HEPATOPROTECTIVE ACTIVITY OF Stevia rebaudiana BERT. LEAVES AGAINST THIOACETAMIDE INDUCED TOXICITY

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Abstract

The leaves of Stevia rebaudiana Bert. is commonly known as hypocaloric bio-sweeteners. It can be used in multiple health diseases and traditionally the leaves of Stevia are used as sugar substitute. The present study was carried out to evaluate the hepatoprotective activity of Stevia rebaudiana leaves against thioacetamide-induced (s.c.) hepatotoxicity. Aqueous leaves extract of Stevia rebaudiana (AESR) at dose levels of 200 and 400 mg/kg/day were evaluated. Oral administration of Stevia leaves extract at 400 mg/kg resulted in a significant reduction in aspartate transaminase, (AST, 131.1±3.0 IU/L), alanine aminotransferase (ALT, 62.2±1.8 IU/L), gamma glutamyl transpeptidase (GGT, 13.0±0.3 IU/L), alkaline phosphatase (ALP, 197.4±3.2 IU/L) and total bilirubin (0.23±0.0 mg/dL) compared to control. The glutathione (GSH) and MDA levels of the liver tissue samples were also measured. Histology of the liver sections of the animals treated with the extract also showed dose-dependent reduction of necrosis. The present investigation revealed the hepatoprotective activity of the aqueous leaves extract of Stevia rebaudiana against thioacetamide induced hepatotoxicity.

Key words: Stevia rebaudiana, Biochemical parameters, Hepatotoxicity, Thioacetamide.

Stevia rebaudiana Bert. Yapraklarının Tiyoasetamid ile İndüklenmiş Toksisiteye Karşı Hepatoprotektif Etkisi

Stevia rebaudiana Bert. yaprakları düşük kalorili biyotatlandırıcı olarak bilinir. Bu yapraklar birçok hastalığın tedavisinde kullanılırlar ve geleneksel olarak da yapraklar şeker yerine olarak kullanılır. Bu çalışmamın amacı Stevia rebaudiana yapraklarının tiyoasetamid ile oluşturulan hepatotoksikite karşı hepatoprotetik aktivitesinin değerlendirilmesidir. Çalışmada Stevia rebaudiana yapraklarının su ekstreleri (AESR) 200 ve 400 mg/kg gün dozlarında değerlendirilmiştir. AESR’nin 400 mg/kg gün dozda oral yolla uygulaması kontrol grubu ile karşılaştırıldığında aspartat transaminaz (AST, 131.1±3.0 IU/L), alanin aminotransferaz (ALT, 62.2±1.8 IU/L), gama glutamyl transpeptidaz (GGT, 13.0±0.3 IU/L), alkalin fosfataz (ALP, 197.4±3.2 IU/L) ve total bilirubin (0.23±0.0 mg/dL) düzeylerinde önemli düşüş tespit edilmiştir. Karaciğer dokusu örneklerinde karaciğer glutatyon (GSH) ve MDA seviyeleri de ölçülmuştur. Ekstrelerle tedavi edilen hayvanların karaciğer bölgelerinin histolojisi nekrozun doza bağlı olarak azaldığını göstermiştir. Bu değerlendirme Stevia rebaudiana yapraklarının su ekstrerinin tiyoasetamid ile oluşturulan hepatotoksikite karşı hepatoprotetik aktivitesi olduğunu göstermektedir.

Anahtar kelimeler: Stevia rebaudiana, Biyokimyasal parametreler, Karaciğer toksisitesi, Tiyoasetamid.

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INTRODUCTION

Stevia, the nature’s sweetest gift belongs to the family Asteraceae. It is an amazing plant from the rain forest of Amazone. The other names of Stevia are sweet leaf, honey leaf, sweet herb, honey yerba. Stevia is a native to South America (Paraguay, Brazil) but extensively grown in places like Central America, Israel, Australia, Japan and China (1). Stevia is distinguished by the presence of sweet diterpene glycosides: rebaudioside A, rebaudioside C, stevioside and dulcoside in its leaf tissue (3). Stevioside a white crystalline compound isolated from Stevia is 150 to 300 times sweeter and rebaudioside A is 250–450 times sweeter than sucrose (4). The glycosides of Stevia rebaudiana leaves have been extracted using classical techniques: maceration, infusion or decoction, either requiring long processing time and low efficiency (maceration), or facing thermal degradation (infusion and decoction) (5). Stevia have versatile medicinal uses without any side effects that focus the interest towards Stevia in worldwide. Stevia is poised for major growth in Indian economic market as domestic crop (6). It is used for the treatment of various conditions such as cancer (7), diabetes (8), obesity, cavities, hypertension (9), fatigue, depression, and in cosmetic and dental preparations (10). It possesses hypotensive (11,12), vasodilating, taste improving, sweetening, antifungal, antiviral, antibacterial (13) properties and increases urination function of the body. Several toxicological studies were carried out to verify the possible mutagenic and genotoxic effects of Stevia extracts on bacterial cells and different mammalian species, and the results were recently reviewed (14,15). These studies and nearly 20 years of use in both Japan and Brazil seem to demonstrate that Stevia extracts are safe. Extracts of Stevia rebaudiana are part of weight-loss programs because of their ability to reduce the cravings for sweet and fatty foods, to treat the diseases, hypoglycaemia, candidiasis, skin abrasions and inhibiting growth and reproduction of bacteria-like plaque (16). It does not affect blood sugar level hence safe for diabetics (17). It also has antiinflammatory and antioxidant properties (18). However, some aspects have not yet been fully elucidated, and further investigations are required. Specifically, scanty reports on its hepatoprotective activity and hence the present study was carried out to evaluated hepatoprotective activity against thioacetamide-induced hepatotoxicity in rat.

EXPERIMENTAL

Plant material

Stevia leaves were collected from the cultivated area of acidic soil zone of Shimoga, Karnataka, India (pH 6.10). Leaves were dried in oven at temperature of 45°C. After drying the leaves were grindred in the blender to reduce the mesh size of 22 ~ 44 μm.

Extraction of Stevia leaves

250 g of dried Stevia leaves was extracted with double distilled water by hot maceration (Reflux) method for 4 hours after standardization of method at oven temperature of 45°C (19). Extracts were collected and concentrated using rotary flash evaporator and the yield was estimated 2.56 g. Finally, presence of active compounds likes stevioside and rebaudosides were determined by phytochemical tests and TLC chromatography (20,21).

Experimental animals

Healthy Wister-Albino rats of either sex were used. Animals weighed 150-250 g, bred in animal house of St. John’s Pharmacy College, Bangalore, India, and were used in this present study. The animals were maintained at standard housing conditions and fed standard pellet diet and water
All procedures were performed according to the Institutional Animal Ethics Committee’s approval (IJAHSM/IAEC/2008/008).

**Assessment of acute toxicity of AESR**

Acute toxicity studies of AESR were performed on mice. Ten male and 10 female mice were divided into control and experimental groups. Each group included five male and five female mice. The experimental group received extract and the control group received water by gavage with the aid of a metal gastric needle at a single dose of 1000 mg/kg of the animal weight. The animals were observed carefully every 2 days to record toxic manifestations, and to measure body mass and water and ration consumption. After 14 days, the mice were sacrificed. The livers, kidneys, lungs, and hearts were observed macroscopically and the relative weights (g organ w./10g body w.) were determined.

**Thioacetamide-induced hepatotoxicity**

Hepatotoxicity was induced by the subcutaneous administration of thioacetamide, at a dose of 100 mg/kg body weight, as a 2% (w/v) solution in distilled water. Six rats were used in each group. Thioacetamide was administered on the sixth day of a total of 7 day-study period to all the groups of animals except for the control group (received vehicle of the extract). Group II served as thioacetamide control and received vehicle of the extract. Group III - V received the treatments for one week of study. Dose of 50 mg/kg of silymarin was selected and administered orally in the study based on some published studies demonstrating the hepatoprotective activity of this dose (22,23). In the present study, silymarin was used as the standard to compare the activity of the extract. Table 1 shows the design of the study.

**Table 1. Information about the study design.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (receive only vehicle of extract)</td>
</tr>
<tr>
<td>II</td>
<td>Thioacetamide + vehicle of extract</td>
</tr>
<tr>
<td>III</td>
<td>Aqueous <em>Stevia</em> extract (200 mg/kg/day)</td>
</tr>
<tr>
<td>IV</td>
<td>Aqueous <em>Stevia</em> extract (400 mg/kg/day)</td>
</tr>
<tr>
<td>V</td>
<td>Silymarin (50 mg/kg/day)</td>
</tr>
</tbody>
</table>

**Assessment of Liver Function Biochemical estimations**

Twenty four hours after administration of drugs, the animals were anaesthetized with anesthetic ether for withdrawing the blood sample by cardiac puncture after over night of fast. The blood was allowed to coagulate at room temperature for 45 minutes and then centrifuged at 2500 rpm for 15 minutes for separation of serum. The serum was used for the present biochemical estimations which includes AST, ALT, GGT, ALP and total bilirubin content by using biochemical estimation kits. The liver of all the animals were taken out, washed with cold saline water and bottled dry the livers between filter papers. The livers were weighed and a 10% homogenate of liver were prepared in ice cold 0.15M potassium chloride solution using glass Teflon tissue homogenizer. The homogenate was then used for determination of liver GSH and MDA levels (24).

**The measurement of GSH**

Liver tissue (200 mg) was homogenized in 0.02 M EDTA (8 mL) and stored in ice bath until use. Homogenate (5 mL) was prepared from liver tissue in tubes with EDTA, then it was
mixed with 4 mL distilled water and 1 mL 50% trichloroacetic acid (TCA). Then it was centrifuged at 3000 rpm (15 min) and 2 mL of liquid supernatant was mixed with 0.4 M Tris buffer (1 mL) (pH 8.9) and 0.1 Ellman reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] (DTNB) (Sigma). The absorbance at 412 nm in spectrophotometer was recorded. The result values were signified as μmol GSH/g tissue (25).

The measurement of MDA

10 mL homogenate was prepared with 1 g liver tissue in 0.15 N KCl. 0.2 mL sample was taken from homogenate and was mixed with 0.8 ml distilled water, 1.5 mL 2-thiobarbituric acid (TBA), 1.5 mL acetic acid and 0.2 mL sodium dodecil sulphate. For standardization of homogenate, 0.2 mL tetrathoxycopropane was used instead of homogenate. Blank was prepared with distilled water. Tubes were vortexed and boiled in water bath at 95°C for 1 h. Tubes were centrifugated at 4000 rpm (10 min) after cooling. Supernatant was collected and the absorbance of supernatant was recorded in spectrophotometer at 532 nm. The results were recorded as nmol/g tissue.

Histopathological studies

Small portion of the liver from each of the six animals in all of the groups were preserved in 10% w/v buffered formol saline (pH 7.4). The paraffin section were then prepared and stained with haemotoxylin-eosin dye for observation of liver damage.

Statistical analysis

Results of biochemical estimations were expressed as mean ± SD. The variations in a set of data have been estimated by one way analysis of variance (ANOVA) followed by Tukey multiple comparison tests. Minimum level of significance was fixed at 0.05.

RESULTS

Preliminary phytochemical studies revealed the presence of tannins, carbohydrate, saponins, diterpines, flavonoids and polyphenolic compounds.

Acute toxicity of AESR

The acute toxicity test after oral administration of 1000 mg/kg of AESR revealed no toxicity at this dose. There were no significant alterations in water or food consumption, or body weight during the experiment. The body weights, relative weights of the kidneys, livers, lungs and hearts were not statistically different from those of the control group. The LD_{50} of the AESR was found to be >1000 mg/kg body wt.

The protective actions of AESR on hepatotoxicity induced by TAA are summarized in Tables 2 and 3. The level of AST, ALT, ALP, GGT and total bilirubin content significantly decreased with the dose level at 400 mg/kg of Stevia extract. The values were 131.06±3.0, 62.2±1.8, 197.4±3.2, 13.0±0.3 and 0.23±0.0 respectively which were significant as compared to control group (p<0.001). These values were much lower than group II where only TAA was administered along with vehicle (p<0.001). Even AESR at dose level of 200 mg/kg decreased the levels of all the biochemical parameters compared to group II but maximum hepatoprotective activity was observed at 400 mg/kg dose level, which was comparable to that of silymarin (Table 2) (p<0.001). Table 3 shows that liver glutathione levels were 2.8 mol/mg in group IV compared to 1.5 mol/mg in group II and at the same time the values of MDA level were higher, i.e 26.8 nmol/g and 36.0 nmol/g
respectively which were significantly comparable with control and silymarin at dose level of 50 mg/kg (p<0.001).

Table 2. Effect of AESR on serum AST, ALT, ALP, GGT and total bilirubin.

<table>
<thead>
<tr>
<th>Group</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>ALP (IU/L)</th>
<th>GGT (IU/L)</th>
<th>Total bilirubin (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>101.4±1.6</td>
<td>34.4±1.6</td>
<td>111.8±2.5</td>
<td>7.2±0.3</td>
<td>0.17±0.0</td>
</tr>
<tr>
<td>TAA treated</td>
<td>174.4±1.3</td>
<td>109.1±0.8</td>
<td>304.7±3.9</td>
<td>22.3±0.5</td>
<td>0.6±0.0</td>
</tr>
<tr>
<td>AESR 200 mg/kg + TAA</td>
<td>155.6±2.4</td>
<td>80.7±0.8</td>
<td>230.2±4.2</td>
<td>18.5±0.6</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>AESR 400 mg/kg + TAA</td>
<td>131.1±3.0</td>
<td>62.2±1.8</td>
<td>197.4±3.2</td>
<td>13.0±0.3</td>
<td>0.23±0.0</td>
</tr>
<tr>
<td>Silymarin 50 mg/kg +</td>
<td>118.1±1.4</td>
<td>53.6±1.8</td>
<td>160.1±4.1</td>
<td>9.9±0.7</td>
<td>0.19±0.0</td>
</tr>
<tr>
<td>TAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N = 6; Values are expressed as mean ± SD

\*p< 0.001, \text{b} p< 0.01 vs Control
\*\*p< 0.001, \text{c} p< 0.05 vs TAA

Data were analyzed by using one way ANOVA followed by Tukey multiple comparison test.

Table 3. Effect of AESR on liver GSH and MDA levels.

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (x 10⁻³ mol/mg protein)</th>
<th>MDA (nmol/g liver wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.05±0.0</td>
<td>17.2±0.1</td>
</tr>
<tr>
<td>TAA</td>
<td>1.5±0.0*</td>
<td>36.0±0.1*</td>
</tr>
<tr>
<td>AESR 200 mg/kg + TAA</td>
<td>2.1±0.0**</td>
<td>31.0±1.1*</td>
</tr>
<tr>
<td>AESR 400 mg/kg + TAA</td>
<td>2.8±0.0**</td>
<td>26.8±1.0*</td>
</tr>
<tr>
<td>Silymarin 50 mg/kg +</td>
<td>3.1±0.0**</td>
<td>12.3±0.8*</td>
</tr>
<tr>
<td>TAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N = 6; Values are expressed as mean ± SD

\*\*p< 0.001 vs Control
\*\*\*p< 0.01 vs TAA

Data were analyzed by using one way ANOVA followed by Tukey multiple comparison test.

The histological observations of the control animals showed normal hepatocytes with well preserved cytoplasm, prominent nucleus, nucleolus and central vein. There was no sign of inflammation, fatty change or necrosis in these animals (Figure 1). In animals treated with TAA only, liver sections showed periportal lymphoplasmacytic infiltration, centrizonal inflammation and necrosis. Inflammatory cells were observed in the portal triad. Cytoplasmic vacuoles and ballooning degeneration of hepatocytes were also observed (Figure 2). Pretreatment with AESR at 200 mg/kg dose showed periportal and centrizonal inflammation and disappearance of centrizonal necrosis. Focal centrizonal hepatocytes also showed cytoplasmic vacuoles and balloon degeneration (Figure 3). AESR at 400 mg/kg dose showed greater reduction in periportal and centrizonal inflammation and no any centrizonal necrosis. Focal areas also showed degenerative changes of periportal hepatocytes (Figure 4). It also showed few portal triad with periportal lymphocytic infiltration, central vein and rest of the hepatic parenchyma appeared unremarkable. No centrizonal necrosis was identified (Figure 5). Pretreatment with silymarin at 50 mg/kg dose showed almost normal liver lobule with no sign of necrosis in the centrizonal area and portal triad. Only focal periportal inflammation was observed (Figure 6).
Figure 1. Photomicrograph of liver from control animals showed normal architecture of liver.

Figure 2. Photomicrograph of liver from animal treated with TAA only showing inflammatory cells in centrilobular area and also around the portal triad. The inflammatory infiltrate is seen spreading into the liver lobule.

Figure 3. Photomicrograph of liver from animal treated with 200 mg/kg of AESR and TAA and showing a inflammation in the periportal and centrizonal area.

Figure 4. Photomicrograph of liver from animal treated with 400 mg/kg of AESR and TAA, showing a minimal inflammation in the periportal and centrizonal area.

Figure 5. Photomicrograph of liver from animal treated with 400 mg/kg of AESR and TAA showing a focal periportal inflammation.

Figure 6. Photomicrograph of liver from animal treated with 50 mg/kg of silymarin and TAA showing a focal periportal inflammation.
DISCUSSION

The present study indicates the potential hepatoprotective activity of Stevia rebaudiana Bert. It is also found to possess free radical scavenging activity in TAA induced hepatotoxicity in Wistar rats. There is no detailed published report on the hepatoprotective activity of Stevia rebaudiana so an initial dose of 200 mg/kg of the aqueous extract of Stevia rebaudiana was chosen considering the high yield of the extract. TAA has been used as a tool to induce hepatotoxicity in experimental animals to produce various grade of liver damage including nodular cirrhosis, liver cell proliferation, production of pseudolobules, and parenchymal cell necrosis (26). Several investigators have reported that a single dose of this hepatotoxin can produce centrilobular hepatic necrosis and chronic administration leads to cirrhosis in rats (27). Mechanism of thioacetamide toxicity is due to the formation of thioacetamide-5-oxide which is responsible for the change in cell permeability, increased intracellular concentration of Ca++, increase in nuclear volume and enlargement of nucleoli and also inhibits mitochondrial activity which leads to cell death (28,29). Several researchers have suggested that part of hepatocellular injury induced by TAA is mediated through oxidative stress caused by the action of cytokines through lipid peroxidation (30,31). Reduced hepatic antioxidant function has also been suggested as one of the mechanism of TAA induced hepatotoxicity (32). In a number of animal models, TAA induced cirrhosis seem to resemble the important features of human diseases (33). Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (28). In the present study, TAA was found to cause significant elevations in the levels of serum AST, ALT, GGT, ALP and total bilirubin. Pretreatment with AESR was found to significantly reverse these toxin induced changes. Hence, a reduction in the levels of these enzymes demonstrates membrane stabilizing activity of the Stevia extract. Flavonoids present in Stevia rebaudiana could be responsible for the membrane stabilizing activity.

Lipid peroxidation occurs to a limited extent under normal physiological conditions, but external factors can augment this process so that it escapes cell control which leads to damage of macromolecules such as lipids in the cell membrane and eventually causing membrane damage and death of cell. Glutathione is an important endogenous antioxidant system that is found in particularly high concentration in liver and it is known to have key functions in protective processes. The extract resulted in significant increase in the liver glutathione levels as compared to TAA group and at higher doses (i.e., at 200 and 400 mg/kg AESR) the increase was statistically significant as compared to control group. Treatment with both of the extracts also resulted in significant decrease in the lipid peroxidation in the liver as shown by decrease in the MDA levels in the liver. Hence, the drug resulted in inhibition of lipid peroxidation which could be due to enhanced liver glutathione content as a result of drug treatment. These results suggest that the hepatoprotective action of Stevia rebaudiana might be due to the presence of antioxidants like flavonoids, phenolic compounds (33). Centrizonal necrosis, which involves the cells around the central hepatic vein, occurs in viral hepatitis, TAA, carbon tetrachloride and chloroform toxicity and anoxic states such as cardiac failure and shock (34). In the present experiment, it indicates the damage caused by TAA to the hepatocytes. The decrease in the necrosed area demonstrated by the extract as well as decrease in the infiltration of the inflammatory cells in the liver lobules is indicative of therapeutic efficacy of the plant extract. Silymarin is a standardized seed extract of Silybum marianum, which contains flavonolignans. Silymarin at doses up to 100mg/kg has been used as a standard hepatoprotective agent by numerous investigators. Our study showed the hepatoprotective potential of the extract of Stevia rebaudiana and silymarin pre-treatment against thioacetamide induced hepatotoxicity is due to multiple mechanisms.
CONCLUSION

It can be concluded that aqueous extract of *Stevia rebaudiana* has potential hepatoprotective activity and attenuates the hepatotoxic effects of TAA by membrane stabilizing effect and acting as an antioxidant.

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REFERENCES