Stevia rebaudiana LEAF EXTRACT REDUCES BLOOD GLUCOSE AND VISCERAL FAT ACCUMULATION IN ALLOXAN-INDUCED DIABETIC MICE

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INTRODUCTION
Diabetes mellitus is a group of diseases characterized by hyperglycemia and varying degrees of insufficient insulin effects. The type 2 diabetes mellitus pathogenesis involves insulin resistance and dysfunctional secretion of insulin by the β cells of the pancreas (Gupta et al., 2010). This condition raises the risk of several complications, mostly by microvascular damage (retinopathy, nephropathy, and neuropathy). It is associated with reduced life expectancy, significant morbidity and diminished quality of life (Chehade et al., 2013). Diabetes mellitus, particularly type 2, is a chronic metabolic disorder with an increasing trend worldwide. It has become an epidemic in some regions of the world and the number of people with the disease is expected to double in the next 10 years due to factors like increase in age population and changes in lifestyle, thereby adding to the burden for public health (Sicree et al., 2006). According to the World Health Organization (1999), there are approximately 177 million people with diabetes worldwide, which powered and maintained the interest of researchers to further understand the disease's pathophysiology, the risk factors associated with it and develop drugs and other treatments to address the condition.

One of the famous ways of treating chronic diseases is the use of complementary/alternative medicine (Eisenberg et al., 1998). People with diabetes tend to use non-prescribed supplements (herbal, vitamin, mineral, or others). These methods are reported to deliver more promising results and lesser side effects. Several clinical trials are also conducted to test the effectiveness and efficiency of the aforementioned treatments, especially herbal remedies. One of the plants that currently receive increasing attention is S. rebaudiana. It is famous for its sweet taste and commercial value all over the world as a sugar substitute in foods, beverages, and medicines. It is a plant that offers sweetness with fewer calories and does not show any side effects after consumption on human health (Gupta et al., 2013). Moreover, it has been reported that S. rebaudiana shows the ability to maintain a blood glucose level with glucose tolerance enhancement in diabetic rats. Stevia could also cause hypoglycemia in patients with diabetes through decreasing glycolysis and gluconeogenesis and absorbing glucose in the duodenum. In addition, anti-hyperglycemic and antioxidative potential of stevia and its glycoseide were detected in several tissues such as the kidney, liver, and pancreas (Misra et al., 2011). In the study of Sytar et al. (2015), stevia leaves contain different phenolic compounds that showed high antioxidant activities.

The current study assessed the ability of S. rebaudiana to reduce blood glucose levels and the accumulation of visceral fats in ICR mice with alloxan-induced diabetes. Specifically, the study evaluated the effects of stevia leaf extracts at different concentrations on changes in body weight, FBGL after and before giving the daily treatments and OGTT. Also, at the end of the treatment period, gross and microscopic changes in the pancreas, liver, and kidney were described and compared.

MATERIAL AND METHODS
Preparation of stevia leaf extract
The stevia plants (S. rebaudiana) were collected from the highland province of Benguet that has an annual temperature that ranges from 15 to 23 °C. About 500 g of dried stevia leaves were subjected to ethanol extraction then subjected to rotary evaporation. The collected extract was stored in a light-proof amber bottle and placed in refrigeration (4 °C) until further used.

Experimental animals
Inbred ICR mice (n=36), about six to eight weeks of age were used in the study. The mice were housed in a temperature-controlled room (21°C±2°C), with a 12-h light/dark cycle (Fawcett, 2012) at the Mouse Research and Breeding Facility at the Animal Control Center, Central Luzon State University (CLSU), and given with ad libitum supply of feeds and water. After one week of acclimatization, the mice were randomly divided into six groups. The protocol for handling and experimentation in laboratory animals employed at the College of Veterinary Science and Medicine followed the institutional animal care and use committee (IACUC) of the university.

Experimental design
One of the six groups was designated as the negative control group, while the other five groups were intraperitoneally given with 3% (w/v) alloxan (70mg/kg) to induce diabetes. Among five groups with induced diabetes, one group served as the positive control (untreated group) while the remaining four groups were orally treated using a gavage needle with the following: glibenclamide...
Monitoring of body weight and blood glucose level

Changes in body weight of mice with alloxan-induced diabetes were determined by getting the difference in body weight before and after treatment within the two weeks experimental window. The IBG was determined using a handheld glucometer (EasyTouch® Glucose Monitoring System). The mice were fasted for eight hours prior to the procedure. A drop of blood sample was drawn from the tail and was directly applied on the glucose strip inserted to the apparatus. The measured glucose level will appear on the monitor.

Oral glucose tolerance test

Oral glucose tolerance test was conducted in mice after the two-week observation period. The mice were fasted for eight hours and their baseline blood glucose levels were determined using the procedure described previously. After this, glucose solution was administered (2 g/kg body weight) orally using a gavage needle. The blood glucose levels were measured at 0, 10, 20, 30, 40 and 50 min after the glucose oral administration. The values were plotted in a graph to make a glucose tolerance curve for each group (Mouse Metabolic Phenotyping Center, 2012).

Gross and histopathological examination

At the end of observation period, selected animals from each treatment were sacrificed. The liver, pancreas, and kidneys were collected from each mouse. The organs were fixed in 10% buffered formalin and prepared for tissue processing. The prepared tissue slides were stained using haematoxylin and eosin stains. The slides were examined under a microscope for lesions and notable observation.

Statistical analysis

The measured body weight was presented as mean ± standard deviation (SD). The data were analyzed using analysis of variance (ANOVA) followed by Fisher’s least significant difference (LSD). The level of significant difference was set at 95% confidence interval at a p-value of ≤0.05.

RESULTS AND DISCUSSION

Body weight and blood glucose level

Table 2 The changes in body weight before and after treatment with glibenclamide and different concentrations of stevia leaf extract

<table>
<thead>
<tr>
<th>Treatments</th>
<th>N</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>6</td>
<td>-1.57 ± 1.40*</td>
</tr>
<tr>
<td>Positive control</td>
<td>6</td>
<td>1.86 ± 1.35*</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>6</td>
<td>1.57 ± 0.98*</td>
</tr>
<tr>
<td>100% stevia leaf extract</td>
<td>6</td>
<td>1.00 ± 1.41*</td>
</tr>
<tr>
<td>60 % stevia leaf extract</td>
<td>6</td>
<td>0.83 ± 0.75*</td>
</tr>
<tr>
<td>10% stevia leaf extract</td>
<td>6</td>
<td>1.50 ± 0.55*</td>
</tr>
</tbody>
</table>

Mean ± SD with different superscript letters in a column are significantly different at 5% level of significance.

Table 2 shows the mean ± SD body weight difference of mice per group before and after treatment. All groups gained weight before the start of treatment, with the positive control group having the highest. All diabetic groups had a decrease in weight after treatment (with positive control having the highest loss), except for the negative control group. Results of the statistical analysis emphasized the significant difference of mean body weight changes of negative control compared with groups treated with alloxan-induced diabetes.

Statistical analysis of the weight changes shows a significant difference (5% level of significance) before and after treatment on all diabetic groups versus the negative control group. Treated and untreated diabetic groups showed no significant difference between each other. As shown in Figure 1, groups with alloxan-induced diabetes showed a decrease in body weight. Same results were obtained from the studies of Assaei et al. (2016), and Singh and Pathak (2015), wherein all groups given with alloxan (diabetic groups), whether treated or not, resulted in weight reduction. American Diabetes Association (2009) also mentioned that diabetic patients suffered several symptoms such as gradual weight loss as a consequence of insulin or insulin mobilization insufficiency. The decrease in body weight of diabetic mice can be attributed to catabolism of protein, even though the food intake was greater in diabetic rats than control. Due to insulin deficiency, protein content was decreased in muscular tissue by proteolysis (Raparia et al., 2012). In the study of Abdellatif (2013), the diabetes caused by STZ administration increases fat and protein mobilization in skeletal muscle, inducing significant weight loss, as was also observed in the present study.

Table 3 Mean percent changes in fasting blood glucose level (mg/dL) of mice with alloxan-induced diabetes and either treated with glibenclamide or different concentration of stevia leaf extract

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Mean percent (% ) difference of IBGL</th>
<th>Day 5, after daily dose</th>
<th>Day 8, before daily dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative control</td>
<td>6.47†</td>
<td>4.47†</td>
<td>17.41†</td>
</tr>
<tr>
<td>2</td>
<td>Positive control</td>
<td>1.80†</td>
<td>17.41†</td>
<td>2.64†</td>
</tr>
<tr>
<td>3</td>
<td>Glibenclamide</td>
<td>18.21†</td>
<td>17.41†</td>
<td>0.97†</td>
</tr>
<tr>
<td>4</td>
<td>100% stevia leaf extract</td>
<td>17.63†</td>
<td>17.41†</td>
<td>1.57†</td>
</tr>
<tr>
<td>5</td>
<td>60% stevia leaf extract</td>
<td>14.53†</td>
<td>17.41†</td>
<td>1.50†</td>
</tr>
<tr>
<td>6</td>
<td>10% stevia leaf extract</td>
<td>23.79†</td>
<td>17.41†</td>
<td>13.69†</td>
</tr>
</tbody>
</table>

Legend: Arrow up (↑) denotes increase in mean percent difference while arrow down (↓) denotes otherwise.

Mice from all groups were tested for IBG at day 5 after administering the daily dose, and at day 8 before giving the daily dose. Mean percent difference of FBGL was determined using day 0 as the baseline value.

Among groups, only negative and positive control groups showed an increase in the post-treatment IBG (6.47 and 1.80%, respectively) at day 5. All groups exhibited a decrease in IBG (ranges from 14.53 to 23.79%) with the group treated with 10% stevia leaf extract as the highest, followed by groups treated with glibenclamide, and 100% and 60% stevia leaf extract. Pretreatment IBG at day 8 shows an increase in mean percent difference in all groups (0.97 to 17.41%), except for groups treated with 100% and 10% stevia leaf extract, (12.57 and 13.69% decrease, respectively). The results showed that there was a remarkable decrease in IBG, if testing was done after giving either glibenclamide or any concentration of stevia leaf extract. On the other hand, giving of treatments after FBGL generally showed an increase in value, except for 100% and 10% stevia leaf extracts.

According to Assaei et al., (2016) during diabetes or insulin resistance, failure of insulin-stimulated glucose uptake by skeletal fat and muscle causes high glucose concentration in blood. Consequently, glucose uptake by insulin-independent tissues, oxidant production and impaired antioxidant defenses occur. The use of glibenclamide in mild to severe diabetes mellitus in rats showed a decreased blood glucose concentration and an increased level of insulin (Sokolovska et al., 2012). It is a sulfonylurea that has been commonly used to treat type 2 diabetes since it can stimulate beta cells of the pancreas to produce insulin (Proks et al., 2002). On the other hand, stevia has anti-hyperglycemic and anti-oxidative potential due to some of its components which may stimulate the beta cells to release insulin (Jepessen et al., 2000) leading to improvement in the carbohydrate-metabolizing enzymes and thus establishing lower to normal blood glucose level. In addition, the beneficial effects of stevia as an anti-hyperglycemia was reported to be associated with the increased in peroxisome proliferator-activated receptor-γ (PPARγ), a nuclear hormone receptor that maintains glucose level, and insulin mRNA levels (Gupta et al., 2010; Assaei et al., 2016).

Oral glucose tolerance test

Fasting plasma or blood glucose concentrations between 100 and 125 mg/dL necessitates the use of glucose tolerance test. The OGTT is an important test for identifying the pre-diabetic metabolic state in humans (Kim et al., 2016). OGTT results in Table 4 showed a remarkable difference of the positive control group compared with other groups.
Figure 1 Oral glucose tolerance test results of alloxan-induced diabetic mice treated either with glibenclamide or different concentrations of stevia leaf extract

As shown in Figure 2, all groups (except for the positive control) manifested a less than 200 mg/dL baseline blood glucose value that eventually increased and peaked after 10 min (20 min for group treated with 100% stevia leaf extract). A gradual decline was observed and eventually returned to the baseline glucose value after 50 min. The positive control group showed a baseline blood glucose level of more than 500mg/dL that peaked after 10 min then drastically decreased, even lower than the baseline value, after 20 min. The blood glucose level then returned to its baseline value.

Lachin and Reza (2012) stated that alloxan treatment evokes a sudden rise in insulin secretion in the presence or absence of glucose and this insulin release occurs for short duration followed by the complete suppression of the islet response to glucose even when high concentrations of glucose were used. Results of the present study indicate that stevia has the ability to prevent hyperglycemia. Stevia has functional components that help in regulating insulin release from pancreatic beta cells, thus assisting and improving carbohydrate metabolism resulting to lower to normal blood glucose level (Assaei et al., 2016).

Gross and histopathological examination

After the 14-day treatment, mice from all the groups were sacrificed and necropsied. Abdominal visceral organs were initially observed in situ for any noticeable changes/lesions associated with diabetes. Liver, pancreas, and kidney were harvested and examined grossly and histologically.

Figure 3 Gross examination of the visceral organs of the negative control group (A), and positive control group (B). Arrow shows a noticeable accumulation of visceral fats

On gross examination, the abdominal organs of the groups with alloxan-induced diabetes were covered and interlaced with a larger amount of visceral fats, especially perirenal fats, as compared with the negative control group. Liver samples from the negative control group have well-defined edges, no foci of necrosis, congestion or hemorrhages and easy to isolate with the surrounding organs. No notable lesions were found.

Figure 4 Liver of a positive control group mouse showing adhesion (red arrow) and blunt edges (black arrowhead)

Upon gross examination, the liver of mice from the positive control group showed paler color (compared with the negative control group) with relatively larger size, rounded edges, and adhesion to the visceral surface of the abdomen. Group treated with 10% stevia leaf extract showed hemorrhagic foci on the diaphragmatic surface while groups treated with 60% and 100% stevia leaf extracts have no noticeable gross lesion. Similar findings were observed in the study of Abdellatif (2013) where the liver of diabetic mice was mildly enlarged with a rounded inferior margin, smooth capsule and a pale-yellow brown greasy cut surface.

Figure 5 Liver of a mouse treated with 10% stevia leaf extract showing localized area of hemorrhage (black arrowhead)

Figure 6 Histologic section of liver from the negative control group (A) showing hepatocyte atrophy (black arrowhead), anisokaryosis (*), and nuclear duplication (encircled); and the positive control group (B) showing cellular swelling (red arrowhead)
Organs extracted from all the groups were subjected to tissue processing and histopathological examination. Histopathological findings in the liver include: (1) hepatocyte atrophy, anisokaryosis and nuclear duplication in the negative control group, and groups treated with 100% and 60% stevia leaf extract; (2) extensive severe acute cellular swelling of hepatocytes in the positive control and glibenclamide-treated groups; while, (3) multiple single cell necrosis (apoptosis) with nuclear duplication and hepatocyte atrophy in the group treated with 10% stevia leaf extract.

**Figure 7** Histologic section of the liver from the group treated with glibenclamide (A), and groups treated with 100% (B), 60% (C) and 10% (D) stevia leaf extract. Cellular swelling (red arrowhead), hepatocyte atrophy (black arrowhead), anisokaryosis (*), nuclear duplication (encircled) and multiple single cell necrosis (red arrow) were observed.

According to Abdellatif (2013) and Honjo et al. (1986), cellular swelling may appear as vacuolation/ cellular lucency, and may appear to displace the nucleus, resulting to “signet rings” appearance, which is the classical sign of fatty change, in cases of diabetes in rodents. Furthermore, Abdellatif (2013) pointed out that liver cell damage can be attributed to hyperglycemic episodes which increase the generation of free radicals by glucose auto-oxidation. The increase in the overall stress-causing oxygen free radicals in diabetes could be primarily due to the increase in blood glucose levels and secondarily due to the effects of the diabetogenic agent used, the alloxan.

**Figure 8** Gross examination of the pancreas of a positive control group mouse

Gross examination of the pancreas showed no outstanding lesion. However, on histopathology, islet degeneration, vacuolation and loss of cytoplasm of pancreatic islet cells were observed. There was a mild islet shape alteration in diabetic groups of which the positive control group showed the most notable changes. Same observations were reported in the studies of Honjo et al. (1986) and Assaei et al. (2016).

**Figure 9** Histologic section (400x magnification) of the pancreatic islet of group treated with glibenclamide (A), and groups treated with 100% (B), 60% (C) and 10% (D) stevia leaf extract. Vacuolation (black arrowhead) and loss of cytoplasm (red arrowhead) of pancreatic islet cells

In the present study, vacuolation and loss of cytoplasm were observed in all groups and more extensive in the groups treated with glibenclamide and 60% stevia leaf extract. The diabetogenic agent alloxan affects the pancreas by its rapid uptake by pancreatic beta cells, which hastens the reduction process and generates reactive oxygen species (ROS) and superoxide radicals causing cellular and tissue damage (Das et al., 2012).
Gross appearance of the kidney may be normal even with an underlying condition. Morphologic changes in diabetic nephropathy grossly were the ultimate effect of damage in renal compartments- glomeruli, tubules, interstitium, and vessels histologically. Pathogenesis of diabetic nephropathy in cases of diabetes mellitus is that hyperglycemia impairs autoregulation within the glomerulus by activating the local, intrarenal renin-angiotensin-aldosterone system, thus, compounding the hyperglycemia-induced injury (Raparia et al., 2012).

CONCLUSION

Even though there is modernization and advancement in treating common ailments, people tend to look at natural and safer but effective ways on alleviating conditions such as diabetes mellitus. Alternative treatment has been making its way back on mainstream medicine, of which stevia is an example. Based on the fore mentioned findings, stevia leaf extract has similar effects (but of different extent) as the pharmacological agent, glibenclamide. Results on weight changes suggest that weight loss by diabetes were not affected by treatment. Post-treatment fasting blood glucose level shows that stevia leaf extracts were effective in lowering BGL. Among treatment levels, 10% stevia shows the best result in OGTT. Stevia leaf extract possibly has histo-protective/ regenerative effects since it was able to lessen the extent of damage in organs of interest, as compared with the untreated positive control.

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