**Anthocephalus cadamba** (Roxb.) Miq., Rubiaceae, extract shows hypoglycemic effect and eases oxidative stress in alloxan-induced diabetic rats

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Abstract: The hydroethanolic extract of the flowering tops of *Anthocephalus cadamba* (Roxb.) Miq., Rubiaceae, a Bangladeshi medicinal plant, was studied for its potential hypoglycemic effect and antioxidant property in alloxan-induced diabetic rats. The extract induced significant reduction in serum glucose, and transaminases, e.g. aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatases (ALP), activities. Significant changes in the thiobarbituric acid reactive substances (TBARS), peroxidase and catalase levels during the experimental period were also observed. The results established that the hydroethanolic extract of the flowering tops of *A. cadamba* possesses hypoglycemic property and is able to protect liver and brain from oxidative damages caused by diabetes.

Introduction

An increased level of lipid peroxides and/ or oxidative stress is present in diabetic subjects, and may contribute to their increased risk of cardiovascular diseases (Haffner et al., 1995). Excessive production of free radicals is believed to be involved in many diabetic complications including diabetic neuropathy in diabetes mellitus (Sima & Sugimoto, 1999). The neurological consequences of diabetes mellitus in the central nervous system (CNS) are now receiving greater attention. Glucose utilization is decreased in the brain during diabetes (McCall, 1992) providing a potential mechanism for increased vulnerability to acute pathological events. Effective therapeutic strategies to prevent or delay the development of this damage remain limited and the American Diabetes Association recommended that antioxidant therapy needs to be improved; either older antioxidants such as vitamin E, lipoic acid, and N-acetyl-

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and wound-healing properties (Umachigi et al., 2007; Alam et al., 2008a,b), the phytochemical investigations resulted in the isolation of indole alkaloids, secoiridoids, triterpenes and saponins from this plant (Banerji, 1977; 1978; Brown & Chapple, 1976; Kitagawa et al., 1996; Sahu et al., 1999; 2000).

In continuation of our phytochemical and pharmacological screening of Bangladeshi medicinal plants (Uddin et al., 2005, 2007a-c; Datta et al., 2007; Nayeem et al., 2006; Saha et al., 2007; Alam et al., 2008a,b; Mazid et al., 2009; Ara et al., 2010; Miah et al., 2010), we report on the hypoglycemic and oxidative stress-reducing activities of the hydroethanolic extract of the flowering tops of *A. cadamba* in alloxan-induced diabetic rats.

**Material and Methods**

**Plant material**

The flowering tops of *Anthocephalus cadamba* (Roxb.) Miq., Rubiaceae, were collected from Bangladesh Agricultural University campus in July 2005 and identified by the experts of the Botanical Garden of Bangladesh, Agricultural University and the National Herbarium, Mirpur, Dhaka, Bangladesh. Voucher specimens (BAU-MAA-2005-AC1 and ACC. No 32497) for this collection have been retained in the herbarium of the Bangladesh Agricultural University, Mymensing, Bangladesh and the National Herbarium, Mirpur, Dhaka, Bangladesh, respectively.

**Extraction**

Shade-dried and powdered flowering tops of *A. cadamba* (200 g) were Soxhlet-extracted using 80% aq. ethanol. The extract was concentrated by evaporation under reduced pressure at 40 °C using a Buchi rotary evaporator to yield a concentrate of reddish black extract (yield 6.32%).

**Animals**

Swiss albino rats of both sexes, 3-4 weeks of age, weighing 120-130 g, were obtained from the Animal House of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). The animals were housed in five groups in stainless steel cages (71 × 56 × 33 cm) under standard laboratory conditions (relative humidity 55-65%, r.t. 24.0±1.0 °C and 12 h light:dark cycle). Soft wood shavings were used as bedding of cages. Husk and excreta were removed from the cages every day. The animals were fed with pellets of rat feed provided by ICDDR, B, and fresh water *ad libitum*. The study involving rats was approved by the Ethical Review Committee (approval number: MAA0007) of the Department of Pharmacy, Stamford University, Dhaka, Bangladesh, and the experiments were carried out strictly in accordance with the guidelines provided by the World Health Organization.

**Experimental design**

Experimental animals were divided into two lots; one for the glucose tolerance test (GTT) and the other for the long term effect of the extract on diabetic rats for a three weeks study periods.

In the glucose tolerance test fifteen non-diabetic rats of either sex were selected and grouped into three different groups. Group 1 served as the control group which received only normal saline. Group 2 and Group 3 received the extract of *A. cadamba* at the doses of 100 and 200 mg/kg, respectively.

Another thirty rats were used for the long term effect of the extract on diabetic rats for a three weeks study periods. The rats were divided into five groups of six rats each. Group 1: normal rats; Group 2: diabetic control rats (alloxan, 150 mg/kg, i.p.); Group 3: diabetic rats given glibenclamide (600 μg/kg body weight) in aqueous solution daily using an intragastric tube for three weeks; Group 4: diabetic rats given *A. cadamba* extract (200 mg/kg body weight) in aqueous solution daily using an intragastric tube for three weeks; and Group 5: diabetic rats given *A. cadamba* (400 mg/kg body weight) in aqueous solution daily using an intragastric tube for three weeks.

The diabetic condition was assessed by determining the blood glucose concentration at three and five days after alloxan treatment. No detectable irritation or restlessness was observed after each drug or vehicle administration. No noticeable adverse effect (i.e., respiratory distress, abnormal locomotion and catalepsy) was observed in any animals after the drug administration.

**Glucose tolerance test**

Animals were fasted overnight and divided into three groups containing five rats each. Control animals (Group 1) were given 1 mL of distilled water orally. *A. cadamba* extract was given p.o. using a syringe at concentrations of 100 and 200 mg/kg body weight (Groups 2 and 3, respectively). After the administration of *A. cadamba* extract, all groups were given glucose (2 g/kg bw) orally. Blood samples were collected from the tail vein just prior to and 30, 60, 120 and 240 min after glucose loading. Serum glucose level was assayed by a glucometer. Results achieved from the glucose tolerance test were taken as a hypothetical reference to extrapolate the dose levels which would be used for evaluating short-
and long-term effects of *A. cadamba* extract on diabetic rats.

**Blood sample collection**

Blood samples were drawn at weekly intervals till the end of study (*i.e.* three weeks). At the end of the 3rd week, all rats were sacrificed by decapitation (pentobarbitone sodium) anaesthesia (60 mg/kg). Blood was collected in two different tubes, one with anticoagulant-dissodium EDTA for membrane stabilizing activity and the other without anticoagulant for serum separation. Serum was separated by centrifugation. After centrifugation, the clear supernatant was used for the assay of enzyme activities. The EDTA containing blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged each time for 10 min at 3000 rpm and RBC was prepared for the membrane stabilizing activity.

**Brain sample collection**

Whole brain was immediately dissected out, washed in ice cold saline to remove blood. The brains were weighed and 10% tissue homogenate was prepared with 0.025 M Tris-HCl buffer, pH 7.5. After centrifugation at 2000 rpm for 10 min, the clear supernatant was used to measure thiobarbituric acid reactive substances (TBARS), hydroperoxides, nitric oxide (NO) level and catalase activity.

**Biochemical analysis**

**Estimation of blood glucose**

Glucose was measured in serum of non-fasted rats. Blood was sampled by tail tip method (Mallick et al., 2006) and thereafter analyzed with an auto analyzer Reflotron Plus (Roche, Germany) using commercial kit.

**Estimation of blood transaminase**

The serum was assayed for aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatases (ALP) with an auto analyser Reflotron Plus (Roche, Germany) using commercial kit (Alam et al., 2008).

**Estimation of lipid peroxidation**

Lipid peroxidation in brain was estimated colorimetrically by thiobarbituric acid reactive substances TBARS by the method previously described by Niehius & Samuelsson (1968). In brief, 0.1 mL of tissue homogenate (Tris-HCl buffer, pH 7.5) was treated with 2 mL of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm.

**Estimation of hydroperoxides**

Hydroperoxides level was estimated by the method described before by Jiang et al. (1992). Tissue homogenate (0.1 mL) was treated with 0.9 mL of Fox reagent (88 mg butylated hydroxytoluene (BHT), 7.6 mg xylenol orange and 9.8 mg ammonium iron sulfate were added to 90 mL of methanol and 10 mL 250 mM sulfuric acid) and incubated at 37 °C for 30 min. The color developed was read at 560 nm colorimetrically. Hydroperoxides was expressed as mM/100 g tissue.

**Assay of catalase**

Catalase was assayed colorimetrically at 620 nm and expressed as μM of H₂O₂ consumed/min/mg protein as described by Sinha (1972). The reaction mixture (1.5 mL) contained 1.0 mL of 0.01 M pH 7.0 phosphate buffer, 0.1 mL of tissue homogenate (supernatant) and 0.4 mL of 2 M H₂O₂. The reaction was stopped by the addition of 2.0 mL of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).

**Assay of nitric oxide (NO)**

The NO was determined according to the method described by Tracy et al. (1995) as nitrate and nitrite. In this study, Griess-Ilosvvy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 mL) containing brain homogenates (2 mL) and phosphate buffer saline (0.5 mL) was incubated at 25 °C for 150 min. After incubation, 0.5 mL of the reaction mixture was mixed with 1 mL of sulfamic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, naphthyl ethylene diamine dihydrochloride (1 mL) was added, mixed and allowed to stand for 30 min at 25 °C. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions.

**Hypotonic solution-induced diabetic rat erythrocyte hemolysis**

Membrane stabilizing activity of the extract was assessed using hypotonic solution-induced rat
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erythrocyte hemolysis (Shinde et al., 1999a,b). The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extract (0.25-2.0 mg/ml). The control sample consisted of 0.5 ml of RBC mixed with hypotonic-buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of hemolysis or membrane stabilization was calculated according to modified method described by Shinde et al. (1999a,b) and Umukoro & Ashorobi (2006).

\[
\text{\% Inhibition of haemolysis} = 100 \times \left\{ \frac{\text{OD}1 - \text{OD}2}{\text{OD}1} \right\}
\]

Where:

OD1: Optical density of hypotonic-buffered saline solution alone
OD2: Optical density of test sample in hypotonic solution

Statistical analysis

Data was analyzed by one way ANOVA using SPSS-12 for Windows. Differences in means were estimated by means of repetitive measures followed by Bonferroni and Dunnet’s post hoc test and expressed as statistical mean±standard deviation. Differences between means were regarded significant at \(p<0.05\).

Results and Discussion

Alloxan(2,4,5,6-tetra-oxohexahydropyrimidine) induces “chemical diabetes” in a wide variety of animal species by damaging the insulin secreting pancreatic beta cells, resulting in a decrease in endogenous insulin release (Lenzen & Panten, 1988; Vijayvargia et al., 2000). In the present study hypoglycaemic activity of the hydroethanolic extract of \textit{A. cadamba} was evaluated on non-diabetic and diabetic rat models. The glucose tolerance test using the non-diabetic rats showed a dose-dependent decrease of glucose in plasma after administration of the extract over 4 h (Table 1). The extract significantly decreased the blood glucose level and prevented the weight loss in alloxan-induced diabetic rats. The effect of the extract on body weight in alloxan-induced diabetic rats is shown in Table 2.

Alloxan administration caused a significant weight loss after three weeks of treatment from 128.00±2.09 to 107.83±5.41 g, whereas rats in the normal group continued to put on weight. Treatment with 200 and 400 mg/kg doses of the extract reversed the weight loss. Serum glucose levels in normal rat group (Group 1) reached steady concentrations 73.17±2.48 mg/dL in the second week, and 75.50±1.52 mg/dL in the third week. On the other hand, a significant increase in serum glucose levels was observed in the diabetes control group (Group 2), 240.67±3.98 and 241.33±2.55 mg/dL in the second and third week, respectively.

The glucose level in the glibenclamide treated group (Group 3) remained unchanged during the experimental period. Multi-dose study revealed that administration of the extract at the dose of 200 mg/kg reduced the elevated glucose level significantly in the second week after alloxan administration to 203.67±7.17 mg/dL, and in the third week glucose level further decreased to 191.50±6.09 mg/dL. The glucose lowering activity of the extract (400 mg/kg) began in the second week with a reduction of 158.67±6.56 mg/dL, and reached to its maximum reduction in the third week by 110.67±5.75 mg/dL. The extract displayed antidiabetic effect in a concentration dependent manner (Table 3), and the activity was quite similar to that of the glibenclamide treated group at a higher dose.

Oxidative stress, leading to an increased production of reactive oxygen species (ROS) as well as lipidperoxidation, increases in diabetes (Wolff, 1993) and also hyperglycemic animals (Liu et al., 1996). Similarly, oxidative damage in rat brain is increased by experimentally induced hyperglycemia (Aragno et al., 1997). It has been suggested that the modified oxidative state induced by chronic hyperglycemia may contribute to nervous tissue damage; free radical species impair the central nervous system, attacking neurons and schwann cells, and the peripheral nerves (Kawai et al., 1998; Kumar & Menon, 1998; Aragno, 2000). Because of high polyunsaturated lipid content, schwann cells and axons are particularly sensitive to oxygen free radical damage;

### Table 1. Glucose tolerance test of the hydroethanolic extract of \textit{Anthocephalus cadamba} (ACE) on non-diabetic rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Group 1 (Control)</td>
<td>85.40±1.34</td>
</tr>
<tr>
<td>Group 2 (ACE 100 mg/kg)</td>
<td>86.40±0.55</td>
</tr>
<tr>
<td>Group 2 (ACE 200 mg/kg)</td>
<td>83.20±1.10</td>
</tr>
</tbody>
</table>

Values are represented as mean±standard deviation. Differences in means were estimated by means of repetitive measures followed by the Bonferroni and Dunnet’s post hoc test (n=5). Statistical significance was considered as \(p<0.05\) in all cases vs. control.
lipid peroxidation may increase cell membrane rigidity and impair cell function. Lipid peroxidation products are also increased in the brains of Type 1 diabetic rats (Makar et al., 1995) and Type 2 diabetic mice (Kumar & Menon, 1998).

Diabetes and stress mediated increases in oxidative stress as well as decreases in antioxidant activity may make the brain more vulnerable to subsequent pathological events. The effects of the A. cadamba extract on brain oxidative parameters in alloxan-induced diabetes are shown in Table 4. The treatment with the extract (200 and 400 mg/kg) over a period of three weeks produced a protective effect on the significantly decreased levels of lipid peroxidation product TBARS and NO. It also increased the activity of the enzyme peroxidase and catalase. TBARS and hydroperoxides (lipid peroxidative markers) showed high lipid peroxidation. This could be due to the fact that the brain contains relatively high concentration of easily peroxidizable fatty acids (Carney et al., 1991). In agreement with previous studies (Kakkar et al., 1998), the induction of diabetes in rats with alloxan resulted in an increase in lipid peroxidation (TBARS), an indirect evidence of intensified free radical production. Most of the tissue damage is thought to be free-radicals mediated by attacking membranes through peroxidation of unsaturated fatty acids. The present findings showed increased lipid peroxidation in rats exposed to alloxan, and its attenuation by the oral administration of the A. cadamba extract at the doses of 200 and 400 mg/kg. The antiperoxidative effect of the extract at the dose of 400 mg/kg exhibited more significant effect than that of glibenclamide. Significantly lower levels of lipid peroxides in brain of the extract treated diabetic rats and increased activities of enzymic and non-enzymic antioxidants in brain suggested that the extract reduced oxidative stress by quenching free radicals.

Catalase is a hemoprotein that catalyzes the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals. Reduced activities of superoxide dismutase and catalase in liver and kidney were observed during diabetes, and implicated to deleterious effects due to the accumulation of $O_2^•$ and $H_2O_2$ (Searle & Wilson 1980). Administration of the extract of A. cadamba increased the activity of these enzymes (Table 4) through free-radical-scavenging activity because of the presence of phenolic compounds in the extract (Niranjan et al., 2000).

Nitric oxide (NO) has been recognized as a biological neural messenger molecule although it is best known as a toxic reactive free-radical in the CNS. NO or NO-derived nitrogen oxides must interact with neuromodulators in order to modify these modulators, especially monoamines, and thereby change their regulatory action on synaptic transmission (Fossier et al. 1999). Thiol (-SH) containing enzymes and proteins are critical targets for NO, so it forms relatively stable nitroso-thiols (-S-NO). Reduction of NO production

Table 2. Effect of the hydroalcoholic extract of Anthocephalus cadamba (ACE) on body weight of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 Normal rats</td>
<td>128.17±2.32</td>
<td>129.83±2.86</td>
<td>134.67±1.75</td>
</tr>
<tr>
<td>Group 2 Diabetes control</td>
<td>128.00±2.09</td>
<td>113.50±6.09</td>
<td>107.83± 5.41</td>
</tr>
<tr>
<td>Group 3 Glibenclamide</td>
<td>127.50±2.88</td>
<td>133.17±1.83</td>
<td>137.83±1.94</td>
</tr>
<tr>
<td>Group 4 ACE 200 mg/kg</td>
<td>127.83±1.94</td>
<td>132.17±1.17</td>
<td>134.33±1.51</td>
</tr>
<tr>
<td>Group 5 ACE 400 mg/kg</td>
<td>128.33±2.06</td>
<td>133.67±1.51</td>
<td>138.17±1.47</td>
</tr>
</tbody>
</table>

Values are represented as mean±standard deviation. Differences in means were estimated by means of Repetitive measures followed by Bonferroni and Dunnet’s post hoc test (n=5). Statistical significance was considered as $p<0.05$ in all cases vs. control.

Table 3. Effect of the hydroethanolic extract of A. cadamba (ACE) on blood glucose level in plasma.

<table>
<thead>
<tr>
<th>Group</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 Normal rats</td>
<td>73.17±2.48</td>
<td>73.17±2.48</td>
<td>75.50±1.52</td>
</tr>
<tr>
<td>Group 2 Diabetes control</td>
<td>239.27±1.28</td>
<td>240.67±3.98</td>
<td>241.33±2.55</td>
</tr>
<tr>
<td>Group 3 Glibenclamide</td>
<td>112.13±2.12</td>
<td>118.83±2.64</td>
<td>132.50±3.08</td>
</tr>
<tr>
<td>Group 4 ACE 200 mg/kg</td>
<td>212.67±1.11</td>
<td>203.67±7.17</td>
<td>191.50±6.09</td>
</tr>
<tr>
<td>Group 5 ACE 400 mg/kg</td>
<td>188.25±2.16</td>
<td>158.67±6.56</td>
<td>110.67±5.75</td>
</tr>
</tbody>
</table>

Values are represented as mean±standard deviation. Differences in means were estimated by means of Repetitive measures followed by Bonferroni and Dunnet’s post hoc test (n=5). Statistical significance was considered as $p<0.05$ in all cases vs. control.
may consequently result in a stimulation of oxidative phosphorylation and increase peripheral oxygen uptake (Kuo & Schroeder, 1995). A critical reaction that NO undergoes in oxygenated biological media is a direct bimolecular reaction with \( O_2 \) S- yielding peroxynitrite (ONOO-). Peroxynitrite and its further products have been linked to several interactions which may contribute to cellular injury, including lipid peroxidation, nitrosylation of some molecules, and inactivation of sodium channels. ONOO- has been shown to oxidize a variety of biological molecules and may be responsible for certain types of NO-mediated toxicity (Szabo et al., 1995). Taking these facts together, NO or closely related molecules are considered to be neurodestructive. The positive correlation between NO and TBARS in the present findings might show the direct or indirect enhancer effect of NO on lipid peroxidation. Alloxan treated diabetic control group (Group 2) produced the highest amount of NO and TBARS among all groups, and the \textit{A. cadamba} extract prevented the production of NO and TBARS (Table 4) in a dose-dependent manner.

Alloxan administration significantly increased liver function biomarkers, aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatases (ALP), in comparison with normal rats (Table 5). The extract at the concentrations of 200 and 400 mg/kg produced an inhibitory effect on elevated liver markers while the diabetes control group and dibenclamide treated group did not show any change in the elevated liver enzyme concentration. The AST and ALT activities are known as cytosolic marker enzymes reflecting hepatocellular necrosis as they are released into the blood after cell membrane damage. In the present study, therefore, both enzyme activities were used as indicators of hepatic damage. Table 3 shows the activities of AST and ALT in experimental rats. Compared with the normal rats, the diabetic rats showed more activities of serum AST and ALT. Further the histopathological study of the liver section confirmed the protection of the hepatic lesion after alloxan treatment (Figure 1).

The major pathological consequence of free-radical induced membrane lipid peroxidation includes...
increased membrane rigidity, decreased cellular deformability, reduced erythrocyte survival, and lipid fluidity (Kolanjiappan et al., 2002). Hunt et al. (1990) reported that glucose oxidation in the presence of transition metals result in excessive generations of reactive oxygen species, which in turn affect biomembrane structure and function by mediating lipid peroxidation process. The enhanced TBARS and the declined antioxidants observed in the erythrocytes of diabetic rats can therefore be attributed to the increased biomembrane lipid peroxidation process, and thereby contributing to alterations in antioxidants status. Previous investigations revealed that various herbal preparations could stabilize the red blood cells membrane and exert their anti-inflammatory activity (Sadique et al., 1989).

Since the membrane of the red blood cell is similar to that of lysosomal membranes, the effect of drugs or extracts on human red blood cell membrane could be extrapolated to the stabilization of lysosomal membranes (Oyedapo & Famurewa, 1995; Shinde et al., 1999a,b). The extract of *A. cadamba* significantly protected the erythrocyte hemolysis (Table 6) which could be attributed to its ability to scavenge free-radical and decrease the NO and TBARS production.

These results suggested that the antidiabetic effect of *A. cadamba* flowering tops might be, at least in part, a consequence of increased glucose metabolism, and an increase in serum insulin concentration as a result of protection of the pancreas from free-radical damages induced by alloxan.

**Figure 1.** Typical photomicrograph of liver. A. Control group showing normal architecture of the hepatocyte; B. Diabetes control group having fatty degeneration and ballooning of the hepatocyte; C. Glibenclamide treating group also showing fat accumulation; D. *Antocephalus cadamba* (200 mg/kg) treating group sowing some fatty degeneration but relatively normal architecture; E. *A. cadamba* (400 mg/kg) treating group sowing relatively normal architecture of the hepatocyte in the surrounded acene.
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