

## Effect of the Standardized Extract of *Holarrhena Antidysenterica* Seeds against Streptozotocin-Induced Diabetes in Rats

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### ABSTRACT

*Holarrhena antidysenterica* is found in Himalayan and sub-Himalayan tracts and is used traditionally for a range of health disorders, including colic, diarrhea, dysentery, constipation, flatulence, and urethrosis, as well as being considered functional as carminative, antispasmodic, astringent, anthelmintic, lithotriptic, diuretic, aphrodisiac, tonic, cardiosuppressant, and antihypertensive, antimutagenic, antibacterial and immunomodulatory properties. In this study, the effects of seeds of *H. antidysenterica* on fasting blood glucose levels in streptozotocin induced diabetic rats were examined, together with its effects on the lipid profile *in-vivo*. The oral glucose tolerance test revealed that animals treated with seeds of ethanolic extract of *H. antidysenterica* showed significant reductions in plasma glucose level compared with control group treated with gum acacia. *H. antidysenterica* seeds induced noteworthy reduction in serum glucose level in streptozotocin diabetic rats after 14 and 21 days, reducing the glucose concentration by 39.7 and 48.0%, respectively, when administered at 300 mgkg<sup>-1</sup>. When administered to streptozotocin induced diabetic rats at 300 mgkg<sup>-1</sup> *H. antidysenterica* seeds had strong effects on their lipid profile by significantly decreasing total lipid, triglyceride and cholesterol. This pharmacological activity investigation has confirmed that *H. antidysenterica* seeds confers moderate defense against diabetes *in-vivo*. In addition, the potential of *H. antidysenterica* seeds to reduce triglyceride and total cholesterol levels while increasing high density lipoprotein may contribute to its beneficial effects in diabetic rats.

**Keywords:** Cholesterol, glucose, *Holarrhena antidysenterica*, streptozotocin, triglyceride

Received 24 Feb 2015

Received in revised form 30 March 2015

Accepted 02 April 2015

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### INTRODUCTION

*Holarrhena antidysenterica* (Apocynaceae), commonly known as bitter oleander and locally as "inderjotulkh" or "kurchi", is a tropical Asian found in Himalayan and sub-Himalayan tracts, ascending up to 1200 m [1]. Its seeds are 1-2cm long, linear-oblong, light brown, marked with linear lines and are bitter in taste [1,2]. The plant is used traditionally for a range of health disorders, including colic, diarrhea, dysentery, constipation, flatulence, and urethrosis, as well as being considered functional as carminative, antispasmodic, astringent, anthelmintic, lithotriptic, diuretic,

aphrodisiac, tonic, cardiosuppressant, and antihypertensive [3-5].

*Holarrhena antidysenterica* has been reported to acquire antimutagenic [6], antibacterial [7] and immunomodulatory [8] properties. In this investigation, the special effects of seeds of *H. antidysenterica* on fasting blood glucose levels in streptozotocin induced diabetic rats were examined, together with its effects on the lipid profile *in-vivo*. A diabetic state induced by streptozotocin in rats is a common model for testing antidiabetic plant extracts and diminished glucose levels are a

measure of restoration of insulin-like activity. It is essential to determine the lipid profile since a change in this is an effect of diabetes that leads to numerous of the severe effects of the disease. There is only a single report about the effect of this plant on the level of blood sugar regulation in experimental diabetic rabbit [9]. In our laboratory, results of the lead experiment were extremely encouraging and exposed the hopeful antihyperglycemic property of ethanolic extract of *H. antidysenterica* seeds (HAE) in streptozotocin-induced diabetic rats.

## **MATERIALS AND METHODS**

### **Preparation of Extract and Fractionation**

The seeds of *Holarrhena antidysenterica* (2.0 kg) were collected from a local herbal store, powdered using electric grinder and percolated with petroleum ether for 3 days to remove fatty substances; the marc was further extracted with 50% ethanol for 4 days (3 X 5L). The extract was separated by filtration and concentrated on rotavapour (Buchi R-200 USA) at 40°C and then freeze-dried in lyophilizer (Labconco, USA) to obtain 282.0 g of solid residue (yield 14.10 % w/w).

### **Phytochemical Screening and HPTLC Analysis**

The ethanolic extract of *H. antidysenterica* (HAE) were analyzed for occurrence of alkaloids, steroids and terpenoids, saponins, flavonoids, tannins and phenolic compounds as described by Trease and Evans, 1989 [10] and Harborne, 1993[11]. HPTLC analysis was processed on pre-activated (100°C) Aluchrosep silica gel 60F254 HPTLC plates (S.D.fine-chem Ltd, Mumbai, India) together with quercetin and HPTLC plates were eluted in solvent system toluene : ethyl acetate : formic acid (5:4:1) for phenols. Then After development, the plates were dried and densitometrically scanned at wavelength 366 nm (WinCats software, CAMAG, Switzerland).

### **Animals**

Sprague-Dawley rats (100-150g) of either sex were purchased from the animal house of the National Laboratory Animal Centre, Lucknow, India. They were kept under controlled conditions of temperature 27 ± 2°C and relative humidity 44-56%, light/dark cycles of 12 hours respectively

for one week before and during the experiments. Animals were provided with standard rodent pellet diet (Amrut, India) and the food was withdrawn 18-24 h before the experiment though water was allowed *ad libitum*. All experiments were performed in the morning accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals [12]. The protocols were approved by Institutional Committee for Ethical use of Animals and Review Board (Reg. No. IAEC/15/CPCSEA).

### **Oral toxicity test**

Groups of six rats were given one of a series of doses of *H. antidysenterica* (50, 500, 1000 or 2000 mgkg<sup>-1</sup>, p.o.) and continuously observed for 2 h to detect changes in gross abnormal behaviour and mortality. Any mortality during the experiment and the following seven days were recorded. A group of animals treated with vehicle (gum acacia 2%) served as control [13].

### **Experimental Induction of diabetes**

Rats were rendered diabetic by injecting a freshly prepared aqueous solution of streptozotocin (50 mgkg<sup>-1</sup>, i.p.). Diabetes was confirmed in streptozotocin rats by measuring the fasting blood glucose concentration after 96 h following the administration of streptozotocin. The rats with a blood glucose level above 200 mgdL<sup>-1</sup> were considered to be diabetic and were used experimentally.

### **Oral glucose tolerance test**

Oral glucose tolerance tests [14] were performed in overnight-fasted (18 h) normal rats. Rats were divided into two groups of six rats each (n=6) and these were administered drinking water or *H. antidysenterica* ethanolic extract (HAE) (300 mgkg<sup>-1</sup>) in water orally, respectively. Rats were loaded with glucose (2 gkg<sup>-1</sup>) 30 min after administration of HAE. Blood was withdrawn from the retro orbital sinus under ether inhalation at 0, 30, 60 and 120 min after glucose administration and serum glucose level was estimated by enzymatic GOD-POD method using a glucose diagnostic kit (Qualigens Diagnostics, Mumbai, India), in which glucose is oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. Hydrogen peroxide in

the presence of enzyme peroxidase oxidizes phenol which combines with 4-aminoantipyrine to produce a red-coloured quinoneimine dye. The intensity of the red colour so developed was measured at 505 nm and was directly proportional to the glucose concentration [15].

#### **Experimental design**

After induction of diabetes, the rats were divided into five groups. Group 1 control rats, received vehicle solution (2% gum acacia); group 2 diabetic control, received streptozotocin (50 mgkg<sup>-1</sup>, i.p.); group 3, diabetic rats treated with HAE 100 mgkg<sup>-1</sup> in 2% gum acacia; group 4, diabetic rats treated with HAE 300 mgkg<sup>-1</sup> in 2% gum acacia; group 5, diabetic rats treated with glibenclamide 1mgkg<sup>-1</sup> in aqueous solution. The vehicle and drugs were administered orally using an intragastric tube daily for three weeks. After three weeks of treatment, the rats were fasted overnight and blood samples were analysed for serum glucose concentration [15].

#### **Plasma lipid profile**

The serum cholesterol level was estimated by the Wybenga & Pileggi method [16] using a cholesterol diagnostic reagent kit (Span Diagnostics, Surat, India). Cholesterol reacts with a hot solution of ferric perchlorate, ethyl acetate and sulphuric acid (cholesterol reagent) and gives a lavender-coloured complex which is measured at 560 nm. The total lipid was estimated by the Phosphovanillin method using a Total Lipids diagnostic reagent kit (Merck, Mumbai) [17]. Lipids formed a coloured complex when treated with Phosphovanillin in sulphuric acid solution, and the absorbance at 520 nm was proportional to the amounts of total lipids present. Triglyceride was estimated by the glycerol phosphate oxidase method using Triglyceride kit (Qualigens Diagnostics, Mumbai) [18]. Triglycerides in the sample were hydrolysed by microbial lipases to glycerol and free fatty acids (FFA). Glycerol was phosphorylated by adenosine 5-triphosphate (ATP) to glycerol-3-phosphate (G-3-P) in a reaction catalysed by the enzyme glycerol-kinase (GK). G-3-P was oxidized to dihydroxyacetone phosphate (DAP) in a reaction catalysed by the enzyme glycerol phosphate oxidase (GPO). In this

reaction H<sub>2</sub>O<sub>2</sub> was produced in equimolar concentration to the level of triglycerides present in the sample. H<sub>2</sub>O<sub>2</sub> reacted with 4-aminoantipyrine (4-AAP) and 4-chlorophenol in a reaction catalysed by peroxidase (POD). The result of this oxidative coupling was a quinonimine red-coloured dye. The absorbance of this dye in solution was proportional to the concentration of triglycerides in the sample.

#### **Statistical analysis**

The statistical analysis of all the pharmacological studies was carried out using Graph pad prism. The *in-vivo* data were presented as mean ± s.e.m. for six rats and as described in the figure legends for *in-vitro* experiments. Differences between treatments were assessed using analysis of variance, followed by Bonferroni's test for multiple comparisons. Differences were considered significant when  $P < 0.05$ .

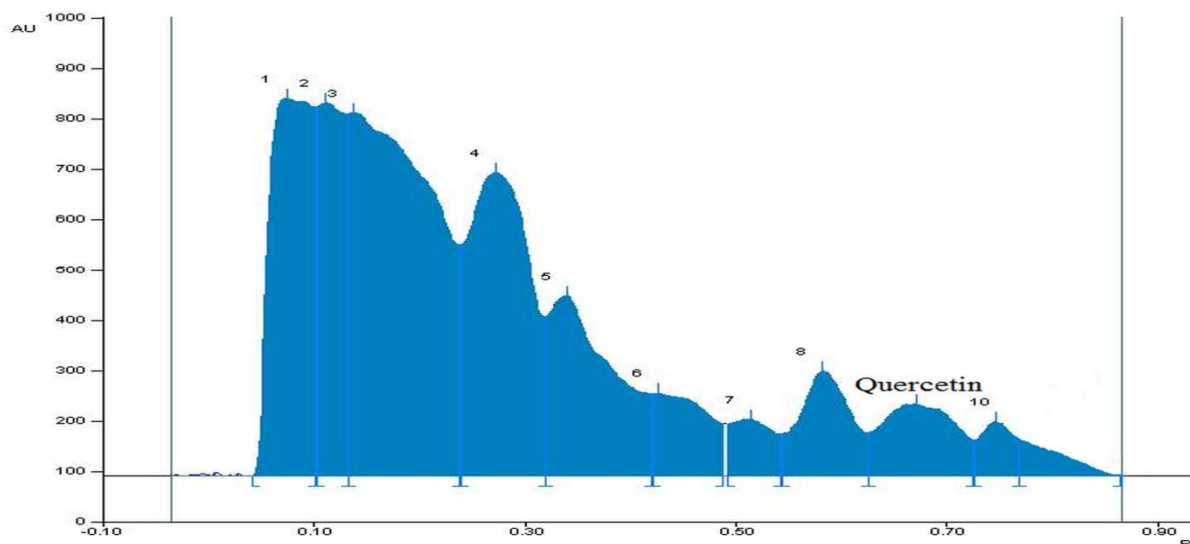
#### **RESULTS AND DISCUSSION**

Phytochemical results showed the presence of alkaloids, carbohydrates, flavonoids, tannins and phenolic compounds. Quantitative HPTLC determination showed the presence of 0.17982% w/w of quercetin (a flavonoid) in ethanolic extract of *H. antidysenterica* seeds (HAE) (**Figure 1**).

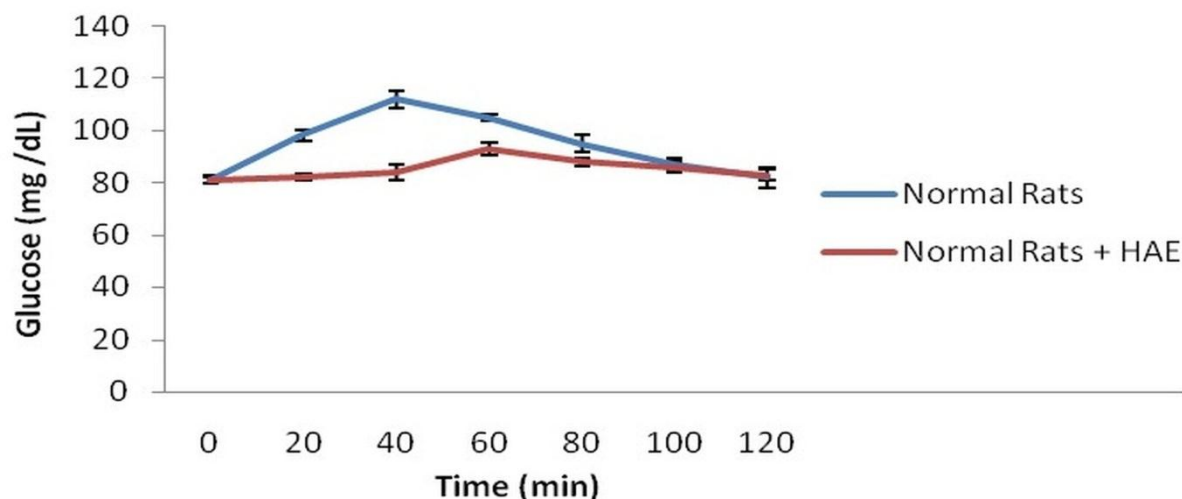
Acute *in-vivo* toxicity studies revealed the non-toxic nature of *H. antidysenterica* seeds. There were no mortality or any toxic observations found at the doses selected up to the end of the study period.

The oral glucose tolerance test revealed that animals treated with seeds of ethanolic extract of *H. antidysenterica* (HAE) showed significant reductions in plasma glucose level compared with control group treated with gum acacia (**Figure 2**).

Streptozotocin has been commonly used for inducing type I diabetes in various animals by promoting degeneration and necrosis of pancreatic  $\beta$ -cells [19]. Diabetes induced by streptozotocin in rats was established by the existence of high fasting plasma glucose levels (**Table 1**, Diabetic Control). *H. antidysenterica* induced noteworthy reduction in serum glucose level in streptozotocin diabetic rats ( $P < 0.001$ ) after 14 and 21 days, reducing the glucose concentration by 39.7 and 48.0%, respectively, when administered at 300 mgkg<sup>-1</sup> (**Table 1**).



**Figure 1: HPTLC finger print profile of ethanolic extract of *H. antidysenterica* seeds (HAE)**



**Figure 2: Effect of the ethanolic extract of *H. antidysenterica* (HAE) on oral glucose tolerance test in rats. Rats were loaded with glucose ( $2 \text{ g kg}^{-1}$ ) 30 min after administration of the extracts. Blood was withdrawn from the retro orbital sinus under ether inhalation at 0, 30, 60 and 120 min after glucose administration and serum glucose level was estimated**

The speedy onset of the glucose-lowering effect of *H. antidysenterica* in diabetic rats was unlikely to be related to  $\beta$ -cell neogenesis. However, it was expected that not all  $\beta$ -cells were damaged by the single streptozotocin dose of  $50 \text{ mg kg}^{-1}$  used in these experiments, since glibenclamide, a sulphonylurea that stimulates insulin secretion by acting at  $\beta$ -cell ATP-sensitive  $\text{K}^+$  channels, restored blood glucose levels to the normal range in streptozotocin-diabetic rats (Table 1). Thus, *H. antidysenterica* may also have exerted its glucose lowering effects by directly stimulating insulin secretion from  $\beta$ -cells

that had not been destroyed by streptozotocin treatment. It is possible that, in the *in-vivo* experiments, residual  $\beta$ -cells following streptozotocin-induced diabetes might be stimulated to secrete insulin, and so lower the level of fasting blood glucose. In patients with severe hypertriglyceridaemia, especially where diabetes is accompanied by genetic hyperlipidaemia, therapy with lipid lowering drug is required. When administered to streptozotocin induced diabetic rats at  $300 \text{ mg kg}^{-1}$  *H. antidysenterica* had strong effects on their lipid profile by significantly ( $P < 0.001$ ) decreasing total lipid, triglyceride and cholesterol (Table 2).

**Table 1: Effects of the ethanolic extract of *H. antidysenterica* (HAE) on serum glucose levels in streptozotocin induced diabetic rats**

Groups	Blood glucose (mm)			
	Day 0	Day 7	Day 14	Day 21
Normal Control	04.56±0.56	04.37±0.68	04.48±0.34	04.25±0.53
Diabetic Control	13.89±1.23	12.58±1.57	12.34±0.51	11.99±0.21
HAE 100	13.31±0.12	11.57±0.16 <sup>a</sup>	09.76±0.19 <sup>b</sup>	07.87±0.18 <sup>b</sup>
HAE 300	14.45±0.08	09.45±0.14 <sup>b</sup>	07.44±0.18 <sup>c</sup>	06.23±0.13 <sup>c</sup>
Glibenclamide	14.52±0.13	08.41±0.14 <sup>b</sup>	05.04±0.06 <sup>b</sup>	05.95±0.09 <sup>c</sup>

The values of blood glucose in the table represent the means ± s.e.m. for six rats per group upon treatment with normal saline, HAE and glibenclamide. *P* values were calculated based on the paired-*t*-test. <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 and <sup>c</sup>*P*<0.001 compared with diabetic control group.

**Table 2: Effect of the ethanolic extract of *H. antidysenterica* (HAE) on the level of serum total lipids, triglycerides and cholesterol in streptozotocin-induced diabetic rats**

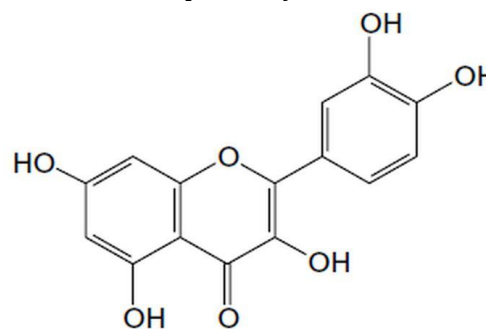
Group	Total lipids (mgdL <sup>-1</sup> )	Triglycerides (mgdL <sup>-1</sup> )	Cholesterol (mgdL <sup>-1</sup> )
Normal Control	082.34±1.73	70.93±1.48	72.29±2.47
Diabetic Control	139.41±0.41	117.43±1.23	160.61±2.87
HAE 100	110.72±2.47 <sup>c</sup>	91.64±1.81 <sup>c</sup>	99.83±2.77 <sup>c</sup>
HAE 300	089.73±2.51 <sup>c</sup>	69.63±1.46 <sup>c</sup>	71.93±2.11 <sup>c</sup>
Glibenclamide	090.28±1.39 <sup>c</sup>	74.56±1.86 <sup>c</sup>	81.81±0.59 <sup>c</sup>

The values of lipid profile in the table represent the means ± s. e. m. for six rats per group upon treatment with normal saline, HAE and Glibenclamide. *P* values were calculated based on the paired-*t*-test. <sup>c</sup>*P*<0.001 compared with diabetic control group.

Insulin resistance in the obesity is apparent before the development of chronic hyperglycaemia. Therefore, at the prediabetic stage, it is improbable that insulin resistance is results from oxidative stress triggered by hyperglycaemia itself. However, the strong involvement of obesity and insulin resistance suggests that a chief mediator of oxidative stress mediated at the prediabetic stage might be a circulating factor secreted by adipocytes. There are informations that hypercholesterolaemia in streptozotocin-induced diabetes in rat results from increased intestinal absorption and cholesterol synthesis. Lipoproteins from diabetic rats are oxidized and demonstrate cytotoxicity, a characteristic which can be prevented by insulin or antioxidant treatment [20].

Phytochemical and HPTLC analysis of the ethanolic extract of *H. antidysenterica* was carried out to standardize the extract, using quercetin as marker component. The concentrations of quercetin in the plant were found to be 0.17982% (w/w), respectively. Quercetin has been reported to prevent and protect against streptozotocin-induced oxidative stress and

$\beta$ -cell damage in rat pancreas [21]. Thus, the significant antidiabetic and lipid lowering activity of *H. antidysenterica* may be attributed partially to the presence of quercetin, although other compounds, as yet unidentified, probably also contribute.

**Figure 3: Quercetin**

### CONCLUSION

This pharmacological activity evaluation has confirmed that *H. antidysenterica* confers moderate defense against diabetes *in-vivo*. In addition, the potential of *H. antidysenterica* to reduce triglyceride and total cholesterol levels while increasing high density lipoprotein may contribute to its beneficial effects in diabetic rats.

**ACKNOWLEDGEMENT**

Authors are thankful to the Director, CSIR-National Botanical Research Institute, Lucknow for providing necessary facilities. One of the authors, SSG is grateful to Department of Science & Technology (DST), Ministry of Science and Technology, New Delhi for providing DST-INSPIRE fellowship. Thanks also due to excellent technical assistance provided by Mr Lalu Prasad, NBRI during the course of study.

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