Original Article

DOI: 10.5582/ddt.2012.v6.6.298

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

Fatema Zohura Talukder[1], Kousik Ahmed Khan[1], Riaz Uddin[1], Nusrat Jahan[1], Md. Ashraful Alam[1,2,*]

[1] *Pharmacognosy and Pharmacology Laboratory, Department of Pharmacy, Stamford University Bangladesh, Dhaka, Bangladesh;*
[2] *School of Biomedical Sciences, The University of Queensland, Brisbane, QLD, Australia.*

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 contain ecdysterone (*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.

**Address correspondence to:*
Md. Ashraful Alam, Lecturer (On study leave), Department of Pharmacy, Stamford University Bangladesh, 51 Siddeshwari Road, Dhaka, Bangladesh.
E-mail: sonaliagun@yahoo.com

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 ± 1.0ºC), 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:

% Scavenging activity =100 × (Absorbance of the control – Absorbance of the test sample)/Absorbance of the control

The inhibition curve was plotted for two experiments and was expressed as the % of the mean inhibition ± standard deviation (SD). IC_{50} 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 $[K_3Fe(CN)_6]$ (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 $FeCl_3$ (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-Illosvoy reaction (*16,20*). In this investigation, Griess-Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1%, w/v) instead of 1-napthylamine (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 (H_2O_2)*

The ability of the extracts to scavenge H_2O_2 was

determined by the method described by Ruch *et al*. (*21*). H_2O_2 (43 mM) was prepared in phosphate buffered saline (pH 7.4). Standard (ascorbic acid) and extract solutions were prepared at concentrations of 50 to 250 mM. Aliquots of standard or extract solutions (3.4 mL) were added to 0.6 mL of H_2O_2 solution. The reaction mixture was incubated at room temperature for 10 min, and the absorbance was determined at 230 nm. The percentage of scavenging was calculated as follows: % H_2O_2 scavenging = 100 × (Absorbance of control − absorbance of sample)/Absorbance of control.

2.5.5. *Assay for total phenolic content*

The concentration of total phenols in extracts was measured with a UV spectrophotometer based on a colorimetric oxidation/reduction reaction (*22*). The oxidizing reagent used was Folin-Ciocalteu reagent. Gallic acid served as the standard. Two-point-five mL of Folin-Ciocalteu reagent (diluted 10 times with water) and 2 mL of Na_2CO_3 (75 g/L) were added to 0.5 mL of diluted extract (1 mg in 4 mL distilled water). The sample was incubated for 20 min at room temperature. A control sample was prepared with 0.5 mL of distilled water. The absorbance was measured at 760 nm. These data were used to estimate the phenolic content based on a standard curve obtained with various concentrations of gallic acid. The results were expressed as µg of gallic acid per mg of extract.

2.6. *Glucose tolerance test*

Animals fasted overnight and were then divided into three groups with five mice each. Control animals were given 1 mL of distilled water orally (Group I). *A. aspera* extracts were administered orally using a feeding syringe at concentrations of 100 and 200 mg/kg (Groups II and III, respectively). After the *A. aspera extract* administration, all groups were given glucose (2 g/kg) orally. Blood samples were collected from the tail vein just prior to and 60, 120, and 240 min after the glucose challenge. Blood glucose concentrations were assayed with a glucometer. Results of the glucose tolerance test served as a hypothetical reference to extrapolate the dose levels that would be used to evaluate short- and long-term effects of *A. aspera* extracts on diabetic mice.

2.7. *Experimental design of a model of alloxan-induced diabetes*

2.7.1. *Animal treatment*

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 were 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 0.025 M Tris-HCl buffer, pH 7.5. After centrifugation at 8,000 rpm for 15 min, the supernatant was used to measure thiobarbituric acid reactive substances (TBARS), hydroperoxides, NO levels, and catalase activity.

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 Niehius 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,

Table 1. Scavenging of free radicals by a crude hydroethanolic extract of *A. aspera* and ascorbic acid according to DPPH, NO, and H_2O_2 scavenging assays

Sample	IC_{50} (µg/mL)			Total phenolic content (µg/mg)[a]
	DPPH scavenging assay	NO scavenging assay	H_2O_2 scavenging assay	
A. aspera extract	243.7	39.0	92.0	80.4
Ascorbic acid	55.9	69.9	158.9	--

[a] Data is presented as µg of gallic acid per mg of extract.

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 H_2O_2 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 H_2O_2. 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-Illosvoy 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.

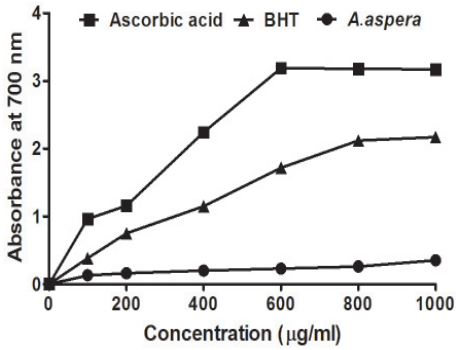

Figure 1. Reducing power of ascorbic acid, BHT, and an extract of *A. aspera*. Values are given for duplicate experiments.

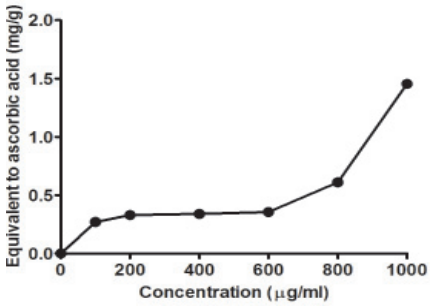

Figure 2. Total antioxidant capacity of an extract of *A. aspera*. Values are given for two consecutive experiments.

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 (IC_{50}: 39.0 µg/mL) and H_2O_2 scavenging (IC_{50}: 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 ($y = 0.0162x + 0.0232$, R^2 =

0.9985) 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 gain weight. Treatment with a 200 or 400 mg/kg dose of *A. aspera* remedied the weight loss.

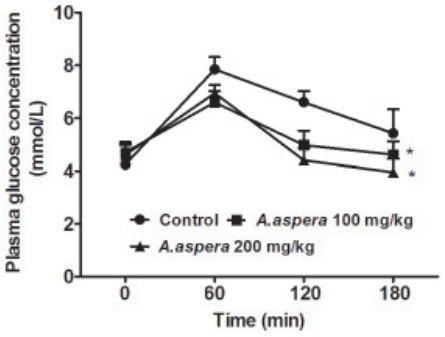

Figure 3. Glucose tolerance test of non-diabetic mice with an extract of *A. aspera*. Values are expressed as mean ± SD. *Statistical significance was defined as $p < 0.05$ in all cases *vs.* control.

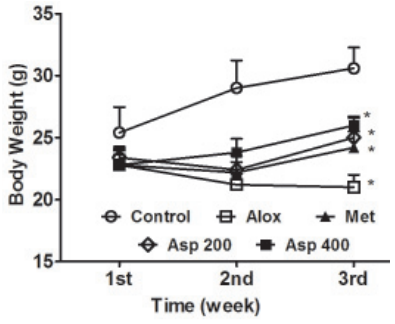

Figure 4. Effect of the hydroalcoholic extract of *A. aspera* on the body weight of mice. Values are expressed as mean ± SD. * Statistical significance was defined as $p < 0.05$ in all cases *vs.* control. Alox, alloxan; Met, metformin; Asp 200, *A. aspera* 200 mg/kg; Asp 400, *A. aspera* 400 mg/kg.

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

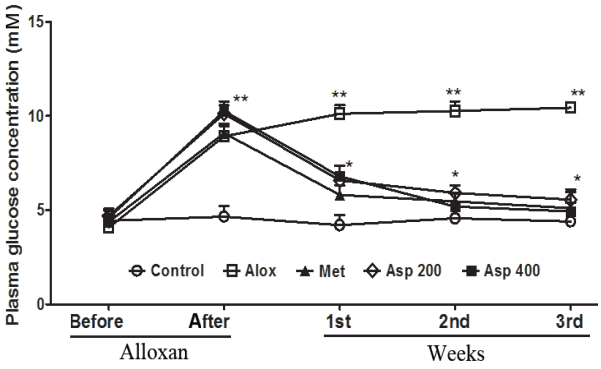

Figure 5. Effect of the hydroethanolic extract of *A. aspera* on blood glucose levels in plasma. Values are expressed as mean ± SD. Statistical significance was defined as $p < 0.05$ in all cases *vs.* control. Alox, alloxan; Met, metformin; Asp 200, *A. aspera* 200 mg/kg; Asp 400, *A. aspera* 400 mg/kg.

Table 2. Effect of an *A. aspera* extract on oxidative markers in brain and liver homogenates from alloxan-induced diabetic mice

Group	TBARS (nmol/g tissue)		Hydroperoxide (mM/g of tissue)		Catalase activity (U^b/mg of protein)	NO (nmol/g of tissue)
	Brain	Liver	Brain	Liver	Liver	Liver
Normal	11.8 ± 1.5^a	14.0 ± 1.6^a	15.1 ± 1.5^a	24.1 ± 2.5^a	41.0 ± 0.6^a	11.5 ± 1.9^a
Diabetes control	32.2 ± 2.2^b	42.0 ± 9.3^b	19.4 ± 2.8^b	28.8 ± 3.3^b	22.4 ± 4.4^b	29.3 ± 1.7^b
Metformin	27.1 ± 2.8^c	27.1 ± 1.7^c	13.1 ± 1.4^c	17.6 ± 1.3^c	31.9 ± 3.9^c	18.3 ± 5.5^c
A. aspera 200 mg/kg	28.5 ± 5.6^c	32.3 ± 4.3^c	16.9 ± 1.1^c	19.5 ± 2.7^c	33.9 ± 1.7^c	22.8 ± 2.4^c
A. aspera 400 mg/kg	21.2 ± 3.1^c	32.0 ± 1.4^c	13.2 ± 1.6^c	9.6 ± 1.2^c	35.3 ± 0.3^c	21.8 ± 2.7^c

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, b] Group normal *vs.* diabetes control, $p < 0.05$; [b, c] Diabetes control *vs.* treatment, $p < 0.05$. U^b = µmol of H_2O_2 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 H_2O_2 generation. NO and H_2O_2 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, oleanolic acid, and quercitin, and these compounds presumably improve diabetes and hyperglycemia (*8,9,32*). Oleanolic 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 H_2O_2, which is generally considered to be a cytotoxic agent. The decreased activity of catalase and SOD may be a response to increased H_2O_2 and O_2^- production as a result of the autoxidation of glucose and non-enzymatic glycation (*40*). Catalase is a hemeprotein that catalyzes the reduction of H_2O_2 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 O_2^- and H_2O_2 (*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 oleanolic acid present in the plant. Oleanolic 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 Ca^{2+} ion concentrations has been noted (*43*). Moreover, oleanolic 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.

Acknowledgement

The authors wish to thank the administration of Stamford University Bangladesh for help with funding and logistics.

References

1. Limaye PV, Raghuram N, Sivakami S. Oxidative stress and gene expression of antioxidant enzymes in the renal cortex of streptozotocin induced diabetic rats. Mol Cell Biochem. 2003, 243:147-152.
2. Watanabe J, Umeda F, Wakasugi H, Ibayashi H. Effect of vitamin E on platelet aggregation in diabetes mellitus. Tohoku J Exp Med. 1984; 143:161-169.
3. Ghani A. Medicinal Plants of Bangladesh with Chemical Constituents and Uses. The Asiatic Society of Bangladesh. 2nd ed., Dhaka, Bangladesh, 2003; pp. 71-72.
4. Datta RM, Mitra JN. Common plants in and around Dacca. Bull Bot Soc Beng. 1953; 7:8-10.
5. Kumar S, Singh JP, Kumar S. Phytochemical screening of some plants of Manipur-I. J Econ Bot Phytochem. 1990; 1:13-16.
6. Banerji A, Chintalwar GJ, Joshi NK, Chadha MS. Isolation of ecdysterone from Indian plants. Phytochemistry. 1971; 10:2225-2226.
7. Banerji A, Chadha MS. Insect moulting hormone from *Achyranthes aspera*. Phytochemistry. 1970; 9:1671.
8. Michl G, Abebe D, Bucar F, Debella A, Kunert O, Schmid MG, Mulatu E, Haslinger E. New Triterpenoid Saponins from *Achyranthes aspera* Linn. Helvetica Chimica Acta. 2000; 83:359-363.
9. Kunert O, Haslinger E, Schmid MG, Reiner J, Bucar F, Mulatu E, Abebe D, Debella A. Three saponins, a steroid, and a flavanol glycoside from *Achyranthes aspera*. Monatshefte für Chemie. 2000; 131:195-204.
10. Tahiliani P, Kar A. *Achyranthes aspera* elevates thyroid hormone level and decrease hepatic lipid peroxidation in male rats. J Ethnopharmacol. 2000; 71:527-532.
11. Edwin S, Jarald EE, Deb L, Jain A, Kinger H, Dutt KR, Raj AA. Wound healing and antioxidant activity of *Achyranthes aspera*. Pharm Biol. 2008; 46:824-828.
12. Rani N, Sharma SK, Neeru Vasudeva N. Assessment of antiobesity potential of *Achyranthes aspera* Linn. seed. Evidence-Based Complementary and Alternative Medicine. 2012. doi:10.1155/2012/715912.
13. Akhtar MS, Iqbal J. Evaluation of the hypoglycaemic effect of *Achyranthes aspera*. J Ethnopharmacol. 1991; 31:49-57.
14. Majumder MM, Mazumder MEH, Alam MA, Akter R, Rahman MM, Sarker SD. Antioxidant and membrane stabilizing properties of *Ichnocarpus frutescens*. J Nat Remedies. 2008; 8:209-215.
15. Alam MA, Ghani A, Subhan N, Rahman MM, Haque MS, Majumder MM, Mazumder MEH, Akter RA, Nahar L, Sarker SD. Antioxidant and membrane stabilizing properties of the flowering tops of *Anthocephalus cadamba*. Nat Prod Commun. 2008; 3:65-67.
16. Alam MA, Nayeem MAB, Awal MA, Mostofa M, Alam MS, Subhan N, Rahman MM. Antioxidant and hepatoprotective action of the crude methanolic extract of the flowering top of *Rosa damascene*. Oriental Pharm Exp Med. 2008; 8:164-170.
17. Subhan N, Alam MA, Ahmed F, Awal MA, Nahar L, Sarker SD. *In vitro* antioxidant property of the extract of *Excoecaria agallocha* (Euphorbiaceae). DARU. 2008; 16:149-154.
18. Braca A, Tommasi ND, Bari LD, Pizza C, Politi M, Morelli I. Antioxidant principles from *Bauhinia terapotensis*. J Nat Prod. 2001; 64:892-895.
19. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. Jpn J Nutr. 1986; 44:307-315.
20. Garrat DC. The Quantitative Analysis of Drugs, vol. 3. Chapman and Hall, Japan, 1964; pp. 456-458.
21. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis. 1989; 10:1003-1008.
22. Majhenic L, Skerget M, Knez Z. Antioxidant and antimicrobial activity of guarana seed extracts. Food Chem. 2007; 104:1258-1268.
23. Niehius WG, Samuelsson D. Formation of malondialdehyde from phospholipids arachidonate during microsomal lipid peroxidation. Eur J Biochem. 1968; 6:126-130.
24. Jiang ZY, Hunt JV, Wolff SP. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low-density lipoprotein. Anal Biochem. 1992; 202:384-387.
25. Sinha KA. Colorimetric assay of catalase. Anal Biochem. 1972; 47:389-394.
26. Tracy WR, Tse J, Carter G. Lipopolysaccharide-induced changes in plasma nitrate and nitrite concentration in rats and mice: Pharmacological evaluation of nitric oxide synthase inhibitors. J Pharmacol Exp Ther. 1995; 272:1011-1015.
27. Collier A, Wilson R, Bradley H, Thomson JA, Small M. Free radical activity in Type II Diabetes. Diabetic Med. 1990; 7:27-30.
28. Yadav JP, Saini S, Kalia AN, Dangi AS. Hypoglycemic and hypolipidemic activity of ethanolic extract of *Salvadora oleoides* in normal and alloxan-induced

diabetic rats. Indian J Pharmacol. 2008; 40:23-27.
29. Osawa T. Protective role of dietary polyphenols in oxidative stress. Mech Ageing Dev. 1999; 111:133-139.
30. Fernandez-Panchona MS, Villanoa D, Troncosoa AM, Garcia-Parrilla MC. Antioxidant activity of phenolic compounds: From *in vitro* results to *in vivo* evidence. Crit Rev Food Sci Nutr. 2008; 48:649-671.
31. Dröge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002; 82:47-95.
32. Blunden G, Yang M, Janicsak G, Mathe I, Carabot-Cuervo A. Betaine distribution in the Amaranthaceae. Biochem Syst Ecol. 1999; 27:87-92.
33. Liu J, Oleanolic acid and ursolic acid: Research perspectives. J Ethnopharmacol. 2005; 100:92-94.
34. Procházková D, Boušová I, Wilhelmová N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia. 2011; 82:513-523.
35. Mahesh T and Menon VP. Quercetin alleviates oxidative stress in streptozotocin-induced diabetic rats. Phytother Res. 2004; 18:123-127.
36. Coskun O, Kanter M, Korkmaz A, Oter S. Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and β-cell damage in rat pancreas. Pharmacol Res. 2005; 51:117-123.
37. Zambare MR, Bhosale UA, Somani RS, Yegnanarayan R, Talpate KA. *Achyranthes aspera* (Agadha): Herb that improves pancreatic function in alloxan induced diabetic rats. Asian J Pharm Biol Res. 2011; 1:99-104.
38. Sepici-Dincel A, Açıkgöz S, Cevik C, Sengelen M, Yesilada E. Effects of *in vivo* antioxidant enzyme activities of myrtle oil in normoglycaemic and alloxan diabetic rabbits. J Ethnopharmacol. 2007; 110:498-503.
39. Logani MK, Davis RE. Lipid peroxidation in biologic effects and antioxidants: A review. Lipids. 1979; 15:485-493.
40. Aragno M, Brignardello E, Tamagno O, Boccuzzi G. Dehydroepiandrosterone administration prevents the oxidative damage induced by acute hyperglycemia in rats. J Endocrinol. 1997; 155:233-240.
41. Searle AJ, Wilson R. Glutathione peroxide effect of superoxide, hydroxyl and bromine free radicals on enzyme activity. Int J Rad Biol. 1980; 37:213-217.
42. O'Donnell VB, Freeman BA. Interactions between nitric oxide and lipid oxidation pathways. Circ Res. 2001; 88:12-21.
43. Teodoro T, Zhang L, Alexander T, Yue J, Vranic M, Volchuk A. Oleanolic acid enhances insulin secretion in pancreatic β-cells. FEBS Lett. 2008; 582:1375-1380.
44. Sato H, Genet C, Strehle A, Thomas C, Lobstein A, Wagner A, Mioskowski C, Auwerx J, Saladin R. Anti-hyperglycemic activity of a TGR5 agonist isolated from *Olea europaea*. Biochem Biophys Res Commun. 2007; 362:793-798.
45. Matsuda H, Li Y, Murakami T, Yamahara J, Yoshikawa M. Structure-related inhibitory activity of oleanolic acid glycosides on gastric emptying in mice. Bioorg Med Chem. 1999; 7:323-327.
46. Kirpichnikov D, McFarlane SI, Sowers JR. Metformin: An update. Ann Intern Med. 2002; 137:25-33.
47. Collier CA, Bruce CR, Smith AC, Lopaschuk G, Dyck DJ. Metformin counters the insulin-induced suppression of fatty acid oxidation and stimulation of triacylglycerol storage in rodent skeletal muscle. Am J Physiol Endocrinol Metab. 2006; 291:E182-E189.

(Received October 6, 2012; Revised December 27, 2012; Accepted December 27, 2012)