Research Article

The Potential Efficacy of Stevia Extract, Glimepiride and Their Combination in Treating Diabetic Rats: A Novel Strategy in Therapy of Type 2 Diabetes Mellitus

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Abstract. Background: The currently therapy of type 2 diabetes is unsatisfactory. Nowadays, there is a great interest in using the medicinal plants for treatment of diabetes. Therefore, we studied the efficacy of stevia extract alone and in combination with commonly used sulfonylureas “glimepiride” in a trial to introduce a new effective therapeutic regimen for type 2 diabetes mellitus. Methods: Nicotinamide (230 mg/kg, IP) followed by streptozotocin (65 mg/kg, IP) were injected for induction of type 2 diabetes in male rats. The diabetic groups were treated for 21 days as the following, the first with stevia extract (300 mg/kg), the second with glimepiride (1 mg/kg), and the third with a combination of glimepiride and stevia extract. Many parameters were measured to evaluate the alleviation of the toxic effect of streptozotocin by stevia and/or glimepiride. Immunohistochemical expression of endothelial nitric oxide (eNOS) was assessed in renal tissues. Results: The stevia extract reduced the blood glucose, triglycerides, cholesterol, ALT, AST, urea, creatinine, tumour necrosis factor (TNFα) levels and malondialdehyde concentration compared to control diabetic group. Stevia treatment improved insulin and adiponectin levels. Stevia reduced eNOS expression in renal tissues compared to the diabetic rats. All these changes were more significant with combined treatment. Conclusion: Stevia and glimepiride may be a new putative therapeutic regimen for management of type 2 diabetes and its complications.

Keywords: Type 2 diabetes mellitus, stevia, glimepiride, streptozotocin and nitric oxide synthase.

1. Introduction

Diabetes mellitus (DM) is a global problem, characterized by high levels of glucose in the blood. The World Health Organization demonstrates that diabetes affects 366 million people all over the world and new predictions estimate that 552 million people will be diabetic on 2030 [1]. With high rates in the incidence of obesity, the prevalence of T2DM is also increased. Thus, identification of new therapeutic antidiabetic regimens that are beneficial for patients with T2DM is of great need [2].

The current therapies for T2DM are unsatisfactory. For regulation of the blood glucose levels as near to normal values as possible, diet control, exercise, and hypoglycemic agents are needed. Most of diabetic patients often complain
of secondary failure after prolonged treatment with sulfonylureas (SUs) [3].

Sulfonylureas, although commonly used as a second line therapy, often cause hypoglycemia and weight gain [4]. Besides, having many adverse effects, none of the oral antidiabetic agents successfully maintain euglycemic state and control the micro- and macrovascular complications. Thus, United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that single therapy of oral antidiabetic drugs usually shows a failure in the regulation of euglycemic state through a long time, and most of diabetics need to change this monotherapy to either combinations of oral antidiabetic drugs or insulin therapy [3].

Moreover type 2 Diabetes show complex pathophysiological changes, so that the ideal antidiabetic combination strategy: provides multiple mechanisms of action that maintain the function of pancreatic beta cell, improve the problem of insulin resistance, be well tolerated with little risk of hypoglycemia or weight gain, as well as provide cardiovascular and renal protective benefits [4, 5]. Therefore, the need for newer and safer agents for treatment of T2DM is impelling. Nowadays, there is a huge interest in the use of herbal agents for the management of T2DM. Thus, many clinical studies are done to estimate the antihyperglycemic action of medicinal plant. Among these herbal plants “Stevia rebaudiana Bertoni” which was extensively used as a traditional medicine to control hypertension, hyperglycemia, and hyperlipidemia. Stevia is famous due to its sweetness property and its usefulness action in blood glucose control [6]. Glycosides of stevia like stevioside and rebaudioside A have been established as natural zero-calorie/low-calorie sweeteners [7]. Awing to the sweetening power and potential therapeutic benefits of its leaves; Stevia has attracted both economic interest and scientific research.

Studies reported that the stevia exhibits significant pharmacological activities such as: antihypertensive, antihyperglycemic, anticancer, antiobesity, anti-diarrheal and enzyme inhibitory activities [7, 8]. Treatment with stevia extract is leading to enough antihyperglycemic action, with a little risk for hypoglycemia, reduced body weight, and other beneficial health effects like antioxidant, hepatic and renal protection [9, 10]. However, thorough investigation of the pharmacological interaction between the stevia and commonly used antidiabetic agents (glimepiride) are needed.

Therefore, this study was designed to provide a new experimental strategy for the effectiveness of stevia extract in combination with glimepiride (tradename Amaryl<sup>TM</sup>) compared to glimepiride alone in treating T2DM.

**2. Materials and Methods**

**2.1. Chemicals.** Streptozotocin powder was purchased from Sigma-Aldrich Chemical Company (Germany) and Glimepiride powder (Amaryl<sup>TM</sup>) was given from pharmaceutical companies (EIPICO) company, Egypt as a gift.

**2.2. Plant materials.** Leaves of *Stevia rebaudiana* Bertoni, family Asteraceae were obtained from farm in Assiut, Egypt during the flowering stage (April to July 2016). Stevia leaves were cleaned with water to eliminate the dust particles. Then, the leaves of stevia were dried.

**2.3. Extraction of the plant.** The dried powdered leaves (5kg) of stevia rebaudiana Bertoni was macerated in 70% ethanol (10 L x 3) for extraction. Followed by concentration of the alcoholic extract and the solvent free residue was (835 g) (16.7% w/w). Then, 500 mL of distilled H2O was added to a Part of the alcoholic extract (425 g) and repeated solvent fractionations with dichloromethane was done till complete exhaustion using Rotary flash evaporator, Heidolph WB 2000 (Germany). Concentration of the dichloromethane fraction and aqueous fraction were done thus, the solvent free residues were (87 g) and (336 g) respectively.

**2.4. Experimental animals.** Forty eight adult male rats weighing 180-200 g obtained from the animal home of Faculty of Medicine, Assuit University and were lived in stainless-steel cages (five rats in each cage). Rats were given water and food *ad libitum*. The animals were housed with ideal conditions of temperature (22 ± 2°C) and humidity (55 ± 5%) with 12-light/12-dark cycle. Animal handling and rights were maintained on accordance with the Ethical Committee guidelines of the Faculty of Medicine, Assuit University.

**2.5. Induction of diabetes.** For induction of T2DM, 230 mg/kg nicotinamide and 65 mg/kg of streptozotocin, which was freshly prepared in citrate buffer (0.1 M, pH 4.5) were injected I.P in male rats [11]. The STZ-injected rats were infused glucose (20%) in the first 24 h to avoid the initial STZ-induced deaths due to hypoglycemia. Occurrence of T2DM was insured by measuring the blood glucose level, 72 h from I.P administration of streptozotocin. STZ-injected rats with blood glucose levels more than 200 mg/dl were experimental models of T2DM and included in the experiment.

**2.6. Experimental design.** Forty eight wistar rats were classified into following groups: Group I: received saline (Control-diabetic); Group II: received saline (control diabetic); Group III: received 0.05% tween 80 (control diabetic); Group IV: diabetic rats treated with 300 mg/kg aquatic extract of stevia; Group V: diabetic rats treated with 1 mg/kg glimepiride; Group VI: diabetic rats treated with 300 mg/kg aquatic extract of stevia combined with 1 mg/kg glimepiride.

Stevia extract was prepared as solution (dissolve in saline), while glimepiride was dissolved in 0.5% tween 80. The aqueous extract of stevia and glimepiride were given orally by stomach tube every day for 21 days. Control rats were treated likewise with pure vehicles. Doses of glimepiride and stevia were determined in accordance with our preliminary tests and from literature review [12, 13].
2.7. Biochemical analysis. At the end of the experiment and before scarifying the experimental animals, blood samples were collected from the orbital sinus for determination of glucose, insulin, adiponectin, TNFα levels, lipid profile, liver and kidney function test. Blood glucose was measured by glucometer (Accu-Chek, Germany). Total cholesterol, triglycerides, high density lipoprotein (HDL), liver enzymes and renal function test were measured colorimetrically by Spectophotometer. The serum insulin, TNF-α and adiponectin were estimated by using an enzyme-linked immunosorbent assay (ELISA) kits purchased from (Calbiotech., USA, Sino Biotech Co., Ltd and Elbascience, USA) respectively. The level of renal and hepatic MDA was evaluated by a kit obtained from Biodiagnostic, Egypt. The hepatic and renal tissue from each rat, which were used for histopathological and immunohistochemical analysis, were kept in 10% formalin. The kidney and part of hepatic tissues were dissected to pieces for homogenization. The renal and hepatic homogenate were putted in the centrifuge for determination of malondialdehyde (MDA) level from the supernatant.

2.8. Histopathological examination. After scarification of the animals, slices from the hepatic and renal tissues were fixed in buffered formalin (10%) and then processed for photomicroscopic assessment [14]. The histopathologic changes were scored according to the percentage of tissue affection into: 0 = (without pathologic changes), mild affection = 1%-30%, moderate affection = 31%-70% and severe affection (>70%).

2.9. Immunohistochemical analysis. Deparaffinization and rehydration of the sections (four µm thick) were done. After that, blocking of the endogenous peroxides activity by H2O2 (3%) was performed. Then, the sections were immersed in 10 mmol/l citrate buffer at pH 6.0 with further exposure to heating in a microwave at 80°C for fifteen minutes to retrieve the antigens. The sections were incubated with the primary antibody (Rabbit Polyclonal Endothelial nitric oxide synthase (eNOS) dilution 1:100, Elabscience Biotechnology Inc, USA) at room temperature overnight. The secondary staining kit (Thermo Scientific Corporation; Fremont, CA, USA) was used based on manufacturer’s instructions. Immunoreactivity score of eNOS was evaluated in the blood vessels, glomeruli and tubules. The staining intensity was scored as: 0: negative, 1: weak, 2: moderate and 3: strong. The percentage of positive cells was scored as: 0 = negative, 1 = (<10%), 2 = (10% - 50%) and 3(> 50%). Then, both scores were added to provide a single score (0-6).

2.10. Statistical analysis. The results are represented as (means ± S.E.M.). The results analyzed by the Graph Pad Prism (Graph Pad; San Diego CA, USA) program. The analysis of difference between groups was done using the (ANOVA) test and post hoc test, Bonferroni test. The significance level was taken at P < 0.05, P < 0.01 and P < 0.001.

3. Results

3.1. The Effect of different treatments on blood glucose levels (BGL). Stevia extract at a dose of (300 mg/kg) produced a significant change in BGL in the form of decreasing the mean blood glucose levels upon daily administration for diabetic rats compared to control rats as represented in (Table 1). These findings were in accordance with previous results [12]. It’s obvious from the (Table 1) that the stevia extract has a significant anti-hyperglycemic action in diabetic rats. Our finding demonstrated a significant reduction in blood glucose level in glimepiride treated rats. The combined group of stevia extract (300 mg/kg) and glimepiride (1 mg/kg) showed a more reduction in the BGL compared to glimepiride alone. It’s evident from the Table 1, that the combination therapy of stevia extract and glimepiride caused an earlier decrease (170.5 ± 9.5) on BGL of diabetic rats at the day15 versus to diabetic rats treated with glimepiride alone (283.8 ± 13).

3.2. Effect of different treatments on serum insulin level. As shown in Figure 1, treatment of the rats with STZ showed a significant change in the serum insulin (p < 0.001) in the form of decreasing the insulin level in comparison to non diabetic rats. While diabetic rats treated with a 300 mg/kg/day of stevia extract orally for three weeks showed a significant increase (p < 0.001) in the serum insulin level in comparison to control rats. Administration of insulin releasing drug like glimepiride (1 mg/kg/day) to diabetic rats for the same duration showed a significant increase (p < 0.001) in the serum insulin level in comparison to control rats. Administration of insulin and stevia extract and glimepiride caused an earlier decrease (170.5 ± 9.5) on BGL of diabetic rats at the day15 versus to diabetic rats treated with glimepiride alone.

3.3. Effect of different treatments on the serum adiponectin level. As shown in Figure 2, administration of 300 mg/kg/day of stevia extract to diabetic rats for three weeks showed a significant elevation (p < 0.001) in the adiponectin level. Combined administration of glimepiride 1 mg/kg/day and stevia extract 300 mg/kg/day to diabetic rats for the same period showed a more elevation in the insulin level as compared to diabetic rats treated with glimepiride alone.

3.4. Effect of different treatment on lipid profile level

3.4.1. Influence on cholesterol level. Treatment of diabetic rats with stevia extract (300 mg/kg/day) caused a significant decrease in the serum cholesterol (P < 0.05, Figure 3) in comparison to untreated rats. Combined administration of stevia
Table 1: Influence of stevia and/or glimepiride on blood glucose levels of diabetic rats.

| Regimens                      | Blood glucose levels (mg/dl) at time intervals | Dose and duration (mg/kg) | Blood glucose levels (mg/dl) at time intervals |
|-------------------------------|-----------------------------------------------|---------------------------|-----------------------------------------------|
| Groups                        | Day 0  | Day 5 | Day 10 | Day 15 | Day 21 |
| 1 Control (non-diabetic)      | 130.8 ± 5.4 | 126.7 ± 6.3 | 121.7 ± 5 | 126.7 ± 4.4 | 116.7 ± 5.7 |
| 2 Diabetic control            | 456.7 ± 23.3 | 440 ± 23.1 | 406.7 ± 22 | 393.3 ± 20.3 | 386.7 ± 18.5 |
| 3 Stevia extract STZ + NA     | 451.7 ± 4.4 | 391.7 ± 4.4 | 350.0 ± 17.3 | 276.7 ± 8.8 | 205 ± 14.4** |
| 4 Glimepiride STZ + NA        | 465 ± 39.6 | 414.5 ± 28.8 | 346.8 ± 24.8 | 283.8 ± 13 | 221.3 ± 15** |
| 5 Glimepiride + Stevia extract | 475 ± 37.5 | 352.5 ± 20.5 | 237.5 ± 17.5 | 170.5 ± 9.5 | 132.5 ± 11.1*** |

Values represent mean ± S.E.M.

** Significant difference at P < 0.01 vs. initial diabetic value

*** Significant difference at P < 0.001 vs. initial diabetic value.

N.B: There is no difference between different control vehicles.

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Figure 1: Influence of daily administration of stevia and/or glimepiride for 21 days to diabetic rats on the insulin level.

extract (300 mg/kg/day) plus glimepiride (1 mg/kg/day) to diabetic rats showed a significant decrease in the cholesterol level (P < 0.001, Figure 3) in comparison to diabetic rats administered glimepiride alone.

3.4.2. Effect on serum triglycerides. Treatments of diabetic rats with stevia extract (300 mg/kg/day) orally for three weeks caused insignificant decrease in serum (TG). Diabetic rats treated with glimepiride (1 mg/kg/day) for the same period showed a significant reduction (P < 0.01, Figure 4) in serum (TG). Concurrent administration of stevia extract (300 mg/kg/day) plus glimepiride (1 mg/kg/day) to diabetic rats for a similar period produced a significant decrease in the (TG) level as compared to untreated diabetic rats (p < 0.001, Figure 4). The stevia enhanced the influence of glimepiride on the level of triglycerides.

3.4.3. Effect on serum high density lipoprotein. Diabetic rats treated with stevia extract 300 mg/kg/day for three
weeks showed a significant elevation \( (p < 0.001) \), Figure 5) in the HDL level as compared to control diabetic rats. However, combined administration of stevia extract 300 mg/kg/day and glimepiride 1 mg/kg/day to diabetic rats orally caused a significant increase \( (p < 0.05) \), Figure 5) in the HDL level as compared to rats treated with glimepiride alone. Stevia extract increase the effect of glimepiride on serum high density lipoprotein.

3.5. Effect of different treatment of liver function test. Table 2, shows that treatment of the rats with STZ caused elevation of the liver function parameters. Administration of 300 mg/kg/day stevia extract to diabetic rats expressed a significant reduction in hepatic parameters (ALT and AST). Co-administration of stevia extract and glimepiride to diabetic rats caused more improvement in liver function.

3.6. Effect on kidney function test. As shown in Table 3, rats treated with STZ exhibited elevation of the kidney function parameters. Treatment of diabetic rats with stevia extract expressed a significant decrease in renal indicators (urea and creatinine). Combined treatment administration of stevia extract and glimepiride to diabetic rats caused more improvement in renal function.

3.7. Effect of different treatments on level of tumour necrosis factor-α (TNF-α). As shown from Figure 6, treatment of the rats with STZ caused a significant elevation \( (p < 0.001) \) in the serum level of TNF-α. Combined administration of glimepiride 1 mg/kg/day and stevia extract 300 mg/kg/day to diabetic rats showed a significant reduction \( (p < 0.001) \) in the tumor necrosis factor level as compared to control rats. Stevia extract increase the inhibitory effect of glimepiride on TNF-α, which support the anti-inflammatory effect of stevia.

3.8. Effect of different treatment on renal and hepatic level of Malondialdehyde (MDA). Diabetic rats administered 300 mg/kg/day stevia extract for three weeks showed a significant reduction \( (p < 0.001) \), Figure 7) in the hepatic MDA level. Combined administration of stevia extract 300 mg/kg/day and glimepiride 1 mg/kg/day to diabetic rats for the same duration expressed a significant reduction \( (p < 0.001) \) in hepatic MDA level as compared to rats treated with glimepiride alone.
Table 2: Influence of stevia and/or glimepiride on liver function test of diabetic rats.

| Groups                      | Treatment                        | Dose and duration (mg/kg)                                      | ALT (U/I)         | AST (U/I)         | Total protein conc. (mg/dl) |
|-----------------------------|----------------------------------|----------------------------------------------------------------|-------------------|-------------------|----------------------------|
| 1 Control (non-diabetic)    | Saline Saline                    | Equal volumes single I.P Equal volumes orally for 3 weeks      | 26.7 ± 2.8        | 18.7 ± 2.3        | 7.7 ± 0.2                  |
| 2 Diabetic control          | STZ + NA Vehicle (Saline/Tween)  | Equal volumes single I.P Equal volumes orally for 3 weeks      | 62.3 ± 4.2        | 51 ± 5.3          | 9.4 ± 0.4                  |
| 3 Stevia extract            | STZ + NA Stevia extract          | STZ 55 mg/kg single I.P + NA 230 mg/kg 300 mg/kg stevia orally for 3 weeks | 32.6 ± 4.6***     | 31.7 ± 3.7**      | 8.6 ± 0.5                  |
| 4 Glimepiride               | STZ + NA Glimepiride             | STZ 55 mg/kg single I.P + NA 230 mg/kg 1 mg/kg glimepiride orally for 3 weeks | 28.5 ± 2.4***     | 23.5 ± 0.9***     | 7.8 ± 0.1**                |
| 5 Glimepiride + Stevia extract | STZ + NA Glimepiride + Stevia extract | -STZ 55 mg/kg single I.P + NA 230 mg/kg (1 mg/kg glimepiride + 300 mg/kg stevia) orally for 3 weeks | 22.3 ± 1.7***     | 18.1 ± 1.2***     | 7.2 ± 0.2***               |

Values represent mean ± S.E.M.

* Significant difference at P < 0.05 vs. diabetic value.

** Significant difference at P < 0.01 vs. diabetic value.

*** Significant difference at P < 0.001 vs. diabetic value.

N.B: There is no difference between different control vehicles.

Table 3: Effects of stevia and/or glimepiride on kidney function test of diabetic rats.

| Groups                      | Treatment                        | Dose and duration (mg/kg)                                      | Urea (mg/dl)       | Creatinine (mg/dl) |
|-----------------------------|----------------------------------|----------------------------------------------------------------|-------------------|--------------------|
| 1 Normal control (non-diabetic) | Saline Saline                    | Equal volumes single I.P Equal volumes orally for 3 weeks      | 39.1 ± 1.8        | 0.8 ± 0.04         |
| 2 Diabetic control          | STZ + NA Vehicle (Saline/Tween)  | Equal volumes single I.P Equal volumes orally for 3 weeks      | 60.6 ± 4.3        | 1.3 ± 0.07         |
| 3 Stevia extract            | STZ + NA Stevia extract          | STZ 55 mg/kg single I.P + NA 230 mg/kg 300 mg/kg stevia orally for 3 weeks | 48.4 ± 2.7*       | 0.87 ± 0.04***     |
| 4 Glimepiride               | STZ + NA Glimepiride             | STZ 55 mg/kg single I.P + NA 230 mg/kg 1 mg/kg glimepiride orally for 3 weeks | 43 ± 1.7***       | 0.8 ± 0.02***      |
| 5 Glimepiride + Stevia extract | STZ + NA Glimepiride + Stevia extract | -STZ 55 mg/kg single I.P + NA 230 mg/kg (1 mg/kg glimepiride + 300 mg/kg stevia) orally for 3 weeks | 40.5 ± 2.6***     | 0.72 ± 0.02***     |

Values represent mean ± S.E.M.

* Significant difference at P < 0.05 vs. diabetic value.

** Significant difference at P < 0.01 vs. diabetic value.

*** Significant difference at P < 0.001 vs. diabetic value.

N.B: There is no difference between different control vehicles.
Similarly, diabetic rats treated with stevia extract produced a significant decrease (p < 0.001, Figure 8) in renal MDA content. Combined daily administration of glimepiride 1 mg/kg/day plus stevia extract 300 mg/kg/day to diabetic rats orally for the same period expressed a significant reduction (p < 0.001) in renal MDA content. The addition of stevia extract to glimepiride significantly (p < 0.05) decreased renal MDA content in comparison to diabetic rats treated with glimepiride alone (Figure 8).

3.9. Histopathological results

3.9.1. Histologic evaluation of the hepatic tissue. Sections of liver tissue from normal control group showed normal liver architecture without any pathological changes (Figure 9A.). Liver tissues from the diabetic control rats revealed significant changes, which appeared as excessive hydropic degeneration (2.66 ± 0.21), lobular inflammation (1.33 ± 0.33) (Figure 9B.), vascular congestion (1.50 ± 0.22) (Figure 9C.), portal inflammation (1.16 ± 0.30) (Figure 9D.), necrosis (0.66 ± 0.21), sinusoidal dilatation (1.66 ± 0.21) and congestion (Figure 9E.). The mean score of hepatic injuries (1.49 ± 0.27) was significantly elevated in diabetic rats in comparison to normal group (p = 0.002). Significant decrease of the liver injuries was appeared in the group of stevia (Figure 9F&G). The mean score of liver injuries (0.38 ± 0.09) was significantly less than diabetic control rats (p = 0.005). Treatment with glimepiride significantly improved all the hepatic injuries except for mild hydropic degeneration (0.66 ± 0.42) and vascular congestion (0.16 ± 0.16) (Figure 9H). The mean score of liver injuries (0.13 ± 0.10) was significantly less than diabetic control rats (p = 0.004). Co-administration of glimepiride and stevia extract produced significant improvement of all the hepatic injuries except for mild hydropic degeneration (0.16 ± 0.16) (Figure 9I). The mean score of liver injuries (0.02 ± 0.02) was significantly lower than diabetic control rats (p = 0.003). However, insignificant difference was detected as compared with glimepiride treatment (p = 0.461).
3.9.2. Histologic evaluation of the renal tissue. The kidney sections from the normal control group showed normal appearance of the glomeruli and tubules without any pathological changes (Figure 10A). In the diabetic control group, the renal tissues showed signs of earlier diabetic changes as hydropic tubular degeneration (2.66 ± 0.21) (Figure 10B), vascular congestion (2.33 ± 0.21) and mild dilation of glomeruli with glomerular capillaries congestion (1 ± 0.0) (Figure 10C). The mean score of the renal injuries (1.99 ± 0.50) was significantly more than normal group (p = 0.037). Improvement of all renal injuries with only mild glomerular enlargement, tubular degeneration and mild vascular congestion was seen in the stevia treated group (Figure 10D). The mean score of renal injuries (0.44 ± 0.05) was significantly decreased in the stevia group than that of diabetic control rats (p = 0.046). Regarding the renal injuries in the glimepiride treated group, reduction of the tubular degeneration (1.33 ± 0.21) and vascular congestion (0.33 ± 0.21) (Figure 10E) together with absence of any glomerular injury was observed (Figure 10). Although the mean score of renal injuries in the glimepiride treated group (0.55 ± 0.40) was lower than untreated diabetic group, the difference was statistically insignificant (p = 0.127). Co-administration of glimepiride and stevia extract produced significant improvement of all the renal injuries except for mild vascular congestion (0.33 ± 0.21) (Figure 10F). The mean score of renal injuries (0.11 ± 0.11) was significantly lower than untreated diabetic rats (p = 0.046). However, insignificant difference was detected in comparison to the glimepiride treated group (p = 0.346).

3.10. Immunohistochemical results

3.10.1. Evaluation of eNOS immunohistochemical expression in the renal tissues of the normal control group. In the normal control group, moderate immunoreactivity of eNOS was seen in the cytoplasm of the endothelial cells in most of the glomeruli, arterioles, interlobular arteries and peritubular capillaries (Figure 11A). While eNOS expression was absent in the proximal and distal convoluted tubules.

3.10.2. Evaluation of eNOS immunohistochemical expression in the renal tissues of the diabetic control rats. The diabetic group demonstrated positive eNOS immunostaining in endothelial cells in most of glomeruli, arterioles, interlobular arteries and peritubular capillaries (Figure 11A). While eNOS expression was absent in the proximal and distal convoluted tubules. The expression of eNOS was significantly higher in diabetic group (5.33 ± 0.21) as compared with the normal group (3.33 ± 0.21) (p = 0.002).
3.10.3. Evaluation of eNOS immunohistochemical expression in renal tissues of the stevia group. Significant decrease of the eNOS protein expression was noted in the Stevia treated group (2.3 ± 0.21) (Figure 11C). The mean of eNOS protein expression was significantly less than diabetic rats (p = 0.003).

3.10.4. Evaluation of eNOS immunohistochemical expression in renal tissues of glimepride group. Treatments with glimepride result in decreased expression of eNOS in the endothelial cells and in the tubules (4.8 ± 0.16) (Figure 11D). Although a lower mean of eNOS protein expression was detected in glimepride group in comparison with the diabetic group, the difference was statistically insignificant (p = 0.092).

3.10.5. Evaluation of eNOS immunohistochemical expression in the renal tissues of the combined stevia and glimepride group. Significant decrease of the eNOS protein expression was noted in group treated by combination of glimepride and stevia extract (3.6 ± 0.42) (Figure 11E). The mean of eNOS protein expression was significantly less than glimepride treated group (p = 0.045) and diabetic control rats (p = 0.014).

4. Discussion

Type 2 Diabetes represents a worldwide challenge for healthcare. The World Health Organization (WHO) revealed that the diabetic prevalence in 2014 increased up to 8.5% in the adult peoples, while most of them were obese. Diabetes mellitus was the 7th cause of mortalities by ranking of the World Health Organization in 2016 and is projected to move up by 2030 [15, 16].
Figure 10: Hematoxylin and eosin stained renal sections of the normal control group showing normal glomeruli and tubules (A, x200). Kidney sections from diabetic control group showing: severe hydropic degeneration in tubules (B, x400), vascular congestion and enlargement in the glomeruli with marked enlarged congested capillary tuft of glomeruli (C, x400). Kidney sections from stevia treated group showing improvement of all the renal injuries with mild glomerular enlargement, mild tubular degeneration and mild vascular congestion (D, x400). Kidney sections from glimepride treated group showing mild improvement of the renal injuries with moderate hydropic degeneration of the tubules and vascular congestion (E, x400). Kidney sections from glimepride and stevia treated group showing marked improvement of all the renal injuries with only mild vascular congestion (F, x400).

Figure 11: Immunohistochemistry of eNOS: kidney sections from the normal control group showing week to moderate expression in endothelial cells of the glomeruli and peritubular capillaries (A, x400). Sections from the diabetic group showing strong expression in endothelial cells of the glomeruli, peritubular capillaries and the proximal convoluted tubules (B, x400). Sections from stevia treated group showing week expression in the glomeruli, peritubular capillaries and the proximal convoluted tubules (C, x400). Glimepiride treated group showing moderated expression in the glomeruli, peritubular capillaries and the proximal convoluted tubules (D, x400). Glimepiride and stevia treated group showing week expression in the glomeruli, peritubular capillaries and the proximal convoluted tubules (E, x400).

The increased prevalence of T2DM and obesity is due to aging, dietary habits, increased consumption of sugars and decreased physical activities [17]. The majority of diabetic peoples take sweeteners with low calories in order to reduce the caloric entry. However, many of artificial sweeteners like aspartame, cyclamates and saccharine, considered high calorie sugars and are potentially carcinogenic. The high prevalence of diabetes and obesity and need for safety of some artificial sweeteners, have enhanced the search for natural little calorie sweetener [18].

Among the new effective antidiabetic agents the herb Stevia rebaudiana Bertoni have been used in management of D.M in South America [19]. Stevioside and its related glycosides have been tested as natural zero-calorie sweeteners [7].
The currently marketed anti-diabetic agents are less effective and having many drawbacks. Besides, these side effects, none of the available anti-diabetic drugs successfully establishing euglycemic state and treating long term micro- and macro-vascular complications. United Kingdom Prospective Diabetes Study (UKPDS) revealed that the treatment of diabetes with single oral hypoglycemic agent mostly failed to establish euglycemic state for long time, and most of diabetics have to use combined antidiabetic agents [3]. Stevia is a medicinal herb with multi potential health benefits [20].

Sulfonylureas as glimepiride lower the high blood glucose levels by inducing insulin release via the closing the ATP-sensitive potassium channels on beta cells. However, sulfonylureas have no influence on insulin resistance (IR), the main cause and complication in type 2 diabetes mellitus. Therefore, many patients with type 2 have to use sulfonylureas in adjunct with other agents that enhance insulin sensitivity and/or diabetic control [21].

In a consistence with other studies, our findings revealed that rats with type 2 diabetes treated with stevia extract (300 mg/kg/day) orally for 21 days showed a good control of blood glucose levels. Similary, combined treatment of diabetic rats with stevia extract (300 mg/kg/day) and glimepiride (1 mg/kg/day) for a similar period caused a more decrease in the BGLs in a comparison to diabetic rats treated with glimepiride alone. This finding is collaborated with the previous results that approved potential benefits of stevia plant on hyperglycemia on diabetic animals [22–24].

The results also showed that diabetic rats administered stevia extract expressed a significant elevation in insulin release in a similar level like insulin releaser drugs like glimepiride. Moreover the diabetic rats administered combination of glimepiride and stevia extract expressed a more enhancement in the insulin release reflecting on decreasing the blood glucose levels. These findings demonstrated that stevia extract having a good efficacy on controlling the hyperglycemia in diabetic rats especially when combined with other hypoglycemic agents, like glimepiride.

Previous data revealed that the adiponectin is a main factor in the development obesity, IR, MS, and T2DM. It cause insulin-sensitization and anti-inflammatory action, and sometimes decreases body weight [25, 26]. Decreased adiponectin level is mostly linked to obesity, MS and T2DM. Thus, it has an important role as a therapeutic agent in management of MS and T2DM.

Several studies have demonstrated that glimepiride increase adiponectin gene expression in adipocytes [27] and other data have also revealed that glimepiride may cause enhancement in insulin sensitivity which linked to increased serum adiponectinemia [28]. However, it is still unknown whether the treatment with glimepiride in T2D could improve the serum adiponectin level for better glycemic control or not. Our findings demonstrate that stevia extract has a significant action on enhancing the serum adiponectin. Also treatment of diabetic rats with glimepiride significantly increases the adiponectin level. Moreover, the diabetic rats administered combination of stevia and glimepiride showed additive effects on elevating the adiponectin level. Thus the combined administration of stevia and glimepiride may be potentially a therapeutic target for management of obesity and type 2 diabetes.

The United Kingdom Prospective Diabetes Study (UKPDS) stated that, the prognosis of diabetics with T2DM depends on management of elevated blood glucose level, and other associated problems, like hypertension and hyperlipidemia [29]. Thus, the pharmacologic treatment for T2DM should be directed to control BGL and improve the lipid profile.

Our diabetic rat model showed the main features of diabetic hyperlipidemia: an elevated triglyceride and total cholesterol levels and a decrease in the HDL-cholesterol. These features is mostly due to elevation in the rate of lipolysis from insulin-resistant adipocytes [30] which, decreases the glucose uptake in the muscles, enhances triglyceride formation, exaggerates glucose synthesis in the liver, and leading to failure of β-cell [31]. Thus, improving the serum lipid profile could help in suppressing the progress of metabolic syndrome and T2DM.

It was obvious from our findings that diabetic rats with treated stevia extract showed a significant reduction in the TC and TG levels and increased HDL level. Glimepiride treatment significantly improved the levels of triglyceride, cholesterol, and HDL-cholesterol and its effect was enhanced when it combined with stevia extract. These results are collaborated with previous result [32–34], that stevia extract decreased total cholesterol level and elevated the HDL-cholesterol level. Also, data on humans revealed that administration of stevia extract elevated the level of HDL and decreased the levels of cholesterol, triglycerides [35]. Ahmad et al. 2018 [36] revealed that stevia extract may be considered as a natural anti-hyperlipidemic agent in management of dyslipidemia and its related conditions. Therefore, it appears that stevia extract and glimepiride could give a potential therapeutic effects in diseases linked to impairment of glucose tolerance, hyperlipidemia and increased insulin resistance.

The pro-inflammatory cytokine tumour necrosis factor TNF-α has been involved as a key element in the development and progression of obesity, insulin resistance and T2DM, doing its effect via the process of inflammation and immunity [37]. Our findings showed that the stevia extract significantly reduced the level of TNF-α in diabetic group. Concurrent administration of glimepiride plus stevia extract to diabetic rats reverses the increase in TNF-α level. The influence of glimepiride on TNF-α was more enhanced with its combination with stevia resulting in a significant reduction in its level in comparison to diabetic rats administered glimepiride alone. This action is consistent with the data obtained from insulin-resistant mice model showed that stevioside was also able to down regulate expression of TNF-α [38] and mice model with cisplatin (CP)-induced kidney injury treated with Stevia ethanolic extract (SE) or stevioside. Both SE and
stevioside inhibited CP nephrotoxicity by reducing the level of TNF-α [39].

The combined treatment of glimepiride and stevia extract remarkably improved insulin resistance, explained by a significant reduction in TNF-α and lipid profile. Moreover, the modification of adipocytokine concentration (i.e., elevated serum adiponectin) may also be the cause of improving the insulin resistance.

Diabetic nephropathy (DN), considered a major end-term micro-vascular complication and is the major cause of morbidity and early mortalities in diabetics. Since that the hyperglycemia is the main cause in the pathogenesis of DN [40]. Management of DN needs controlling of many factors like: blood glucose level, hypertension and kidney function. DN was also identified by testing certain biochemical indices in the blood. STZ induced-renal damage is confirmed by both diagnostic biochemical parameters and histopathological changes [10]. Our results showed that the levels of renal biomarkers like urea and creatinine were elevated in the diabetic rats with impairment in the renal structure and this collaborated with previous results [41]. The levels of renal biomarkers were significantly reduced in stevia and combined stevia & glimepiride groups. Moreover, the histopathological analysis of the kidney showed improvement in renal tissue with lower mean of injuries was seen in stevia and combined stevia & glimepiride groups as compared to control diabetic rats. These results are in agreement of previous observed data that concerning the effect of stevia has a amelioration of renal injury in the STZ-injected rats in addition to its antihyperglycemic action [10, 42]. Our findings support the potential role of stevia in combination with other hypoglycemic agent for treatment of T2DM and other diabetes-related renal conditions.

Diabetic nephropathy represents a complex metabolic process characterized with patho-physiological events that both stimulate and depress intra-renal nitric oxide (NO) production. The net effect on renal NO level relies on the stage of the disease. Most of literature has built up abnormalities and the role of intra-renal nitric oxide release in the development of diabetic nephropathy. These differences could be explained by the different methods used for evaluation of NO, the NOS isoforms studied and the stage of the disease [43].

The studies demonstrate that early diabetic nephropathy is associated with increased constitutive NO production derived mainly from eNOS and possibly iNOS and this effect may be related to intra glomerular hemodynamic changes noted in early diabetic renal disease [44]. On the other hand, later stages of diabetic nephropathy characterized by hypertension, progressive renal insufficiency is associated with decreased NO production (especially iNOS derived) resulting in a NO deficient state [45].

Our immunohistochemical results are in line with these data with regard to strong eNOS protein expression in the renal tissue of STZ-injected rats. While, significant decrease of eNOS protein expression was noted in stevia treated group. Importantly, the mean of eNOS protein expression was significantly lower in glimepiride & stevia treated group compared to glimepiride treated group.

Both T2DM and non-alcoholic fatty liver disease (NAFLD) are global problems [46]. The prevalence of NAFLD was very high among diabetic patients reaching upto 70% between these patients [47]. Thus, T2DM is used as estimator for the development of NAFLD. The main problems in diabetes that leading to hepatogenous diabetes are insulin sensitivity and β-cell failure [48].

Our findings demonstrated that stevia extract reduced the serum level of hepatic enzymes in diabetic rats. There is a significant reduction of the level hepatic biomarkers in stevia and combined stevia & glimepiride groups, which was represented in the form of improvement in the hepatic function and structure. The combination of stevia & glimepiride significantly reduced the mean score of hepatic injuries than those treated with glimepiride alone. These results are in accordance with [10] and [49] who revealed the hepatoprotective effect of stevia on diabetes induced hepatic changes in diabetic rats. Glimepiride and stevia extract could improve both metabolic and hepatic dysfunction as a class effect. The alleviation of hepatic dysfunction was mediated partly through the control of hyperglycemia, reduction of elevated hepatic enzymes, antioxidant effect and partly via improving in insulin resistance.

Oxidative stress is also claimed to have an important role in pathogenesis of T2DM. It is reported that the permanent elevation of blood glucose levels occurring in most of diabetics causes glucose autoxidation and glycation of proteins end products [50, 51], which leading to depletion of the antioxidant defense system and thus enhancing free radical production. MDA has a main role in pathogenesis of many chronic diseases like diabetes [24]. Thus, attention has been directed toward natural antioxidants that able to inhibit free radical production [52], especially, the plant-based medicines which currently investigated in the prevention and treatment of diabetes.

Our findings demonstrated that diabetic rats treated with extract from the plant leaves of stevia rebaudiana significantly decreased the hepatic and renal content of MDA. Combined administration of glimepiride plus stevia extract to diabetic rats showed a marked reduction of renal and hepatic MDA in comparison to diabetic rats treated with glimepiride alone. In consistence with the results of the current work, many experimental studies revealed that stevia extract has a potent antioxidant property, as the plant contains large amounts of flavonoids and total phenols [32, 49]. Taken together with the antidiabetic action and antioxidant properties of stevia leaves, the extract of stevia could provide a new therapeutic antidiabetic agent in prevention and management of T2DM.
5. Conclusion

The combined use of glimepiride and stevia extract considered as a novel approach to glycemic control and is accompanied by multiple demonstrable metabolic and renal benefits beyond its glucose-lowering effect. The co-administration of stevia and glimepiride represents a new effective therapeutic strategy for better controlling diabetes and its complications. Our study results provide further support to the recent use of stevia rebaudiana bertoni as antidiabetic agent either alone or combined with the commonly used oral hypoglycemic agents like glimepiride.

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Abbreviations

(A.E.): Aqueous extract
(ALT/GPT): Alanine aminotransferase
(AST/GOT): Aspartate aminotransferase
ATP: Adenosine triphosphate
(BGL): Blood glucose level
(CP): Cisplatin
(DCM): Dichloromethane
(DM): Diabetes mellitus
(DN): Diabetic nephropathy
(E.E.): Ether extract
(eNOS): Endothelial nitric oxide
(EtOH): Ethanol
(HDL-C): High density lipoprotein cholesterol
(I.P): Intraperitoneal injection
(iNOS): Inducible nitric oxide
(IR): Insulin resistance
(MDA): Malondialdehyde
(MS): Metabolic syndrome
(NA): Nicotinamide
(NAD (P) H: Nicotinamide adenine dinucleotide phosphate
(NAFLD): Non alcoholic fatty liver disease
(NO): Nitric oxide
(ROS): Reactive oxygen species
(SE): Stevia ethanolic extract
(SG): Steviol glycosides
(STV): Stevioside
(STZ): Streptozotocin
(TC): Total cholesterol
(TGs): Triglycerides
(TNFα): Tumour necrosis factor-α
(Type 2D.M): Type 2 diabetes mellitus
(UKPDS): United Kingdom Prospective Diabetes Study
(WHO): World Health Organization

Competing Interests

The authors declare no competing interests.

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