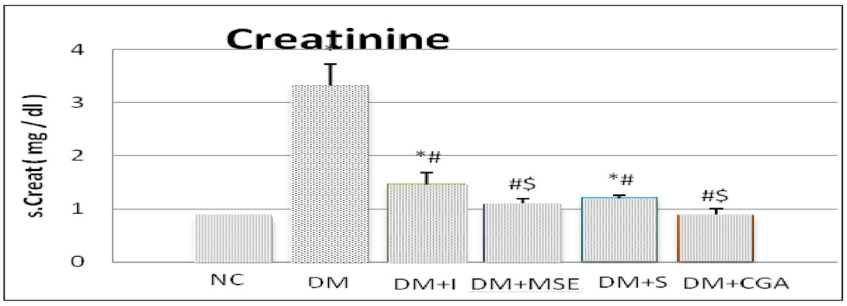


Figure 1B

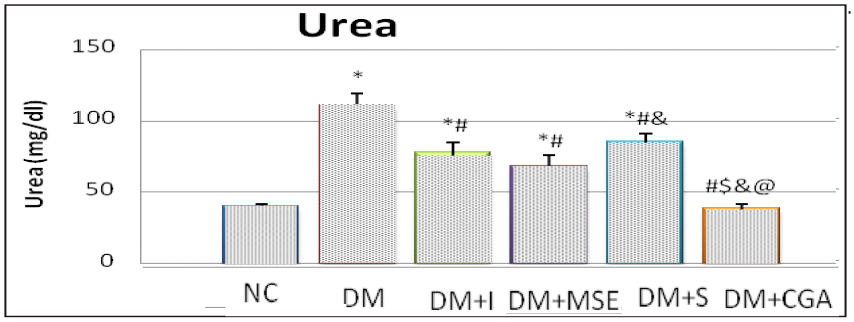


Figure 1C

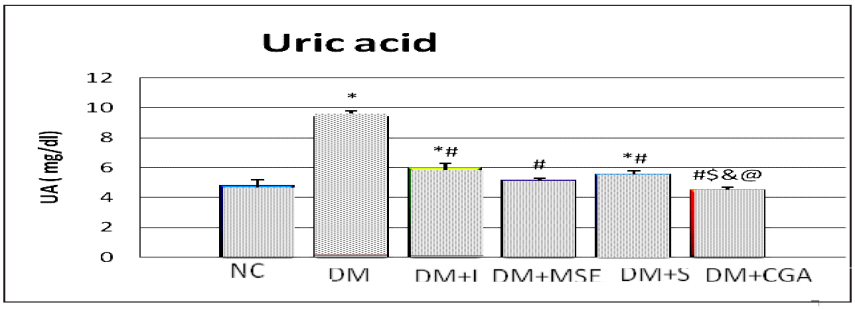


Fig . (1A,B,C) Effect of methanolic stevia extract, Stevioside and Chlorogenic acid on creatinin, urea and uric acid in STZ treated rats . All results are expressed as mean ± SD. One way ANOVA with Tukeys post hoc test (significance at p≤ 0.05). * = vs NC group, # = vs DC group, $= vs Insulin group, & = vs (DM+ methanolic stevia extract) group, @= vs(DM+ pure stevioside) group.

^{1}H- NMR Spectral Data (DMSO-d6) δ (ppm) ; Caffeoyl moiety : δ (ppm) = 6.9 (broad s, Δν1/2 =4 Hz,H-2'), 6.7 (d, J=8.5, H-5'), 6.94 (dd, J=1.2 , J=8.5, H-6'), 7.42(d, J=16, H-7'), 6.1(d, J=16, H-8'). Quinic acid moiety: 1.86 (m, H-2 and H-6), 3.7 (m, H-3), 3.4 (m, H-4) 5.16 (m, H-5). ^{13}C-NMR spectral data (DMSO-d6) of caffeoyl moiety : δ (ppm) = 126 (C-1'), 115.1(C-2'), 148.6(C-3'), 146.0(C-4'), 121.6(C-5'), 126.1(C-6'), 144.9 C-7'), 115.5 (C-8'), 199.9 (C-9') Quinic acid moity : δ (ppm) = 71.31(C-1), 39.4(C-2), 71.33(C-3), 73.4(C-4), 68.2(C-5), 39.5(C-6), 177.4(C=O).

Effect of Stevia Extract, Stevioside and Chlorogenic Acid (CGA) on Kidney Function Tests

Anti-diabetic activity of Stevia extract, stevioside and chlorogenic acid explained in previous studies ~~which~~ suggested that stevia extract and some of its pure compounds have hypoglycemic affect, control lipid profile and contractile dysfunctions in slow twitch (soleus) muscle in type 1 diabetic rats, these effects were associated with up regulation of GLUT4 in soleus muscle [20].

Figure (1A) showed significant increase in serum creatinine compared to NC group ($p<0.05$), where non-significant elevation in treated groups DM+MSE and DM+CGA) groups related to NC group ($p>0.05$). DM+I, DM+MSE, DM+S. and DM+CGA show significant decrease in creatinine levels than DM group ($p<0.05$). Groups DM+MSE and DM+CGA show significant creatinine decreasing compared to DM+I group ($p<0.05$), but no significance has shown between DM+S and DM+I ($p>0.05$) in creatinine. No significant decrease in creatinine showed between MD+MSE, DM+S. and DM+CGA groups ($p<0.05$).

Figure (1B) described that blood urea level has significant elevation in DM, DM+I DM+MSE, and DM+S groups compared to NC group ($p<0.05$), but no significant increases in DM+CGA group compared to NC ($P>0.05$). DM+I, DM+MSE, DM+S. and DM+CGA showed significant low urea levels compared to DM group ($p<0.05$). Blood urea appeared with no significant decrease in DM+MSE and DM+S compared to DM+I group ($P>0.05$). Significant decrease in urea has shown in DM+CGA group comparing to DM+I group ($p<0.05$).DM+S group represent a significant increase in urea value than DM+MSE ($P<0.05$). Blood urea found to be more significant decrease in DM+CGA group compared to others ($p<0.05$)

Figure (1C) uric acid measurement presented which shown significant increase in DM, DM+I, DM+S and compared to NC group ($p<0.05$) except DM+MSE and DM+CGA groups show no significant values comparing to NC group ($P>0.05$). Significant decrease observed in DM+I, DM+MSE, DM+S and DM+CGA compared with DM group ($P<0.05$). DM+MSE and DM+S have no significant decrease showed than DM+I group ($P>0.05$), but significance decrease showed in DM+CGA group compared to DM+I group ($p<0.05$). DM+MSE and DM+S. groups revealed no significance decreased in uric acid between each other ($P>0.05$), while DM+CGA group showed significant decrease in uric acid level compared to DM+MSE and DM+S groups ($P<0.05$).

Our data provided evidence which agrees with Shivanna, et al. who predicted that Streptozotocin was also found to induce kidney damage as evidenced by decreased glomerular filtration rate; this change was however alleviated in the stevia leaves and extracted polyphenol fed groups [21]. The results also agree with Lou**,** et al, suggested that CGA could reduce the protein urea, blood urea nitrogen and blood creatinine in 5/6 Nx animals significantly [22], and with Nishi**,**et al, investigated that oral administration of CGA to HFD/STZ induced Type-2 diabetic rat's demonstrated prominent normalization of blood sugar level, HbA1c level, BUN, creatinine clearance, proteinuria and antioxidant status of kidney[23]. Also agree with Ye HY, et al. supported fact that CGA attenuated oxidative stress in streptozocin-induced DN rats [24].

Conclusion

Diabetic nephropathy (DN) is the most common cause of chronic kidney disease occurring by diabetes mellitus. The early abnormalities of DN are glomerular hyperfiltration, increased renal albumin permeability followed by the development of glomerular mesangial cell proliferation, extracellular matrix accumulation and glomerulosclerosis. In summary, we found that experimental diabetic rats were accompanied by impaired renal function. *Stevia* extracts have a significant role in protecting against kidney damage in the STZ-diabetic rats due to its hypoglycemic effect, especially chlorogenic acid which has therapeutic benefits, supporting the use

of botanicals dietary supplements to improve the quality of life.

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الحمايه الكلويه لمستخلص الميثانول لأوراق الستيفا ريبوديوسيد ومركباتها الفينوليه فى مرض السكرى من النوع الأول

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هدفت الدراسة إلى التعرف على المحتوى الكيميائي لبعض مكونات مستخلص الميثانول لأوراق الستيفا باستخدام تحليل كروماتوجرافي سائل عالي الأداء وفصل الستيفيوسد وحمض الكلوروجينيك بصورة نقية من المستخلص الكحولى والتأثير الوقائي والفعال لهم فى مرض السكرى من النوع الأول . وأظهرت النتائج التعرف على جليكوسيدات ديتيربين وهى ستيفيوسيد والذى يحتل التركيز الأعلى فى النبات، ستيفيول بيوسيد، ريبوديوسيد أ، ب، ج، ودولكوسيد كما أوضحت الدراسة أن مستخلص الميثانول ومركبى الستيفيوسيد و حمض الكلوروجينيك لهم القدرة على التحكم فى الاعتلال الكلوى الناتج من ارتفاع معدلات السكر فى الدم وذلك بحماية الأنسجة الكلوية والحفاظ على كفاءتها بخفض تركيز الكرياتينين واليوريا وحمض البوليك فى الدم وبالتالى تعتبر أوراق ستيفيا مصدر جيد للمحليات منخفضة السعرات الحرارية.