2-Hydroxy 4-methoxy benzoic acid isolated from roots of *Hemidesmus indicus* ameliorates liver, kidney and pancreas injury due to streptozotocin-induced diabetes in rats

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Protective effect was evaluated in streptozotocin (STZ)-induced diabetes rats. 2-Hydroxy 4-methoxy benzoic acid (HMBA) was isolated from the roots of *Hemidesmus indicus* and administered (500 \( \mu \)g/kg body weight) orally for 7 weeks to STZ-induced diabetic and non-diabetic rats to study its effect on protein metabolism, serum electrolytes and on liver and kidney lipid peroxides. Oral administration of HMBA restored the altered biochemical parameters such as urea, uric acid, creatinine, plasma proteins and serum electrolytes to near-normal levels. HMBA treatment significantly decreased lipid peroxidation and malondialdehyde levels in diabetic liver and kidney. Effect of HMBA was equivalent to that of the standard drug, tolbutamide (100 mg/kg body wt). The histological changes were also in correlation with the biochemical findings. The present study showed that HMBA isolated from *H. indicus* roots had ameliorative effect on liver, kidney and pancreatic injury in STZ-induced diabetic rats.

**Keywords**: Diabetes, 2-Hydroxy 4-methoxy benzoic acid, Lipid peroxidation, Streptozotocin, Diabetes.

*Hemidesmus indicus* (Asclepiadaceae) locally called as anantamul (Indian sarsaparilla) is widely distributed throughout India. *H. indicus* root extract is traditionally used as antioxidant, antinflammatory, immunomodulatory and antidote in the Indian system of medicine\(^1\). *H. indicus* root extract has been reported to protect DNA from radiation induced strand breaks\(^2\). Hypoglycemic effect of *H. indicus* root extract in STZ-induced experimental diabetic rats has already been reported\(^3\).

2-Hydroxy 4-methoxy benzoic acid exists in *H.indicus*, is a white needle-shaped crystal which is soluble in water, methanol and chloroform and has a melting point of 155°-158°C and lambda max 260 nm\(^4\). The presence of a benzene ring, methoxy group and hydroxyl group has been confirmed by spectral analysis. The molecular formula is C\(_8\)H\(_8\)O\(_4\) and the molecular weight of the pure compound is 168 (Ref. 5). The concentration of HMBA is in the range of 0.03-0.54% in the roots of *H. indicus* \(^6\).

Diabetes mellitus is a metabolic disorder featured by hyperglycemia and alterations in carbohydrate, fat and protein metabolism associated with absolute or relative deficiency of insulin secretion and/or insulin action\(^7\). The underlying mechanism of diabetic complications is not clear, but much attention has been focused on the role of oxidative stress in diabetes\(^7\) and pathogenesis of different diabetic complications\(^8\). Diabetic experimental animal models have shown that oxidative stress mediated injury to the pancreatic cells causes persistent and chronic hyperglycemia, thereby depleting the activities of the antioxidant defense system and otherwise promoting free-radical generation\(^9\). It has been reported that an abnormally elevated blood glucose level leads to oxidative stress and formation of advanced glycation end product (AGE), which have been closely associated with onset of diabetic complications\(^10\). Increased generation of oxygen radicals has been reported in STZ-induced diabetic conditions\(^11\). Polyunsaturated fatty acids are probably the most susceptible target to free radical attack. The reaction of free radicals with the membrane lipid components leads to lipid peroxidation. This process can eventually cause increased membrane permeability and cell death\(^12\). To counteract these oxidants, cells have several antioxidants and these antioxidants are depleted when cells are exposed to oxidative radicals\(^4\).

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Hyperglycemia not only generates more reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, but also attenuates antioxidative mechanism through glycation of antioxidant enzymes. It has been demonstrated that renal hydrogen peroxide overproduction and lipid peroxide accumulation occur at very early stages of STZ-diabetes and are associated with impairment of antioxidative defense. Diabetic oxidative stress has been alleviated by several traditional prescriptions and extracts through inhibition of lipid peroxidation in STZ-induced diabetic rats.

The aim of the present study was to investigate the effect of HMBA on protein metabolism, electrolytes and STZ mediated oxidative stress in diabetic rats.

Materials and Methods

Chemicals and reagents—2-hydroxy 4-methoxy benzoic acid was purchased from Sigma-Aldrich Co (USA). All other chemicals, reagents, kits and solvents used in this study were of analytical grade and procured locally.

Plant material—The root of *H. indicus*, was identified and collected from the Morappur forest area, Dharmapuri District, Tamil Nadu, India. The roots were identified and a voucher specimen was submitted to VIT University, India. Roots were washed with distilled water, shade dried, powdered and stored in an air-tight container until further use.

Extraction, isolation and purification of pure compound—The root powder of *H. indicus* (100 g) was extracted with methanol using Soxhlet apparatus and concentrated in rotary evaporator and then purified by silica gel (Merck,100 to 200 mesh) column chromatography and eluted with benzene-chloroform and the purity was checked by thin layer chromatography. The purity of the isolated compound was analyzed by spectroscopic techniques (UV, 1H NMR, 13C NMR and GC MS) and compared with the standard.

Animals—Male albino rats (Wistar strain, weighing 150-200 g) were purchased from Tamil Nadu Veterinary Animal Science University, Madhavaram, Chennai, and housed under standard husbandry conditions (30±2°C, 60-70% RH, and 12:12 h day/night cycle) and allowed standard pellet rat feed and water *ad libitum*. The animal experiments were designed and conducted in accordance with the guidelines of the Institutional Animal Ethical Committee (IAEC), VIT University, India.

Induction of experimental diabetes—Diabetes was induced experimentally in rats by a single intraperitoneal injection of freshly prepared solution of STZ (Sigma, USA) at a dose of 35 mg/kg, body wt in 0.1 M citrate buffer (pH 4.5). The STZ treated animals were considered to be diabetic, if the blood glucose values were above 250 mg/dl and stabilized a period of 7 days. Such animals alone were selected for the study.

Experimental design—Animals were divided into six groups of six animals each. Group I served as a control; group II had STZ-treated surviving diabetic rats; group III served as a positive control and received a standard hypoglycemic agent, tolbutamide (100 mg/kg body wt); group IV diabetic rats treated with the HMBA (500 μg/kg body wt/day) for 7 weeks by oral intubation. Blood samples were collected in heparinised vials from the tail vein in all animals. After blood collection, all animals were sacrificed by cervical dislocation. Pancreas, liver and kidney were dissected out immediately and stored at -20°C. Liver and kidney thiobarbituric acid reactive substances, malondialdehyde and hydroperoxides were estimated.

Histopathological study—Pancreas, liver and kidney tissues were preserved in formalin (10%), processed and embedded in paraffin wax. Thin sections (5 μm) were cut on glass slides and stained with hematoxylin and eosin (H & E). After de-waxing, the tissues were examined under light microscope. Histopathological observation of the tissues was carried out at the Department of Veterinary Pathology, Tamil Nadu Veterinary and Animal Sciences University, Chennai.

Statistical analysis—The values were analyzed by one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using SPSS software package, version 9.05. All the results were expressed as mean ±SD for six rats in each group. P values <0.05 were considered statistically significant.

Results and Discussion

Effect of HMBA on protein metabolism is given in Table 1. The levels of total protein and albumin in plasma were reduced significantly in STZ-induced diabetic rats compared to untreated control rats (Table 1). The levels of total protein and albumin recovered to normal after treatment with HMBA for 7 weeks. Decrease in total protein and albumin might be due to microproteinuria and albuminiuria, which are
important clinical markers of diabetic nephropathy, and/or due to increased protein catabolism. Blood levels of urea, uric acid and creatinine increased significantly in STZ-induced diabetic rats compared to untreated control rats. HMBA treatment for 7 weeks normalized the levels of non-protein nitrogenous substances. The observed effect of HMBA was equivalent to that of standard drug, tolbutamide. The observed increase in plasma levels of urea, uric acid and creatinine, might be due to STZ-induced metabolic disturbances as well as renal dysfunction. The levels of non-protein nitrogenous substances are always used as significant markers for the assessment renal dysfunction. Increased protein glycation in STZ–induced diabetic condition has been reported to be associated with increased muscle wasting and thereby, increased release of purines. The elevated levels of purine under diabetic condition have been reported to be the main source of uric acid as well as the activity of xanthine oxidase.

In diabetic rats, there was a significant increase in the serum electrolytes; sodium, potassium and calcium. Oral administration of HMBA significantly reduced the levels of serum electrolytes compared to normal rats (Table 2). Effect of HMBA on the liver and kidney lipid peroxidation has been given in Table 3. Elevated levels of thiobarbituric acid reactive substances (TBARS), tissue hydroperoxides and tissue malondialdehyde in diabetic rats were reduced significantly to near-normal levels upon treatment with HMBA.

### Table 2—Effect of HMBA isolated from H. indicus on serum electrolytes in normal and streptozotocin-induced diabetic rats [Values are mean ± SD of 6 rats]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Na⁺ (mEq/l)</th>
<th>K⁺ (mEq/l)</th>
<th>Ca²⁺ (mEq/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>145.00±5.90</td>
<td>6.80±1.25</td>
<td>7.38±0.15</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>162.00±2.36</td>
<td>17.90±0.23</td>
<td>9.10±0.23</td>
</tr>
<tr>
<td>Diabetic + tolbutamide (100 mg/kg body wt/day)</td>
<td>143.12±2.12</td>
<td>5.13±0.62</td>
<td>7.91±0.56</td>
</tr>
<tr>
<td>Diabetic + HMBA (500 μg/kg body wt/day)</td>
<td>145.34±2.4</td>
<td>5.55±0.43</td>
<td>7.50±0.56</td>
</tr>
</tbody>
</table>

*Significant as compared to control at $F > 0.05$ (ANOVA) and $P < 0.05$ (DMRT)

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*Significant as compared to control at $F > 0.05$ (ANOVA) and $P < 0.05$ (DMRT)

### Table 3—Effect of HMBA on liver and kidney TBARS, hydroperoxides and malondialdehyde in normal and streptozotocin-induced diabetic rats [Values are mean ± SD of 6 rats]

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS (mmol/mg protein)</th>
<th>Hydroperoxides (mmol/ 100g tissue)</th>
<th>Tissue MDA (nmol/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Kidney</td>
<td>Liver</td>
</tr>
<tr>
<td>Normal</td>
<td>0.68±0.21</td>
<td>1.53±1.08</td>
<td>70.64±2.10</td>
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<tr>
<td>Diabetic control</td>
<td>1.45±0.01*</td>
<td>2.64±0.36*</td>
<td>115.57±1.45*</td>
</tr>
<tr>
<td>Diabetic + tolbutamide (100 mg/kg body wt/day)</td>
<td>1.12±1.01*</td>
<td>1.91±0.27a</td>
<td>84.13±1.32a</td>
</tr>
<tr>
<td>Diabetic + HMBA (500 μg/kg body wt/day)</td>
<td>0.68±1.3a</td>
<td>1.55±0.05a</td>
<td>71.2±0.16a</td>
</tr>
</tbody>
</table>

*Significant as compared to control at $F > 0.05$ (ANOVA) and $P < 0.05$ (DMRT)

*Significant as compared to diabetic control at $F > 0.05$ (ANOVA) and $P < 0.05$ (DMRT).
Increased glucose oxidation in presence of transition metals has been shown to produce membrane damage by membrane lipid peroxidation and protein glycation. This could be the reason for altered flux in electrolyte balance that resulted in elevated extracellular concentration of sodium, potassium and calcium in STZ-induced diabetic rats. HMBA, the active principle isolated from *H. indicus* roots has been shown to possess venom inhibitory activity and antioxidant activity. Streptozotocin selectively destroys pancreatic insulin secreting β-cells and causes enhanced level of ROS in pancreas, liver and related tissues. Increased levels of ROS results in tissue damage and enhanced lipid peroxidation.

Lipid peroxidation is a free radical-induced process leading to oxidative deterioration of polyunsaturated fatty acids. Under physiologic conditions, low concentrations of lipid peroxides are found in tissues. Elevated level of lipid peroxides in the plasma of diabetic rats and lipid peroxidation mediated injury has been considered to be one of the characteristic features of chronic diabetes. Lipid peroxides mediated tissue damage has been observed in the development of both Type 1 and 2 diabetes. The plasma level of MDA is considered to be an important marker of lipid peroxidation. In this study, lipid peroxidation in liver and kidney was evaluated by measuring levels of MDA. Significant increase in MDA levels in tissues of diabetic rats compared to normal control rats was in agreement with earlier reports. There are ample evidences on the damaging consequences of oxidative stress and its role in experimental diabetes caused by overproduction and/or insufficient removal of ROS.

The normal healthy control group showed a normal beta cell structure under light microscope. The cells were well granulated and the cell organelles such as cisternae of the rough endoplasmic reticulum and Golgi complex were of normal size (Fig. 1A). However, in the STZ-induced diabetic rats, there was atrophy of islet cells and characteristic changes in beta cells with disappearance of granules and malformation of the cells (Fig. 1B). Treatment of diabetic rats with HMBA normalized the islet cells (Fig. 1C).

The liver of control rats had normal histological appearances (Fig. 2B). The hepatocytes of experimentally induced diabetic rats showed shrunken nuclei, granular cytoplasm, dilatation in the sinusoids and inflammation (Fig. 2B). The hepatocytes of diabetic rats treated with HMBA showed minimal degenerative changes (Fig. 2C). The normal architecture of kidney is shown in (Fig. 3A), inflammation and mild necrosis was seen in kidney of STZ-induced rats (Fig. 3B) which was normalized by HMBA treatment (Fig. 3 C).

The present study was an attempt to identify the compound having ameliorative potential in reducing the diabetes mediated oxidative injury. It is suggested that *H. indicus* may be used for diabetic treatment and recovery of organ damage in diabetic subjects.
Fig. 2—Histopathological examination of liver in experimental animals. (A)—Normal healthy control showing normal hepatic cells (10 ×); (B,C)—STZ-induced diabetic rats showing shrunken nuclei [B—granular cytoplasm (arrow GC), C—dilated sinusoids (arrow DS) and inflammation (arrow IN) (10 ×)]; and (D)—HMBA (500 μg/kg body weight/day) treated rat showing partially reduced degenerative changes in hepatic cells (10 ×).

Fig. 3—Histopathological examination of kidney cells in experimental animals. (A)—Normal healthy control showing normal renal cells (10 ×); (B)—STZ-induced diabetic with slightly modified renal capsules (arrow RC) (10 ×); and (C)—HMBA (500 μg/kg body weight/day) treated rat showing partially reduced degenerative changes in kidney (10 ×).

Acknowledgement
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References


