In vitro free radical scavenging and anti-hyperglycemic activities of *Achyranthes aspera* extract in alloxan-induced diabetic mice

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ABSTRACT: Medicinal plants have played an important role in the treatment and prevention of diseases since ancient times. They are also potential sources of nutrients and drugs. This study evaluated *Achyranthes aspera* ethanolic extracts for their in vitro antioxidant activity and anti-hyperglycemic effects on alloxan-induced diabetic mice. Diabetes was induced in Swiss albino mice through intra-peritoneal administration of alloxan and their blood glucose levels and weight were measured weekly. At the end of the experiment, all animals were sacrificed and tissue samples were collected. *A. aspera* extracts had potent antioxidant activity compared to reference standard compounds. Treatment with an *A. aspera* extract at doses of 200 mg/kg and 400 mg/kg significantly reduced blood glucose levels in alloxan-induced diabetic mice. *A. aspera* extract also prevented lipid peroxidation as gauged by thiobarbituric acid reactive substances (TBARS) and hydroperoxides. Moreover, *A. aspera* extract increased the activity of catalase and reduced NO levels in alloxan-induced diabetic mice. Results revealed significant anti-hyperglycemic activity of *A. aspera* extracts in alloxan-treated mice that may be mediated by diminished oxidative stress.

Keywords: *Achyranthes aspera*, anti-hyperglycemia, oxidative stress, antioxidant, diabetes

1. Introduction

Hyperglycemia induces glucose auto-oxidation and protein glycation, and subsequent oxidative degradation of glycated proteins leads to enhanced production of reactive oxygen species (1). In diabetes, endogenous antioxidants and scavenging protectors such as vitamin E and glutathione are depleted and antioxidant enzymes such as superoxide dismutase (SOD) and catalase are less active (2). Thus, supplementation with a natural antioxidant from a plant source would help to prevent organ damage. *Achyranthes aspera* (Amaranthaceae) is commonly found as a weed throughout Bangladesh and the Indian subcontinent (3,4). The ethanol extract of the plant contains alkaloids and saponins (5). Various parts of the plant, seeds, stem, leaves, and root are reported to containecdysterone (6,7). Phytochemical investigations of this plant also revealed the presence of long-chain fatty acids, triterpenoids, saponins, and flavonol glycosides (8,9). Upon acidic hydrolysis, it yields an aglycone in the form of oleanolic acid or quercitin (8,9).

Several pharmacological studies also investigated *A. aspera*. *A. aspera* extracts were reported to have thyroid-stimulating and antiperoxidative properties (10). Recent reports suggest that *A. aspera* may benefit wound healing (11) and prevent obesity in mice (12). The aqueous and methanol extracts of the plant also decreased blood glucose levels in normal and alloxan-induced diabetic rabbits (13). *A. aspera* is also used by traditional healers to treat diabetes (3). However, literature on the effect of *A. aspera* extracts on oxidative stress in diabetes is lacking. Previous pharmacological studies encouraged the current authors to explore the therapeutic value of *A. aspera* in the face of oxidative stress. In continuation with phytochemical and pharmacological investigations of Bangladeshi medicinal plants (14-17), the current study reports on the antioxidant and anti-hyperglycemic activities of *A. aspera* extracts in diabetic mice.

2. Materials and Methods

2.1. Reagents

Alloxan, 2,2-diphenyl-1-picrylhydrazyl (DPPH), naphthyl ethylene diamine dihydrochloride, Folin-Ciocalteu reagent, gallic acid, and thiobarbituric acid were purchased from Sigma-Aldrich, St. Louis, MO, USA. All other reagents are of standard laboratory grade.
2.2. Plant material

*A. aspera* was collected from the Bangladesh Agricultural University campus in July 2007 and identified by an expert at the National Herbarium, Mirpur, Dhaka, Bangladesh. Accession No. 32067 was retained there for further reference and a specimen has been preserved in the Pharmacognosy Laboratory, Stamford University Bangladesh.

2.3. Extraction

An extract was yielded by placing a dried powder of stem and leaves (200 g) in 80% ethanol in a Soxhlet apparatus at an elevated temperature. The extract was concentrated by evaporation under reduced pressure at 40°C using a Buchi rotary evaporator to yield a gummy concentrate that was greenish in color.

2.4. Animals used

Male Swiss albino mice, 3-4 weeks of age, weighing between 20-30 g were used for in vivo pharmacological screening. The mice were collected from the Animal Research Branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDR, B). They were housed in five groups in stainless steel cages with dimensions of 28 × 22 × 13 in. Soft wood shavings were used as bedding. The mice were acclimatized to the new environment for one week prior to the investigation and lived at constant room temperature (24.0 ± 1.0°C), humidity 55-65%, and 12 h light/12 h dark cycles. Remaining feed and excreta were removed from cages daily. Rat feed pellets from ICDDR, B were given to the mice with fresh water *ad libitum*. The University’s Animal Research Ethical Committee approved the study protocol.

2.5. In vitro antioxidant activity test

2.5.1. DPPH radical scavenging activity

The free radical scavenging capacity of the extracts was determined using DPPH (14-16,18). A DPPH solution (0.004%, w/v) was prepared in 95% ethanol. *A. aspera* extracts were mixed with ethanol to prepare a stock solution (5 mg/mL). A freshly prepared DPPH solution (0.004%, w/v) was placed in test tubes and *A. aspera* extracts were added followed by serial dilution (1 μg to 500 μg) of every test tube to reach a final volume of 3 mL. After 10 min, absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DU UV–visible spectrophotometer). Ascorbic acid was used as a reference standard and dissolved in distilled water to prepare a stock solution with the same concentration (5 mg/mL). Control samples were prepared with the same volume of distilled water without any extract and reference ascorbic acid. Ninety-five percent ethanol served as the blank. The % scavenging of the DPPH free radical was measured using the following equation:

\[
\text{% Scavenging activity} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100
\]

The inhibition curve was plotted for two experiments and was expressed as the % of the mean inhibition ± standard deviation (SD). IC50 values were obtained by probit analysis.

2.5.2. Reducing power

The reducing power of *A. aspera* was determined by the method previously described by Oyaizu (1986) (19). Different concentrations of *A. aspera* extracts (100 to 1,000 μg) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 mL, 1%). The mixture was then incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl₃ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid served as the standard. Phosphate buffer (pH 6.6) served as the blank solution. The absorbance of the final reaction mixture of two parallel experiments was read and expressed as mean ± standard deviation (SD).

2.5.3. Nitric oxide (NO) radical inhibition assay

NO radical inhibition was estimated using a Griess-Ilosvay reaction (16,20). In this investigation, Griess-Ilosvay reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1%, w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (0.5 mL), and *A. aspera* extract (10 to 320 μg) or standard solution (ascorbic acid, 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 sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min to complete diazotization. Then, 1 mL of naphthyl ethylene diamine dihydrochloride was added. After mixing, the mixture was allowed to stand for 30 min at 25°C. A pink colored chromophore formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions.

2.5.4. Scavenging of hydrogen peroxide (H₂O₂)

The ability of the extracts to scavenge H₂O₂ was
A total of 25 mice (20 diabetic surviving mice, 5 normal mice) were used in the experiment. Group 1 consisted of normal mice. Group 2 consisted of diabetic control mice (alloxan, 150 mg/kg, i.p., in citrate buffer, pH 4.4). Group 3 consisted of diabetic mice given metformin (600 μg/kg body weight) in aqueous solution daily for 3 weeks via intraperitoneal administration. Group 4 consisted of diabetic mice given A. aspera plant extract (200 mg/kg body weight) in aqueous solution daily for 3 weeks via an intragastric tube, and Group 5 consisted of diabetic mice given A. aspera (400 mg/kg, body weight) in aqueous solution daily for 3 weeks via an intragastric tube.

The diabetic state was assessed by determining the blood glucose concentration 3 and 5 days after alloxan treatment. No detectable irritation or restlessness was observed after each drug or vehicle administration. No noticeable adverse effects (i.e., respiratory distress, abnormal locomotion, and catalepsy) were observed in any of the animals after the drug administration.

2.7.2. Blood sample collection

Blood samples were drawn at weekly intervals till the end of study (i.e., 3 weeks) by the tail tip method and used to assay glucose levels in plasma. At the end of the 3rd week, all mice were sacrificed by decapitation after they were anaesthetized with pentobarbitone sodium (60 mg/kg).

2.7.3. Brain and liver sample collection

The brain and liver were completely removed and washed in ice-cold saline to remove blood. The brains were weighed and 10% tissue homogenate was prepared with sodium (60 mg/kg). The diabetic state was assessed by determining the blood glucose concentration 3 and 5 days after alloxan treatment. No detectable irritation or restlessness was observed after each drug or vehicle administration. No noticeable adverse effects (i.e., respiratory distress, abnormal locomotion, and catalepsy) were observed in any of the animals after the drug administration.

2.7.4. Estimation of blood glucose

Glucose was measured in the serum of non-fasting mice. Blood was sampled by the tail tip method and then analyzed with a Reflotron Plus auto analyzer (Roche, Germany) using a commercial kit.

2.7.5. Estimation of lipid peroxidation

Lipid peroxidation in the brain and liver was estimated colorimetrically using TBARS by the method previously described by Niehuis and Samuelsson (23). 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 M HCl, and 15% TCA) and placed in a water bath for 15 min. It was then allowed to cool. The absorbance of the clear supernatant was measured against a reference blank at 535 nm.

2.7.6. Estimation of hydroperoxide

Hydroperoxide levels were estimated by the method previously described by Jiang et al. (24). Specifically,
0.1 mL of tissue homogenate 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 sulphate were added to 90 mL of methanol and 10 mL of 250 mM sulphuric acid) and incubated at 37°C for 30 min. The color that developed was read colorimetrically at 560 nm. Hydroperoxide was expressed as mM/100 g tissue.

2.7.7. Assay of catalase

Catalase activity was assayed colorimetrically at 620 nm and expressed as μmoles of H2O2 consumed/min/mg protein using the method described by Sinha (25). The reaction mixture (1.5 mL, vol) contained 1.0 mL of 0.01 M phosphate buffer (pH 7.0), 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 mixed at a 1:3 ratio).

2.7.8. Assay of NO

NO in the form of nitrate and nitrite was determined according to the method described by Tracy et al. (26). In the current study, Griess-Ilosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1%, w/v) instead of 1-napthylamine (5%). The reaction mixture (3 mL) containing liver homogenate (2 mL) and phosphate buffer saline (0.5 mL) was incubated at 25°C for 150 min. The remaining steps of the NO scavenging assay were as previously described. A pink chromophore formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. The NO level was measured using a standard curve and was expressed as nmol/g of tissue.

2.8. Statistical analysis

All data sets were presented as mean ± SD. Comparison between groups was done by statistical analysis of data sets using one-way analysis of variance (ANOVA) followed by a Newman-Keuls multiple-comparison post hoc test. A p-value of < 0.05 was considered statistically significant. All statistical analyses were performed using Graph Pad Prism version 5.00 for Windows.

### 3. Results

#### 3.1. Antioxidant activity of A. aspera extracts

A. aspera extracts had strong free radical scavenging activity in vitro according to various antioxidant assays. Significant antioxidant activity was evident in terms of NO scavenging (IC50: 39.0 μg/mL) and H2O2 scavenging (IC50: 92.0 μg/mL) (Table 1). However, DPPH scavenging (Table 1) and reducing power assays revealed that the extracts had little to no activity (Figure 1). Total antioxidant capacity also increased with an increase in the concentration of the assay medium (Figure 2).

#### 3.2. Total phenolic content

The total phenolic content in the extracts of A. aspera was determined using Folin-Ciocalteu regents. Phenolic content was calculated based on a regression equation for the calibration curve (y = 0.0162x + 0.0232, R2 =

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Table 1. Scavenging of free radicals by a crude hydroethanolic extract of A. aspera and ascorbic acid according to DPPH, NO, and H2O2 scavenging assays

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH scavenging assay (μg/mL)</th>
<th>NO scavenging assay (μg/mL)</th>
<th>H2O2 scavenging assay (μg/mL)</th>
<th>Total phenolic content (μg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. aspera extract</td>
<td>243.7</td>
<td>39.0</td>
<td>92.0</td>
<td>80.4</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>55.9</td>
<td>69.9</td>
<td>158.9</td>
<td>--</td>
</tr>
</tbody>
</table>

* Data is presented as μg of gallic acid per mg of extract.
and was expressed as gallic acid equivalents (80.4 μg/mg extract) (Table 1).

3.3. Effect of A. aspera on plasma glucose levels in normal mice

The oral glucose tolerance test of non-diabetic mice revealed a dose-dependent decrease in plasma glucose over a period of 3 h after administration of the extract of A. aspera, as summarized in Figure 3. The maximum reduction in glucose levels was noted with both doses in the second hour of the study. Control animals had increased glucose levels in the first hour that returned to normal within three hours. This is attributed to glucose homeostasis.

3.4. Body weight after 3 weeks of A. aspera administration

The body weight of the diabetic group decreased significantly (p < 0.05) compared to that of the normal control. The body weight of diabetic rats treated with A. aspera at a dose of 200 and 400 mg/kg body weight almost returned to normal (Figure 4). Alloxan administration caused significant weight loss after 3 weeks of treatment whereas mice in the normal group continued to gainweight. Treatment with a 200 or 400 mg/kg dose of A. aspera remedied the weight loss.

3.5. Effect of A. aspera extracts on glucose levels in alloxan-treated rats

As shown in Figure 5, a single intraperitoneal injection of alloxan at a dose of 150 mg/kg body weight increased glucose levels > 8 mM after 5 days. A. aspera extracts significantly decreased blood glucose levels. Serum glucose levels in normal mice (Group 1) were unaltered throughout the study but increased significantly (p < 0.05) in the diabetic control group (Group 2) during the second and third week of the study. Glucose levels in the metformin treatment group (Group 3) were almost normal during the study period. Administration of A. aspera extracts at a dose of 400 mg/kg significantly reduced elevated glucose levels in the second week after alloxan administration; in the third week, glucose levels were almost normal. Like the higher dose, a lower dose of A. aspera also reduced blood glucose levels (Figure 5). Findings were similar to those for the metformin treatment group.

3.6. TBARS, catalase, hydroperoxide, and NO levels

The level of malondialdehyde (MDA) as a lipid peroxidation product was gauged by TBARS (42.0 ± 9.3 nmol/g tissue) and hydroperoxides (28.8 ± 3.3 mM/g tissue) in the liver of alloxan-induced diabetic control mice, which are significantly higher (p < 0.05) than those of normal mice (14.0 ± 1.6 nmol/g tissue and 24.1 ± 2.5 mM/g tissue, respectively) (Table 2). TBARS (32.2 ± 2.2 nmol/g tissue) and hydroperoxides (19.4 ± 2.8 mM/g tissue) in the brain also increased in alloxan-induced diabetic mice compared to normal mice (11.8 ± 1.5 nmol/g tissue and 15.1 ± 1.5 mM/g tissue, respectively). Treatment with A. aspera significantly decreased the level of lipid peroxidation products (TBARS and hydroperoxides) (Table 2).

Moreover, a significant decrease (p < 0.05) in the activity of antioxidant enzyme catalase was also observed in the liver of alloxan-induced diabetic mice (22.4 ± 4.4 U/mg of protein) when compared to normal mice (41.0
Table 2. Effect of an A. aspera extract on oxidative markers in brain and liver homogenates from alloxan-induced diabetic mice

<table>
<thead>
<tr>
<th>Group</th>
<th>TBARS (nmol/g tissue)</th>
<th>Hydroperoxide (mM/g of tissue)</th>
<th>Catalase activity (U/mg of protein)</th>
<th>NO (nmol/g of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
<td>Liver</td>
<td>Brain</td>
<td>Liver</td>
</tr>
<tr>
<td>Normal</td>
<td>11.8 ± 1.5a</td>
<td>14.0 ± 1.6a</td>
<td>15.1 ± 1.5b</td>
<td>24.1 ± 2.5b</td>
</tr>
<tr>
<td>Diabetes control</td>
<td>32.2 ± 2.2a</td>
<td>42.0 ± 9.3a</td>
<td>19.4 ± 2.8b</td>
<td>28.8 ± 3.3b</td>
</tr>
<tr>
<td>A. aspera 200 mg/kg</td>
<td>27.1 ± 2.8a</td>
<td>27.1 ± 1.7a</td>
<td>13.1 ± 1.4b</td>
<td>17.6 ± 1.3b</td>
</tr>
<tr>
<td>A. aspera 400 mg/kg</td>
<td>28.5 ± 5.6a</td>
<td>32.3 ± 4.3a</td>
<td>16.9 ± 1.1b</td>
<td>19.5 ± 2.7b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. Differences in means were estimated by means of ANOVA followed by a Newman-Keuls post hoc test (n = 5). Statistical significance was defined as p < 0.05 in all cases, a vs. b, Group normal vs. diabetes control, p < 0.05; b, c Diabetes control vs. treatment, p < 0.05. U = μmol of H2O2 consumed/min.

± 0.6 U/mg of protein) (Table 2). Administration of A. aspera extracts restored catalase activity in the liver (33.9 ± 1.7 and 35.3 ± 0.3 U/mg of protein, respectively, for a dose of 200 and 400 mg/kg body weight).

Furthermore, as shown in Table 2, the NO levels also increased in the liver of alloxan-induced diabetic mice (29.3 ± 1.7 nmol/mL) compared to normal mice (11.5 ± 1.9 nmol/mL). Treatment with A. aspera significantly decreased the NO levels in diabetic mice (22.8 ± 2.4 and 21.8 ± 2.7 nmol/mL, respectively, for a dose of 200 and 400 mg/kg body weight).

4. Discussion

Oxidative stress in diabetes coexists with a reduction in antioxidant status (27). In the current study, A. aspera had potent antioxidant activity in vitro and lowered blood glucose concentrations and prevented oxidative stress in alloxan-treated diabetic mice. A pancreatic β-cell toxin, alloxan is responsible for oxidative damage to the pancreas. A low dose of alloxan (120 mg/kg) causes partial destruction of pancreatic β-cells in laboratory rodents and thereby produces glucose intolerance and hyperglycemia (28).

A. aspera extracts had potent free radical scavenging activity in a dose-dependent manner in vitro according to various assays. Phytochemical screening revealed the presence of flavonoids and phenolic compounds in the extracts. Free radical scavenging activity of plant extracts is dependent on the presence of such polyphenolic compounds (29,30). In the current study, A. aspera extracts had potent inhibitory activity against NO and H2O2 generation. NO and H2O2 are both strong free radicals in biological systems that are readily converted into more reactive peroxynitrite and superoxide anions (31). Previous reports suggest that A. aspera has saponin glycosides with aglycones in the form of betaine, oleancic acid, and quercetin, and these compounds presumably improve diabetes and hyperglycemia (8,9,32). Oleancic acid and quercetin are potent antioxidant compounds (33,34). Quercetin is also effective against diabetes in experimental animals and prevents oxidative stress and β-cell damage in streptozotocin-induced diabetic rats (35,36). The current study found that A. aspera extracts reduced the elevated blood glucose levels in alloxan-treated diabetic mice in a dose-dependent manner. Lowering of blood glucose levels is probably mediated by improvement or protection of the pancreas' structure in alloxan-treated mice (37).

Decreased antioxidant enzyme levels and increased lipid peroxidation were also seen in alloxan-induced diabetic animal (38). Thus, supplementation with antioxidants may have a chemoprotective role in diabetes (39). The reoxidation of dialurate to alloxan by molecular oxygen yields H2O2, which is generally considered to be a cytotoxic agent. The decreased activity of catalase and SOD may be a response to increased H2O2 and O2 production as a result of the autodioxidation of glucose and non-enzymatic glycation (40). Catalase is a hemeprotein that catalyzes the reduction of H2O2 and protects tissue from highly reactive hydroxyl radicals. SOD and catalase activity decrease in the liver and kidneys during diabetes and are implicated in the accumulation of O2− and H2O2 (41). Administration of A. aspera extract increased the activity of catalase and decreased hydroperoxide production by scavenging free radicals because of the presence of phenolic compounds in the extract. Moreover, increased NO production from inducible NO synthase may form peroxynitrite with superoxide and contribute to cellular injury, including lipid peroxidation and nitrosylation of some molecules (42). The positive correlation between NO and TBARS concentrations in diabetic mice may be responsible for the direct or indirect effect of NO on increased lipid peroxidation. In this study, alloxan-treated diabetic mice had the largest amounts of NO and TBARS among the groups. A. aspera extracts had NO scavenging activity in vitro and prevented the production of NO and TBARS in a dose-dependent manner in alloxan-treated diabetic animals.

However, alternate pathways of glucose disposal are also possible with A. aspera administration due to the presence of triterpenoid oleancic acid present in the plant. Oleancic acid enhances insulin secretion in response to a glucose challenge in both INS-1 832/13 cells and rat islets; no increase in cAMP and intracellular Ca2+ ion concentrations has been noted (43). Moreover, oleancic acid inhibits α-glucosidase.
and activates TGR5 G-protein-coupled receptors, which may help to lower glucose levels by increasing insulin sensitivity (44). This triterpenoid molecule also affects glucose absorption in the gastrointestinal tract by suppressing gastric emptying (GE) in rats and it inhibits the Na+/glucose co-transport system at the intestinal brush border membrane (45). Metformin, a biguanide used in the current study as a standard drug, also had hypoglycemic action (46). Metformin acts by decreasing hepatic glucose production and intestinal absorption as well as by increasing peripheral glucose uptake and insulin sensitivity (46). Metformin also increases fatty acid oxidation and decreases absorption of glucose from the gastrointestinal tract (47).

The current study substantiates the use of A. aspera in traditional medicine as a nonspecific hypoglycemic agent. Its hypoglycemic activity is due to its ability to scavenge free radicals and prevent oxidative stress in diabetic mice. Further studies are required to establish the safety of the extract and possibly isolate the active principle responsible for the observed activity of A. aspera extracts.

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References


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