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In-vivo Assessment of Antihyperglycemic and Antioxidant Activities of *Holarrhena Antidysenterica* Leaves in Alloxan-Induced Diabetic Rats

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ABSTRACT

In past there have been many medicinal plants, which have been used in traditional medicines for their antidiabetic properties without any scientific support and pharmacological evidence. The present study was undertaken to evaluate the antihyperglycemic activity of the crude extracts of leaves of *Holarrhena antidysenterica*. The pet ether, chloroform and ethanolic extracts have been subjected to estimate the anti-hyperglycemic activity in alloxan-induced diabetic rats. Blood glucose levels were measured using the commercially available glucometer. Glibenclamide was used as a reference drug at a dose of 0.6 mg/kg. The antioxidant activity of the test samples was studied in the liver tissue of diabetic rats by measuring catalase and lipid peroxidation levels. The results showed that ethanolic extract possessed a significant antihyperglycaemic and antioxidant activity equipotent with the reference drug (glibenclamide), when evaluated in diabetic rats.

Keywords: *Holarrhena antidysenterica*, Antidiabetic, Antioxidant, Glibenclamide, Alloxan diabetic rats

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INTRODUCTION

Medicinal plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments. The demand for plant based medicines, health products, pharmaceuticals, food supplement, cosmetics etc are increasing in both developing and developed countries, due to the growing recognition that the natural products are non-toxic, have less side effects and easily available at affordable prices[1].

Diabetes is an important human ailment afflicting many from various walks of life in different countries. Diabetes mellitus is the third leading cause of death (after heart disease and cancer) in many developed countries[2].

The therapeutic measurements include use of insulin and other agents like amylin analogues, alpha glycosidase inhibitors like acarbose, miglitol and voglibiose, sulphonylureas, biguanides for the treatment of hyperglycemia. These drugs also have certain adverse effects like causing hypoglycemia at higher doses, liver problems, lactic acidosis and diarrhoea[3].

Thus even though there are various approaches to reduce the ill effects of diabetes and its secondary complications, herbal formulations are preferred due to lesser side effects and low cost[4]. Therefore, investigation on such agents from traditional medicinal plants has become more important[5].

Numerous studies have been demonstrated that oxidative stress, mediated mainly by hyperglycaemia-induced generation of free radicals, contributes to the development and progression of diabetes and its complications[6]. Pancreatic β-cells are particularly susceptible to the deleterious effects of reactive oxygen species (ROS), because of their low expression of the antioxidant enzymes genes as compared to other tissues[7]. In view of this, the present investigation is focused on the leaf part of the plant *Holarrhena antidysenterica* for its unexplored anti hyperglycemic action.

Holarrhena antidysenterica belongs to the family Apocyanaceae[8-13] and is distributed throughout the tropical and subtropical regions of the world. It is a small deciduous tree with white flowers and found throughout the dry forests of India even as far as Travancore. In Indian traditional medicine, the plant has been considered a popular remedy for the treatment of dysentery, diarrhoea, intestinal worms and the seeds of this plant are also used as an antidiabetic drug in Asian countries[14,15]. The efficacy of *H. antidysenterica* leaves in the modulation of oxidative stress associated with Diabetes Mellitus in experimental animals is not discussed in detail in previous literature. Hence, the current study was undertaken to investigate possible anti

hyperglycaemic and the *in vivo* antioxidant effects of crude extracts of *Holarrhena antidysenterica* leaves in alloxan–induced diabetic rats.

MATERIALS AND METHODS

Plant material

The fresh leaves of *Holarrhena antidysenterica* were collected from surrounding places of Shivamogga, Karnataka. The botanical identity was confirmed using the standard herbaria maintained at Bangalore having the voucher specimen number No-biomed/V.U/H.A/16/08. The plant was dried under shade and the dried leaves of the plant were grounded with a blender. The powdered part was kept in nylon bags in a deep freezer until the time of use.

Preparation of plant extract and preliminary phytochemical analysis

The powdered leaves were subjected to soxhlet extraction using petroleum ether (60- 80^0C), chloroform and ethanol in successive mode respectively for 48 hours. The solvent was then recovered using Rotary Vacuum Evaporator and the concentrated extract was preserved in an airtight bottle. The crude extracts thus obtained were subjected to different tests for the identification of phytochemical constituents[16].

Animals

Adult Wistar albino rats of either sex, weighing 150-200 g were procured from National College of Pharmacy, Shivamogga, Karnataka. The animals were housed in polypropylene cages in standard environmental conditions of temperature (21±2^0C), humidity (55±10%) and a 12-hour light-dark cycle. They were supplied with commercial diet and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee of National College of Pharmacy, Shivamogga (Registration No. 144/1999/CPCSEA/ dtd: 10/04/2000).

Chemicals

The different chemicals used during the study were alloxan monohydrate (Sigma Aldrich Pvt Limited, Bangalore) and glibenclamide (Cipla Pharma Limited, Bangalore). Glucometer with Blood gluco-strips (Roche Diagnostics India Private Limited, Mumbai) and all other reagents used were of analytical grade.

Induction of diabetes by Alloxan

Rats were fasted for 16 hours and were made diabetic by a single intraperitoneal injection of 150 mg/kg body weight alloxan monohydrate (5% (w/v)) in normal saline. The fasting blood glucose levels were checked after 2 days. The rats with blood glucose level above 250 mg/dl were considered to be diabetic and were used for the acute and sub-acute efficacy studies.

Acute effect of the *Holarrhena antidysenterica* extract in alloxan-induced diabetic rats

The healthy and diabetic rats were divided into five groups of six animals each. The healthy rats of Group I received only vehicle (Tween 80 in distilled water, 10% (v/v)) orally in a volume of 10 ml/kg. Group I served as control group. The remaining four groups consisted of the diabetic animals. Group II received glibenclamide as reference drug (0.6 mg/kg, p.o.) suspended in vehicle (10 ml/kg). Group II was the standard group. The *Holarrhena antidysenterica* extracts, suspended in vehicle, was administered at the doses of 250 mg/kg of pet ether and chloroform and 200 mg/kg of ethanol extracts orally in a volume of 10 ml/kg to the animals of group III, IV and V respectively. The blood samples were collected from the marginal ear vein just prior to and at zero, first, fourth and sixth hours after dosing. The blood glucose levels were determined using a glucometer[17].

Subacute effect of the *Holarrhena antidysenterica* extract in alloxan-induced diabetic rats

In the sub-acute method, the diabetic rats were divided into five groups (I–V) of six animals each. Group I received only vehicle (Tween 80 in distilled water, 10% (v/v)) orally in a volume of 10 ml/kg for seven days and served as diabetic control. Group II received glibenclamide as reference drug (0.6 mg/kg, p.o.) suspended in vehicle (10 ml/kg) for seven days. The *Holarrhena antidysenterica* crude extracts, viz., pet ether, chloroform and ethanol extracts, suspended in vehicle, was administered at the doses of 250 mg/kg, 250 mg/kg and 200 mg/kg orally in a volume of 10 ml/kg for 7 days to the animals of groups III, IV and V respectively. During the study, fasting blood glucose levels were checked on first, third and seventh day after each treatment. The glucose levels were expressed as mg/dl.

In vivo antioxidant study

The in vivo antioxidant activity of the plant extracts were assessed by the estimation of lipid peroxidation and catalase enzyme levels after sacrificing the animals on seventh day of the experiment. The method explained[18] and modified[19] was adopted in the present study to determine lipid peroxidation levels in tissue samples. Rats were sacrificed with an overdose of diethyl ether. The liver tissue was immediately excised and chilled in ice-cold 0.9% NaCl. After washing with 0.9% NaCl, 0.5 g of wet tissue was weighed exactly and homogenized in 4.5 ml of 0.25 M sucrose using a Teflon homogenizer to obtain a 10% suspension. The cytosolic fraction was obtained by a two-step centrifugation first at 1000×*g* for 10 mins and then at 2000×*g* for 30 min at 4^0C. A 0.5 ml homogenate was transferred to a vial and was mixed with 2.5 ml of Thiobarbutyric acid (TBA). Each vial was tightly capped and heated in boiling water bath for 80^0C for 10 mins. After cooling the vials to room temperature, equal volume of tissue and 10%

of 2.5 ml Trichloroacetic acid (TCA) were transferred into a centrifuge tube and centrifuged at 1000×*g* for 10 min. The absorbance of the supernatant fraction was measured at 532 nm (UV-1609, Japan). The experiment was carried out for the control group using the same protocol by replacing the Thiobarbutyric acid (TBA) solution with distilled water to compare the peroxidative effect of alloxan on liver tissue of diabetic rats. Alloxan administration produced elevated level of lipid peroxidation examined by the increased levels of MDA (Malondialdehyde)[20]. The results were expressed as *n* mol/ml. Catalase (CAT) activity was determined in serum using the modified method[21]. CAT activity was expressed as kU/l.

Statistical analysis

Values are presented as mean ± S.E.M. Statistical differences between the treatments and the controls were tested by one-way analysis of variance (ANOVA) followed by the Student-T test. A difference in the mean values of $p < 0.05$ or less was considered to be statistically significant.

RESULTS AND DISCUSSION:

The preliminary phytochemical analysis of *H. antidysenterica* leaves extract exhibited the presence of alkaloids, tannins, glycosides and flavonoids. As these phytoconstituents are known to possess antidiabetic properties, the antihyperglycemic study of the selected plant extracts has been carried out.

In the acute oral toxicity study, the pet ether and chloroform extracts at a dose of 1 g/kg body weight and the ethanol extract at a dose of 0.8 g/kg body weight exhibited no mortality. Also there was no change in the animal behaviour observed. Therefore 0.25 g/kg body weight of pet ether and chloroform extracts whereas 0.2 g/kg body weight of ethanol extract was considered safe and used for further investigation.

In acute effect study of various doses of the *H. antidysenterica* extracts in alloxan-diabetic rats, the 250 mg/kg dose of pet ether extract does not cause significant change in blood glucose levels in alloxan-induced diabetic rats at any time point.

Table 1:Acute study in alloxan-induced diabetic rats

Group No.	Dose (mg/kg)	Mean blood glucose concentration in mg/dl ±standard error			
		0 hour	1 hour	2 hour	6 hour
I	---------	437±22.0	454±26.0	477.50±17.50	520.0±8.00
II	0.6	396±6.0	415±6.0	372±10.0	277±17.50***
III	250	455±8.60	508±18.99	424±20.48	444±16.01
IV	250	450±14.87	530±12.35	418±11.39	316±9.89**
V	200	417±12.5	459±9.25	387±6.32	297±8.25***

n=6 in each group. *, ** and *** indicate significant results $p < 0.05$, $p < 0.01$ and $p < 0.001$ in comparison with control group respectively.

The chloroform and ethanol extracts at 250 mg/kg and 200 mg/kg doses showed a significant reduction in glucose level at 4 hour, while glibenclamide showed more pronounced antidiabetic activity in alloxan-diabetic rats. At 6 hour the magnitude of reduction was much closer to glibenclamide where the blood glucose was significantly reduced Table 1.

In the sub-acute treatment, the three extracts of the *H.antidysenterica* were administered throughout 7 days consecutively. The blood glucose level of each animal was monitored on first, third and seventh day after the administration of the test samples. The observed effect with a dose of 200 mg/kg of the *H.antidysenterica* ethanolic extract was more potent than that of the other extracts. The highest reduction in blood glucose was observed on the seventh day for 200 mg/kg dose of the ethanol extract. This result was highly significant ($P< 0.001$) when compared with diabetic control. Similarly standard drug caused significant fall in glucose level on seventh day in 7 days' treatment Table 2.

Table 2: Subacute study in alloxan-induced diabetic rats

Group No.	Dose (mg/kg)	Mean blood glucose concentration in mg/dl± standard error		
		1 day	3 days	7 days
I	----------	437±19.0	454±22.0	477±16.30
II	0.6	320±16.0	307±11.0	277±9.0***
III	250	440±12.60	422±18.50	407±19.84
IV	250	370±18.78	350±12.53	316±9.0
V	200	387±13.35	338±9.75	297±8.5***

In the study of *in vivo* antioxidant effect, the Malondialdehyde (MDA) levels were increased significantly in the liver tissue of the diabetic control group. However in alloxan-diabetic rats, the *H. antidysenterica* chloroform and ethanolic extract (250 and 200 mg/kg) treatment significantly ($P< 0.001$) inhibited the increase in the MDA level on the third and seventh day of treatment. In contrast the pet ether-treated group did not prevent the augmentation of MDA level during the 7 days treatment. Similar effect was observed in the catalase level of the liver tissue. The group treated with standard drug (Glibenclamide), ethanol and chloroform extract showed great significance by decreasing the catalase levels**.** However the level was seen increased in the pet ether-treated group. The results were compared to the diabetic control Table 3.

Table 3:*In vivo* antioxidant study

Group No.	Dose (mg/kg)	MDA(nmol/gwet wt) ±standard error	Catalase level (µmol/g) ±standard error
I	-----	356±19.0	97.56±2.3
II	0.6	263±16.0	77.42±2.6
III	250	440±12.60	95.21±2.8
IV	250	320±18.78	84.61±2.0
V	200	278±13.35	79.52±3.5

n=6 in each group. *, ** and *** indicate significant results $p < 0.05$, $p < 0.01$ and $p < 0.001$ in comparison with control group respectively.

Diabetes is a metabolic disorder which can be considered as a major cause of high economic loss which can in turn impede the development of nations[22]. Moreover, uncontrolled diabetes leads to many chronic complications such as blindness, heart failure, and renal failure. In order to prevent this alarming health problem, the development of research into new hypoglycaemic and potential antidiabetic agents is of great interest. Numerous studies have been demonstrated that oxidative stress, mediated mainly by hyperglycaemia-induced generation of free radicals, contributes to the development and progression of diabetes and its complications[6,23,24]. Abnormally high levels of free radicals which cause membrane damage due to peroxidation of membrane lipids and protein glycation and the simultaneous decline of antioxidant defence mechanisms leads to cell and tissue damage[7,25]. As a new strategy for alleviating the oxidative damage in diabetes, interest has grown in the usage of natural antioxidants.

Repeated administration of chloroform and ethanolic extracts proved to be very useful and produced remarkable antihyperglycemic activity. In alloxan-induced diabetic rats, due to the elevated levels of lipid peroxidation, examined by the increased levels of MDA (Malondialdehyde), leads to a clear manifestation of excessive formation of free radicals and activation of lipid peroxidation system resulting in tissue damage[26]. The significant decline in the concentration of lipid peroxidation level in the liver tissues of extract-treated rats provide a substantial evidence for possessing effective antioxidant property *in vivo*.

CAT constitutes a mutually supportive team of defense against reactive oxygen species (ROS). CAT is a hemeprotein, localized in the peroxisomes or the microperoxisomes, which catalyses the decomposition of H_2O_2 to water and oxygen and thus protects the cell from oxidative damage produced by H_2O_2[26]. Decline in the activity of this enzyme in alloxan-induced diabetic animals and attainment of near normalcy in the chloroform and ethanol-treated rats indicate oxidative stress elicited by alloxan had been nullified due to the effect of the extract.

CONCLUSION

In our present preclinical study, the *H. antidysenterica* leaves extracts have proved to be antihyperglycemic and antioxidant in action. The remarkable antihyperglycemic effect of the ethanolic extract of the plant was quite competent with the standard drug. The extracts were in crude form hence synergistic action of antihyperglycemic principles of leaves might have been responsible for the effect. Further studies are necessary to elucidate the active phytoconstituents

responsible for antihyperglycemic and *in vivo* antioxidant activity. Isolation and study of active principles are under process.

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