

ANTIHYPERGLYCEMIC, ANTIHYPERLIPIDEMIC AND ANTIOXIDANT ACTIVITIES OF ETHANOLIC EXTRACT OF *HELLEBORUS NIGER* LINN ROOTS IN STREPTOZOTOCIN - NICOTINAMIDE INDUCED DIABETIC RATS

Kishor Kumar V¹, Lalitha KG^{2*}

¹Department of Phytopharmacy and Phytomedicine, J K K Munirajah Medical Research Foundation's Annai J K K Sampoorani Ammal College of Pharmacy, B. Komarapalayam – 638 183, Namakkal District, Tamil Nadu, India.

²Department of Pharmaceutical Chemistry, Ultra College of Pharmacy, Madurai – 625 020, Tamil Nadu, India. Cell: +91 9894893301, +91 9842723368

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ABSTRACT

Helleborus niger L (HN) is widely used in the treatment of diabetes mellitus in the Indian traditional system of medicine. Therefore, the present study was done to evaluate the antihyperglycemic, antihyperlipidemic and antioxidant activities of ethanol extract of HN root (EHN) in streptozotocin (STZ) - nicotinamide (NC) induced diabetic rats. *in vitro* α -amylase inhibition assay, normoglycemic study and oral glucose tolerance test were carried out in different root extracts of HN. Antihyperglycemic effect was assessed in EHN using diabetic rats, which was identified as the most effective extract by initial screening. EHN (50, 100 and 200 mg/kg) and glibenclamide (5 mg/kg) orally administered daily for 28 days and the animal was observed in next 14 days. Fasting blood glucose (FBG) and body weight was determined weekly basis up to 42 days. On the 42nd day, various biochemical parameters were estimated. The three doses of EHN shows significantly decrease ($p < 0.01$) in blood glucose levels. The effect was more pronounced in 200mg/kg (71.53%) than 100mg/kg (67.46%) and 50mg/kg (66.63%). In addition, decreased HbA_{1c} and improved Hb level were evidenced clearly in diabetic rats. Simultaneously, improvements in serum lipid profile, serum liver profile in diabetic rats were also evidenced clearly. Moreover, body weight and protein levels were increased in diabetic rats. On the other hand antioxidant activity was restored in diabetic rats. Increased glycogen content, glucokinase and decreased glucose - 6 phosphatase, fructose 1, 6 - biphosphatase effects in liver tissues were observed. EHN preserved islet architecture and prevented hypertrophy of β -cells. The EHN is capable of managing hyperglycemia and complications of diabetes in STZ-NC induced diabetic rats. Hence this plant may be considered as one of the potential sources for the isolation of new oral antihyperglycemic agent(s).

Keywords: Antihyperglycemic; Antihyperlipidemic; Antioxidant; *Helleborus niger*; Nicotinamide; Streptozotocin.

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by elevated blood glucose level resulting from defects in insulin secretion, insulin action or both. According to a projection of the International Diabetes Federation (IDF), estimates that the numbers of diabetic patients in India were 382 million in 2013. It is projected to increase to 592 million by 2035¹. At present, a number of hypoglycemic agents, including insulin and oral drugs such as sulphonylureas, biguanides and glycosidase inhibitors are popularly used in clinics to keep blood glucose at normal levels. Unexpectedly, these agents produced serious side effects in the clinical application, such as weight gain, gastrointestinal disturbances, edema, hypoglycemia and insulin resistance². Therefore, it is important to discover alternative therapies that may have less or no side effects³. Medicinal plants used by folk medicinal healers are successfully used in many countries to control diabetes, and have become the most important sources for

seeking a safe, specific and effective hypoglycemic agents⁴. Moreover, many hypoglycemic components have been obtained from the medicinal plants, mainly including flavonoids, alkaloids, polysaccharides, saponins, terpenoids and unsaturated fatty acids⁵.

Helleborus niger L (HN) belongs to family Ranunculaceae, commonly called as 'Kadagaruganie' in Tamil. HN has been widely used in the Indian traditional system of medicine for treatment of diabetes mellitus^{6, 7}. A number of pharmacological studies have shown that, the roots possess cytotoxic^{8,9} and immunostimulatory properties¹⁰. Helleborin and veratrin (steroidal saponins), Hellebrin or Helleborein (steroid glycoside), hellebrin, desgluco-hellebrin, hellebrigenin, bufate-traeno-lide, beta-ecdysterone and 5-beta-hydroxyecdysterone are the main constituents of the roots and rhizomes⁶. Interestingly, bioactive chemical constituents reported from the rhizomes are hellebrigenin 3-acetate¹¹ and the leaves are helleborus glycosides¹². A Unani drug QS, containing HN and *Anacyclus pyrethrum* DC, (3:1),

*Correspondence : email: kg.lalitha@gmail.com; kishorkpm2006@gmail.com

reduces the dose of insulin in patients with insulin-dependent diabetes mellitus and also it decrease the plasma glucose level^{6,7}. The plant extract, an anti-inflammatory agent, induces inhibition of the enzyme in the androsterone oxidation and 5- andro-stane -17 β -ol-3-one reduction reaction in rat liver *in vitro*⁷.

Traditionally, several plants as herbal remedies for the treatment of diabetes mellitus, thus making such plants possible sources of hypoglycemic agents¹³. In malevolence of this claim, the plant HN root has the capability to cure diabetes, there is no report of any investigation of the hypoglycemic activity of this root. Therefore, the present study was aimed to evaluate the antihyperglycemic, antihyperlipidemic and antioxidant activities of ethanol extract of HN root (EHN) using streptozotocin - nicotinamide (STZ - NC) induced type 2 diabetic rat model.

MATERIALS AND METHODS

Drugs and chemicals

Streptozotocin (Himedia, Mumbai), Nicotinamide (Himedia, Mumbai), Alpha amylase (Himedia, Mumbai), Glibenclamide (Cipla, Mumbai), Acarbose (Cipla, Mumbai), Petroleum ether, chloroform, ethyl acetate, acetone, ethanol and water used for the extraction. All other chemicals and reagents were of analytical grade and enzymatic kits used in this study were obtained commercially.

Plant material

The HN root was obtained from a local traditional healer in Erode, Tamil Nadu (India). The plant was identified by Prof. P. Jayaraman, Director, National Institute of Herbal Science, Chennai and a voucher specimen (Ref. No: PARC/2012/2177) was stored in the Pharmacognosy Department herbarium, JKKMMRF's - Annai JKK Sampoorani Ammal College of Pharmacy, B. Komarapalayam, Tamil Nadu.

Preparation of extracts

The HN root powder (600gm) was extracted with different solvents using soxhlet apparatus by successive extraction method. The extracts were concentrated to dryness in a rotary evaporator under reduced pressure and controlled temperature (37-40°C) and the percentage yield was calculated. The dried extracts were used for further studies.

Preliminary phytochemical screening

The dried different extracts of HN root were screened for the presence of various phytoconstituents¹⁴.

in vitro α -amylase inhibitor assay

The assay was carried out using the reported method with slight modification¹⁵.

Experimental Animals

Wistar albino rats of both sexes weighing (150 - 200 g) were used for the study¹⁶. All animals were maintained under standard laboratory conditions [temperature (22 $\pm$ 2°C) and humidity (45 $\pm$ 5°C)] with a 12 h day: 12 h night cycle. The animals were fed with normal laboratory diet and allowed to drink water *ad libitum*. All the experimental protocols were approved by the Institutional

Animal Ethics Committee (IAEC) (JKKMMFECF/-IAEC/2012/007) and all the animal experiments were conducted according to the principles and guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experimentation on Animals), India.

Acute Toxicity

Acute oral toxicity study was performed as per organization for economic cooperation and development (OECD) guidelines 425¹⁷.

Experimental design

Initial screening of the HN different extracts of the roots for evaluating its hypoglycemic potential was done with a dose of 100 mg/kg given orally by gavage in normal rats by conducting fasting blood glucose (FBG) and oral glucose tolerance test (OGTT) studies. The antihyperglycemic effect of the EHN was also assessed in diabetic rats, with a range of varying doses of 50, 100 and 200 mg/kg which was identified as the most effective extract by initial screening.

Assessment of hypoglycemic activity in normal healthy rats

Forty eight rats were divided into eight groups of six rats each, and used in the experiment. The Group I served as normal control received distilled water. Group II, III, IV, V, VI and VII were received different root extracts of PEHN, CHN, EAHN, AHN, EHN and WHN at the dose of 100 mg/kg. Group VIII was received standard drug glibenclamide 5mg/kg. FBG was taken initially (0h) and then blood samples were collected from the tail vein at 1-4h after administering the extracts¹⁸.

Assessment of hypoglycemic activity by OGTT in normal healthy rats

A different group of forty eight normal rats was divided and treated on the same pattern as mentioned above. All the animals were given oral administration of glucose (2g/kg) 60 min after dosing. Blood samples were collected from the tail vein just prior to (0h) and at 30min, 60min, 90 min and 120 min after the glucose loading and blood glucose levels were estimated¹⁸.

Assessment of antihyperglycemic activity in STZ-NC diabetic rats

The animal model of type 2 diabetes mellitus (NIDDM) was induced by single intraperitoneal injection of 60mg/kg of STZ, and thereafter 120 mg/kg NC was injected after 15min. Hyperglycemia was confirmed by the elevated blood glucose levels determined at 72 h and then on day 7 of the injection. Only rats confirmed with permanent NIDDM (Glucose level between 250 and 300) were used in the antidiabetic study¹⁹⁻²¹. Long term study of 42 days was conducted in severely diabetic rats. Thirty six rats were divided into six groups of six rats each.

Group I: Normal control + distilled water,
Group II: Diabetic control + distilled water,
Group III: Diabetic + EHN (50mg/kg),
Group IV: Diabetic + EHN (100mg/kg),
Group V: Diabetic + EHN (200mg/kg) and
Group VI: Diabetic + glibenclamide (5mg/kg).

The freshly prepared suspension in 2% gum acacia and

were orally administered via oral gavage daily for 28 days (treatment days) and the animal was observed on the next 14 days (post treatment days). Body weights and blood glucose level analysis were done weekly on overnight fasted animals. At the end of the experimental period, the animals were fasted an overnight and blood was collected for various biochemical estimations. The animals were sacrificed by cervical decapitation. Organs like liver, kidney and pancreas were dissected out, immediately rinsed in ice cold saline and stored for further biochemical estimations²².

Evaluation of biochemical parameters

Serum was analyzed for haemoglobin (Hb), glycosylated haemoglobin (HbA_{1c}), total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), serum alkaline phosphatase (ALP), total proteins, albumin, globulin, a:g ratio, were analyzed. Very low density lipoprotein (VLDL) levels and low density lipoprotein (LDL) were determined by following formula $VLDL = TG/5$; $LDL = TC - [HDL + VLDL]$ ²³.

Evaluation of antioxidant activity

A portion of liver and kidney tissue were homogenized in buffer containing 50mM Mannitol, 2 mM Tris HCl, pH 7 (10%) in a Potter Elvehjem homogenized fitted with a polyteflon plunger at high speed. The homogenate thus obtained was centrifuged at 25000 rpm at 4°C. The supernatant obtained from a homogenate of liver and kidney were used for the estimation of superoxide dismutase (SOD), catalase (CAT), glutathione Peroxidase (GPx)¹⁸, reduced glutathione (GSH)²⁴, glutathione-S-transferase (GST)²⁵, lipid peroxidation (LPO) it includes thiobarbituric acid reactive substance (TBARS) and hydroperoxides (HP)²³.

Evaluation of glucose metabolic enzyme activities in liver tissues

The supernatant obtained from a homogenate of liver was used for the estimation of glucokinase²⁶, glucose-6-phosphatase²⁷, fructose 1, 6- biphosphatase and glycogen levels were analyzed²⁸⁻²⁹.

Histopathology

The dissected pancreas was collected in 10% formalin solution, and immediately processed by the paraffin technique. Sections of 5µ thickness were cut and stained by haematoxylin and eosin for histological examination¹⁸.

Statistical analysis

All data were expressed as mean $\pm$ standard error mean (SEM). Results were analyzed by one-way analysis of variance (ANOVA), and significant differences were determined by Dunnett's post hoc test using Graphpad Instat version 3.06 computer software. Differences between groups were considered significant at $p < 0.05$.

RESULTS

Preliminary phytochemical screening

The extraction yield was found to be 5.88% for petroleum ether (PE), 6.15% for chloroform (C), 7.63% for ethyl acetate (EA), 1.57% for acetone (A), 27.79% for

ethanol (E) and 8.34% water (W). The phytochemical analysis of the different extracts of HN root revealed the presence of carbohydrates, glycosides, alkaloids, phytosteroids, flavonoids, saponins and tannins and phenolic compounds.

In vitro α -amylase inhibitor assay

The *in vitro* α -amylase assay was determined using porcine pancreatic amylase enzyme. The IC₅₀ value of different root extracts of HN is given in Fig. 1. The entire extracts exhibit the α -amylase inhibitor activity was compared with the reference standard acarbose. The IC₅₀ value of the PEHN, CHN, EAHN, AHN, EHN and WHN was found to be 68.06, 75.25, 59.20, 80.48, 29.08 and 45.12 μ g/ml, respectively was compared with acarbose (92.38 μ g/ml).

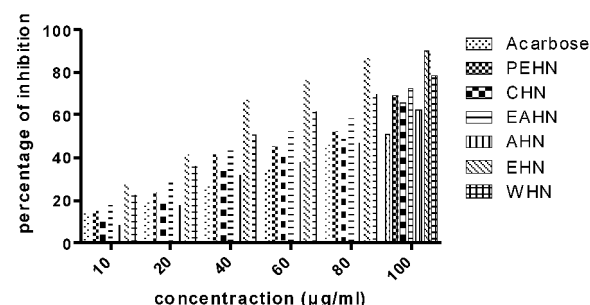


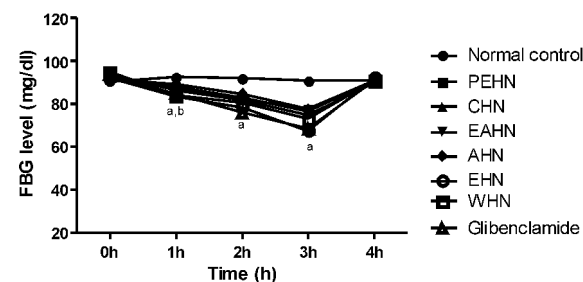
Fig. 1: Effects of different extracts of HN root on α -amylase inhibitory assay

Acute toxicity study

In acute toxicity studies, a single dose of 2000 mg/kg HN root extracts did not indicate modification of behavior. No mortality and signs of toxicity were recorded during 24 h and up to 14 days observation. The oral LD₅₀ value of HN root extracts must be greater than 2000 mg/kg.

Antihyperglycemic activity

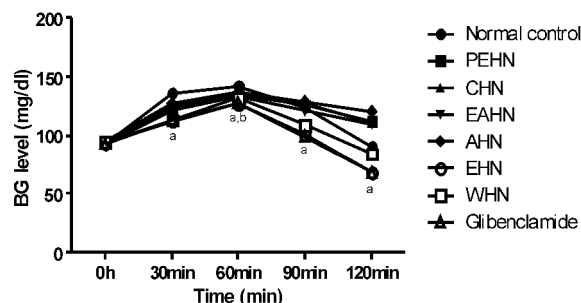
The results of the effect of different root extracts of HN at the dose of 100mg/kg on the fasting blood glucose of normal healthy rats are presented in Fig. 2. In normoglycemic rats, FBG was found to be reduced by PEHN (16.60%), CHN (16.94%), EAHN (19.89%), AHN (15.43%), EHN (26.39%), WHN (22.98%) and glibenclamide (26.73%) at 3h, respectively when compared to rats in the control groups. Blood glucose levels were restored in all treatment groups by 4h.



Data are presented as mean $\pm$ SEM, (n=6). * $p < 0.01$, ^b $p < 0.05$ when compared with corresponding values of the control group.

Fig. 2: Effect of different extracts of HN root on hypoglycemic activity in normal healthy rats.

The results for different root extracts of HN at the dose of 100 mg/kg on the OGTT of healthy rats are presented in Fig. 3. Sixty minutes after glucose load (2g/kg) the blood glucose levels in all groups increased rapidly and gradually decreased thereafter. Interestingly, EHN (32.33%) caused a maximum significant reduction of the rise of blood glucose levels after 60 min when compared to the other extracts likely, PEHN (11.09%), CHN (12.81%), EAHN (9.50%), AHN (6.58%) and WHN (22.63%). From this study, it could be concluded that APE showed the maximum improvement in glucose tolerance test.



Data are presented as mean $\pm$ SEM, (n=6). * $p < 0.01$, * $p < 0.05$ when compared with corresponding values of the control group.

Fig. 3 : Effect of different extracts of HN root on oral glucose tolerance test (OGTT) in normal healthy rats.

The antihyperglycemic effect of repeated oral administration of EHN on fasting blood glucose levels in STZ-NC diabetic rats are presented in Table 1. The administration of different doses of EHN and glibenclamide to STZ-NC treated diabetic rats caused significantly ($P < 0.01$) decline the blood glucose level when compared to normal control rats, which was related to dose and duration of treatment. Maximum reduction was observed on day 42 by 66.63%, 67.46%, 71.53% and 70.21%, respectively. EHN at the dose of 200 mg/kg had the best effect to alleviate the hyperglycemia than the 50, 100 mg/kg.

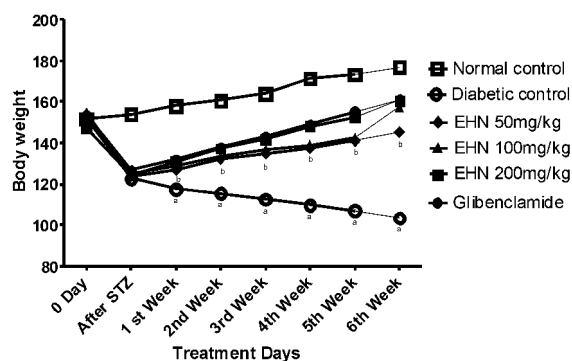
Table 1: Effect of EHN on fasting blood glucose level of control and experimental groups of rats.

Group	Treatment	Dose (mg/kg)	Fasting blood glucose level (mg/dL)							
			0 day	After STZ	1 st week	2 nd week	3 rd week	4 th week	5 th week	6 th Week
I	Normal Control	—	88.50 $\pm$ 2.51	91.00 $\pm$ 0.68	92.83 $\pm$ 0.74	97.00 $\pm$ 1.21	98.16 $\pm$ 0.47	94.66 $\pm$ 1.85	95.50 $\pm$ 2.27	92.33 $\pm$ 3.23
		—	86.50 $\pm$ 4.90	296.33 $\pm$ 4.91	286.66 $\pm$ 7.34*	297.50 $\pm$ 5.86*	315.50 $\pm$ 4.43*	330.50 $\pm$ 3.95*	325.00 $\pm$ 6.09*	304.33 $\pm$ 5.80*
III	EHN	50	95.66 $\pm$ 1.35	311.66 $\pm$ 1.28	267.00 $\pm$ 5.30*	202.66 $\pm$ 2.37*	153.33 $\pm$ 3.60*	102.33 $\pm$ 0.78*	105.66 $\pm$ 1.28*	104.00 $\pm$ 1.93*
IV	EHN	100	93.00 $\pm$ 2.14	305.33 $\pm$ 3.19	222.00 $\pm$ 2.63*	184.00 $\pm$ 3.34*	136.00 $\pm$ 0.73*	98.66 $\pm$ 0.91*	100.00 $\pm$ 0.73*	99.33 $\pm$ 0.55*
V	EHN	200	95.00 $\pm$ 1.52	312.66 $\pm$ 2.43	195.66 $\pm$ 2.92*	155.00 $\pm$ 2.55*	118.33 $\pm$ 1.47*	90.00 $\pm$ 0.73*	92.00 $\pm$ 0.96*	89.00 $\pm$ 0.96*
VI	Glibenclamide	5	92.50 $\pm$ 2.68	303.83 $\pm$ 1.83	150.16 $\pm$ 0.98*	130.83 $\pm$ 1.27*	109.00 $\pm$ 2.75*	89.16 $\pm$ 0.74*	89.83 $\pm$ 1.66*	90.50 $\pm$ 2.93*

Data are presented as mean $\pm$ SEM, (n=6). * $p < 0.01$ when compared to the corresponding values of the normal control. * $p < 0.01$, * $p < 0.05$ when compared to the corresponding values of the diabetic control.

Body weight

STZ-NC produced significant loss in body weight as compared to normal animals during the study. Diabetic control continued to lose weight till the end of the study while different doses of EHN and glibenclamide showed significant improvement ($P < 0.01$) in body weight by 14.91%, 20.54%, 22.61% and 21.32%, respectively compared to diabetic control (Fig. 4).

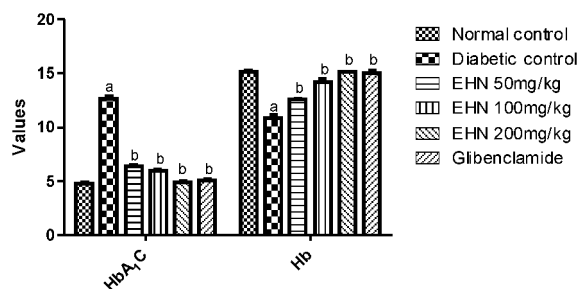


Data are presented as mean $\pm$ SEM, (n=6). * $p < 0.01$ when compared to the corresponding values of the normal control. * $p < 0.01$ when compared to the corresponding values of the diabetic control.

Fig. 4 : Effects of EHN on Body weight of control and experimental groups of rats.

HbA_{1c} and Hb levels

STZ-NC induced diabetic rats showed a significantly ($p < 0.01$) increase in the level of HbA_{1c} and reduction of Hb level when compared with normal control rats. Treatment with different doses of EHN and glibenclamide to STZ-NC treated diabetic rats caused significantly ($p < 0.01$) reduction in HbA_{1c} level by 49.52%, 52.93%, 60.93%, respectively. At the same time, increased in Hb level by 13.78%, 23.64%, 28.53%, respectively when compared with diabetic control rats. The standard drug glibenclamide showed a marked reduction of the HbA_{1c} level by 63.78% and elevation of Hb level by 27.86%, which was similar to the extract treated with 200mg/kg (Fig. 5).



Data are presented as mean $\pm$ SEM, (n=6). * $p < 0.01$ when compared to the corresponding values of the normal control. * $p < 0.01$ when compared to the corresponding values of the diabetic control.

Fig. 5: Effect of EHN on HbA_{1c} and Hb of control and experimental groups of rats.

Antihyperlipidemic activity

The concentrations of serum TC, TG, HDL, LDL and VLDL in control and experimental groups were shown in Table 2. The results showed that the TC, TG, LDL and VLDL concentrations in the serum were significantly increased ($p < 0.01$), whereas the serum HDL level was significantly decreased ($p < 0.01$) in the STZ-NC induced diabetic rats as compared to a normal control group. After the administration of different doses of EHN and glibenclamide, the alteration in lipid metabolism was partially attenuated as evidenced by significant ($p < 0.01$)

reduction in serum TC (31.12%, 38.59%, 48.97% and 48.14%), TG (27.11%, 42.71%, 59.41% and 53.07%), LDL (43.89%, 51.61%, 65.97% and 69.62%) and VLDL (27.12%, 42.40%, 58.94% and 53.08%) levels and by elevation in HDL level (29.90%, 32.52%, 41.75% and 48.20%), respectively when compared with diabetic control rats. Amongst all the doses of EHN 200 mg/kg was more efficient in improvement in the level of lipid parameter as compared to other doses of EHN 50, 100mg/kg and glibenclamide 5mg/kg.

Table 2: Effect of EHN on TC, TRG, HDL, LDL, VLDL, SGOT, SGPT and ALP of control and experimental groups of rats.

Group	Treatment	Dose (mg/kg)	TC (mg/dL)	TG (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)
I	Normal Control	-----	125.66± 2.43	82.83± 1.19	50.66± 1.89	58.43± 2.69	16.56± 0.23	33.66± 0.88	40.50± 0.76	105.66± 2.14
II	Diabetic Control	-----	251.00± 3.55 [*]	211.66± 2.53 [*]	28.83± 1.30 [*]	179.83± 4.17 [*]	42.33± 0.50 [*]	153.83± 3.60 [*]	159.83± 2.61 [*]	288.16± 2.07 [*]
III	EHN	50	172.88± 2.87	154.26± 1.03 [*]	41.13± 0.82 [*]	100.90± 3.90 [*]	30.85± 0.20 [*]	61.80± 0.90 [*]	69.16± 0.82 [*]	146.36± 1.26 [*]
IV	EHN	100	154.13± 2.10 [*]	121.26± 1.00 [*]	42.73± 0.87 [*]	87.01± 2.44 [*]	24.38± 0.20 [*]	47.40± 0.79 [*]	55.53± 0.68 [*]	128.56± 0.82 [*]
V	EHN	200	128.07± 1.51 [*]	85.90± 1.24 [*]	49.50± 0.27 [*]	61.19± 1.10 [*]	17.38± 0.15 [*]	35.96± 1.01 [*]	42.53± 1.15 [*]	107.16± 0.83 [*]
VI	Glibenclamide	5	130.16± 1.74 [*]	99.33± 1.82 [*]	55.66± 0.91 [*]	54.63± 1.52 [*]	19.86± 0.36 [*]	31.83± 0.74 [*]	41.83± 1.07 [*]	107.83± 2.85 [*]

Data are presented as mean ± SEM, (n=6). ^{*}p< 0.01 when compared to the corresponding values of the normal control. ^{*}p<0.01 when compared to the corresponding values of the diabetic control.

SGOT, SGPT and ALP levels

The activity of hepatic marker enzymes like SGOT, SGPT and ALP levels were significantly (p<0.01) increased in the diabetic control group compared to the normal control group. In treatment with different doses of EHN and glibenclamide, there was a significant (p<0.01) reduction of SGOT (59.82%, 69.18%, 76.62% and 79.30%), SGPT (56.72%, 65.25%, 73.39% and 73.82%) and ALP (49.20%, 55.38%, 62.81% and 62.57%), respectively as compared to the diabetic control (Table 2). The maximum lowering of hepatic enzymes like SGOT, SGPT and ALP in STZ- NC induced diabetic rats was appeared in EHN 200 mg/kg dose than 50, 100 mg/kg.

Protein levels

The levels of serum total protein, albumin, globulin and a:g ratio were significantly (p<0.01) reduced in diabetic control rats as compared with normal control rats. Treatment with different doses of EHN and glibenclamide to the diabetic rats, the level of total protein (13.49%, 22.85%, 33.37% and 34.48%), albumin (21.26%, 24.93%, 33.00% and 32.34%), globulin (2.04%, 20.33%, 33.61% and 36.77%) and a:g ratio (19.01%, 5.73%, 2.60% and 6.08%), respectively were found to be restored when compared to diabetic control rats. EHN at the dose of 200 mg/kg was more effective than 50, 100 mg/kg in elevating the protein levels (Table 3).

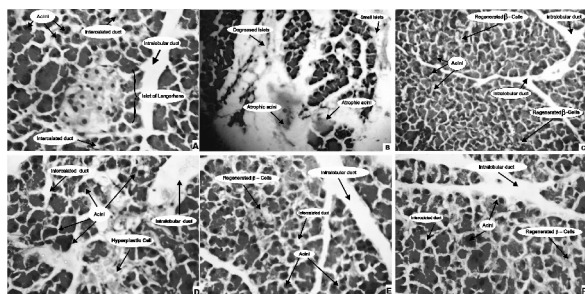
Table 3: Effect of EHN on total protein, albumin, globulin and a:g ratio of control and experimental groups of rats.

Group	Treatment	Dose (mg/kg)	Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	A:G ratio
I	Normal Control	-----	7.99 ± 0.14	4.12 ± 0.12	3.86 ± 0.12	1.06 ± 0.05
II	Diabetic Control	-----	5.13 ± 0.10 [*]	2.74 ± 0.10 [*]	2.39 ± 0.09 [*]	1.15 ± 0.07
III	EHN	50	5.93 ± 0.01 [*]	3.48 ± 0.01 [*]	2.44 ± 0.02	1.42 ± 0.01 [*]
IV	EHN	100	6.65 ± 0.07 [*]	3.65 ± 0.03 [*]	3.00 ± 0.10 [*]	1.22 ± 0.05
V	EHN	200	7.70 ± 0.04 [*]	4.09 ± 0.02 [*]	3.60 ± 0.02 [*]	1.12 ± 0.01
VI	Glibenclamide	5	7.83 ± 0.14 [*]	4.05 ± 0.05 [*]	3.78 ± 0.18 [*]	1.08 ± 0.06

Data are presented as mean ± SEM, (n=6). ^{*}p< 0.01 when compared to the corresponding values of the normal control. ^{*}p<0.01 when compared to the corresponding values of the diabetic control.

Antioxidant activity

At the end of treatment, SOD, CAT, GPX, GST, GSH, TBARS and HP levels were analysed. In comparison to the normal control, the diabetic control group has shown a significantly (P<0.01) decrease of SOD by 58.22% in the liver and 46.84% in the kidney. Treatment with different doses of EHN and glibenclamide exhibited a significant increase in SOD at all doses. EHN at the dose of 200 mg/kg produce most significant (P<0.01) elevation of SOD by 57.03%, respectively in the liver and by 45.40%, respectively in the kidney, whereas the doses of 50 and 100mg/kg produce slight significant (p<0.01) raise of SOD by 34.87% and 48.40%, respectively in the liver and by 27.39% and 33.98%, respectively in the kidney when compared with diabetic control rats (Fig. 6A). Diabetic control rats were also characterized by a decrease in CAT by 28.42% and 58.30%, respectively in the liver, kidney. Administration of three different doses of EHN and glibenclamide induced a significant (P<0.01) enhance of CAT in the liver by 18.85%, 23.06%, 26.46% and 28.39%, respectively, and kidney by 42.95%, 52.15%, 56.59% and 57.14%, respectively as compared to the diabetic control (Fig. 6B). The GPx level of the diabetic control group was significant (p<0.01) decreased by 37.82% and 37.13%, respectively in the liver and kidney. Treatment with different doses of EHN and glibenclamide induced a significant (P<0.01) raise of GPx in the liver by 20.58%, 29.59%, 36.13% and 37.12%, respectively, and kidney by 25.43%, 29.72%, 36.97% and 36.97%, respectively as compared to the diabetic control (Fig. 6C). Diabetic rats were significant (p<0.01) decreased in GSH level by 45.37% and 53.04%, respectively in the liver and kidney. Administration of three different doses of EHN and glibenclamide exhibited a significant (P<0.01) increase of GSH in the liver by 28.33%, 37.02%, 44.18% and 44.37%, respectively, and kidney by 38.93%, 44.01%, 51.51% and 53.42%, respectively as compared to the diabetic control (Fig. 6D). GST of liver and kidney was significant (p<0.01) decreased by 39.15% and 41.98%, respectively, in the diabetic rats when compared to normal control rats. After treatment with the different doses of EHN and glibenclamide induced a significant (P<0.01) increase of GST in the liver by 22.07%, 26.78%, 36.69% and 34.27%, respectively, and kidney by 27.79%, 33.03%, 41.19% and 41.31%, respectively as compared to the diabetic control (Fig. 6E). On the other hand, the TBARS and HP levels were significantly (p<0.01) elevated in diabetic control rats by 58.69%, 32.05% and 48.00%, 41.68%, respectively in the liver, kidney compared to the normal control rats. In comparison to the diabetic control rats, the different doses of EHN and glibenclamide significant (p<0.01) reduced TBARS by 41.30%, 50.00%, 60.32% and 58.69%, respectively in the liver and 34.66%, 41.77%, 51.11% and 49.77%, respectively in the kidney (Fig.6F). The HP level also significantly (p<0.01) reduced by 22.89%, 29.84%, 33.86% and 32.12%, respectively in the liver and 34.82%, 38.16%, 43.32% and 42.07%, respectively in the kidney as compared to the diabetic control rats (Fig. 6G). Amongst all the doses of EHN 200



A = Normal control (Normal acini with islets of β -cells), B = Diabetic control (Atrophic acini and reduction of β -cell size; Shows degenerated islets), C = Glibenclamide (Markedly normal regenerated and preserved cells; with marked proliferated and regenerated β -cells), D = EHN 50mg/kg (Atrophic pancreas with acini and reduction of β -cell size; Shows hyperplastic cells), E and F = EHN 100 and 200 mg/kg (Normal regenerated and preserved cells; with marked proliferated and regenerated β -cells).

Fig. 8: Effect of EHN on cellular damage in pancreas of control and experimental groups of rats 400X

DISCUSSION

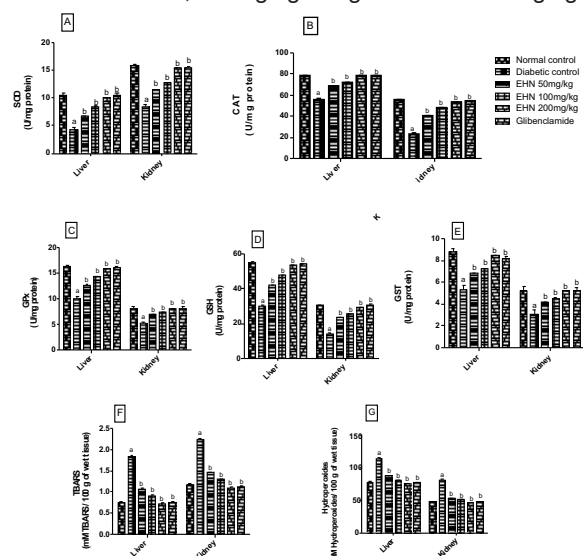
The ethno botanical information, reports many plants that may possess antihyperglycemic potential, of which *Momordica charantia*, *Pterocarpus marsupium*, and *Trigonella foenum graecum* have been reported to be beneficial for treatment of type 2 diabetes. More than 1200 species of plants have been screened for activity on the basis of ethnomedicinal uses³⁰. Based on the above perspectives, in the present study was undertaken to assess the antihyperglycemic potency of HN by means of *in vitro* and *in vivo* model.

The results of the *in vitro* α -amylase inhibition test displayed that HN all the root extracts exhibited inhibitory effects which was compared to that of the standard drug acarbose. The EHN possess noteworthy α -amylase inhibitor activity than the other extracts (Fig.1). In normoglycemic rats, different extracts of HN root showed a dose dependent hypoglycemic effect at 3 h (Fig.2). From oral glucose tolerance test, it could be concluded that doses of EHN showed the maximum improvement in glucose tolerance (Fig.3). Based on the *in vitro*, normoglycemic and OGTT test results, the *in vivo* effect of EHN at the varying doses of 50, 100 and 200mg/kg was studied in STZ-NC induced diabetic rats.

In our study, we used STZ-NC for induction of type 2 DM. STZ causes selective cytotoxicity effect on pancreatic beta cells and thus it affects the endogenous insulin release and as a result increases blood glucose level.³¹ Due to an antioxidant property of NC, it exerts protective effect on the cytotoxic action of STZ by scavenging free radicals and causes only minor damage to pancreatic β -cell mass producing type 2 DM.¹⁹ Oral administration of different doses of EHN to the diabetic rats showed significant reduction of blood glucose levels in a dose dependent manner and also, at 200 mg/kg does the level of EHN exhibited the parallel effect to that of glibenclamide (Table 1). Glibenclamide is a standard antihyper-glycemic drug that stimulates insulin secretion from β -cell of islets of Langerhans. From the results of the present study, it may be suggested that the mechanism of action of EHN may be similar to glibenclamide action³².

In STZ-NC induced diabetes rats, there is a loss in body weight due to muscle destruction or degradation of structural proteins³³. Diabetic rats received different doses of EHN and glibenclamide significantly improve the body weight comparability to the diabetic control rats and all doses of EHN and glibenclamide showing a protective effect in controlling muscle wasting (reversal of gluconeogenesis). The EHN at the dose of 200 mg/kg showed more improvement in the body weight in comparison to the diabetes control and glibenclamide tested groups (Fig.4). The glycosylated haemoglobin is an essential biochemical parameter in diabetes, which helps to establish the degree of protein glycation during diabetes³⁴. In STZ-NC induced diabetic rats, significantly decreased Hb and increased HbA_{1c} levels were noticed than control rats. After the treatment with different doses of EHN and glibenclamide showed decline of HbA_{1c} and upgrading in Hb levels, and it might be due to blood glucose lowering effect of EHN probably through reversal of insulin resistance or rising insulin secretion by regeneration of pancreatic β -cells (Fig.5). Hypercholesterolaemia and hypertriglyceridaemia are most essential factors of diabetic state involved in the progression of atherosclerosis and coronary heart disease which are the secondary complications of diabetes³⁵. Dyslipidaemia is characterised by high plasma levels of total cholesterol, LDL-cholesterol and triglycerides, with low plasma levels of HDL-cholesterol. Our results specify that, treatment with different doses of EHN and glibenclamide administered to diabetic rats reduced total cholesterol, triglycerides, LDL-cholesterol, VLDL-cholesterol and lowered serum levels of HDL-cholesterol (Table 2). Thus, EHN at the dose of 200mg/kg could have a potential to reduce long term cardiovascular complications in diabetic conditions. In STZ- NC induced diabetic rats the liver was necrotized. An increase in the activities of SGOT, SGPT and ALP in plasma might be mostly due to the leakage of these enzymes from the liver cytosol into the blood stream which gives an indication of the hepatotoxic effect of STZ³⁶. Hence our study was also focused to know the protective activity of EHN against hepatic and renal damage caused by diabetes (Table 2). Treatment of the diabetic rats with different doses of EHN and Glibenclamide reduced the activity of these enzymes when compared to the diabetic control rats and consequently alleviated liver damage caused by STZ-NC induced diabetes rats. Significant reductions in the activities of these enzymes in EHN treated diabetic rats indicated the hepato protective role in preventing diabetic complications. Additionally, our data showed that total protein, albumin, globulin and a:g ratio concentration in the serum of STZ- NC induced diabetic rats was significantly decreased ($P < 0.01$) compared to normal control rats which is a reflection of cytotoxicity as reported in the independent studies³⁷⁻³⁸. Interestingly, treatments with different doses of EHN and glibenclamide significantly reversed the protein concentrations in diabetic rats (Table 3). The increased level of serum protein, albumin, globulin and a:g ratio levels in STZ-NC induced diabetic rats were able to perturb the STZ-NC induced cytotoxic effects in erythrocytes

mg/kg was more efficient enhancement in antioxidant intensity of liver and kidney tissues as compared to other doses of EHN 50, 100mg/kg and glibenclamide 5mg/kg.



Data are presented as mean $\pm$ SEM, (n=6). * $p < 0.01$ when compared to the corresponding values of the normal control. $p < 0.01$ when compared to the corresponding values of the diabetic control.

Fig. 6: Effect of EHN on SOD (A), CAT (B), GPx (C), GSH (D), GST (E), TBARS (F) and HP (G) in liver and kidney of control and experimental groups of rats.

Glucokinase activities in liver tissues

The level of glucokinase in diabetic rats was observed significantly ($p < 0.01$) decrease in liver tissues of 67.26% as compared to the normal group. Upon oral administration of different doses of EHN and glibenclamide was significantly ($P < 0.01$) boosting the level of glucokinase in liver by 56.90%, 62.54%, 66.58% and 67.15%, respectively as compared to diabetic control rats. EHN 200mg/kg produces superior glucokinase activity than the other doses of EHN 50, 100mg/kg and glibenclamide 5mg/kg (Figure. 7A).

Glucose-6-phosphatase activities in liver tissues

To evaluate the potency of the EHN on diabetic rats on glucose-6-phosphate on diabetic rat. The level of glucose-6-phosphate was significant ($p < 0.01$) increased by 57.38% in diabetic groups rat when compared to the normal rat. Oral administration of different doses of EHN and glibenclamide was significantly ($P < 0.01$) decline the increased level of glucose-6-phosphate by 39.96%, 42.81%, 49.19% and 56.11%, respectively as compared to diabetic control rats. Different doses received groups rats significantly decreased the level of glucose-6-phosphate, but the dose of EHN with 200 mg/kg was more effective to decline the increased level of glucose-6-phosphate (Figure. 7B).

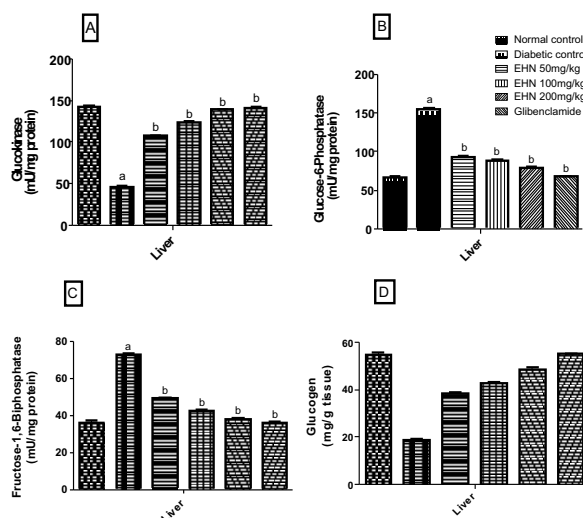
Fructose-1, 6-bisphosphatase activities in liver tissues

The oral administration of different doses of EHN and glibenclamide was significantly ($P < 0.01$) decreases the level of fructose-1-6-biphosphatase by 32.05%,

41.66%, 47.66% and 49.97% in diabetic control rats. The level of fructose-1-6-biphosphatase enhance in STZ-NC induced diabetes. Treatment with different doses of EHN was sharp decrease the level of fructose-1-6-biphosphatase to normalize rat. The EHN with dose 200 mg/kg shown the supreme diminish levels of fructose-1-6-biphosphatase in comparison to other diabetic treated group rats receiving doses of 50, 100mg/kg of EHN and 5 mg/kg of glibenclamide respectively (Figure. 7C).

Glucogen content in liver tissues

The glucogen content in diabetic rats was significantly ($p < 0.01$) reduced by 65.63% when compared to normal control rats. After administration of different doses of EHN and glibenclamide were significantly ($p < 0.01$) elevated in glucogen content by 50.88%, 55.79%, 61.18% and 65.69%, respectively as compared to diabetic control rats. EHN at the dose of 200 mg/kg was more effective than 50, 100 mg/kg in elevating the level of glucogen content (Figure. 7D).



Data are presented as mean $\pm$ SEM, (n=6). * $p < 0.01$ when compared to the corresponding values of the normal control. $p < 0.01$ when compared to the corresponding values of the diabetic control.

Fig. 7: Effects of EHN on glucokinase (A), glucose-6-phosphatase (B), fructose-1, 6- bisphosphatase (C) and glucogen content (D) in liver of control and experimental groups of rats.

Histopathological studies

Histology of pancreas in experimental rats was evaluated at the end of the study. Normal control rats showed normal acini with islets of β -cells (Fig.8A). Diabetic control rats showed atrophic acini and reduction of β -cells size with degreased islet cells (Fig.8B). Diabetic treated with glibenclamide showed markedly normal regenerated and preserved cells with marked proliferated and regenerated β -cells (Fig.8C). Diabetic rats treated with EHN 50mg/kg showed atrophic acini and reduction of β -cell size and population with abnormal architecture of hyperplastic cells (Fig.8D). Diabetic rats treated with EHN 100 and 200mg/kg showed normal regenerated and preserved cells with marked proliferated and regenerated β -cells (Fig. 8E-F).

possibly by inducing the synthesis of some soluble proteins localized in the erythrocyte and also increased protein catabolism and gluconeogenesis during diabetes³⁹.

Earlier studies have reported that there was an increased lipid peroxidation in the liver and kidney of diabetic rats⁴⁰. In the present study, an elevated in the levels of lipid peroxides and hydroperoxides was found and these levels significantly declined after the supplementation of three different doses of EHN and glibenclamide (Fig.6F-G). This indicates that the plant extract inhibit oxidative damage due to the antiperoxidative effect present in EHN. This could be associated with previous study reported that *Tinospora cardifolia* has antiperoxidative and antihyperlipidemic effect of diabetic animals⁴¹. The reduced activities of SOD and CAT in the liver and kidney have been observed during diabetes and this may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxides⁴². However, administration of different doses of EHN and glibenclamide could reverse progress of the disease (Fig.6A-B). The above observations may clearly suggest that increased levels of SOD and CAT of EHN has free radical scavenging activity, which may exert a beneficial effect against pathological alterations caused by reactive oxygen species. Reduced activities of GPx and GST in the liver and kidney have been observed during diabetes and this may result in a number of deleterious effects due to the accumulation of toxic products. Administration of different doses of EHN and glibenclamide to the diabetic rats elevated activity of GPx and GST in the liver and kidney may be due to the suppression of peroxidative stress (Fig.6C-E). Decreased glutathione levels in diabetes have been considered to be an indicator of increased oxidative stress⁴³. In this context, several researchers have also reported decreased levels of tissue GSH in experimental diabetic rats⁴⁴. Administration of different doses of EHN and glibenclamide increased the content of GSH in the liver and kidney of diabetic rats due to control the oxidative stress. Impairment of glucokinase activity leads to the impaired oxidation of glucose via glycolysis leading to its accumulation resulting in hyperglycemia. In the present study, decreased activity of glucokinase was observed in STZ-NC induced diabetic rats. The enzymatic activity was increased with different doses of EHN and glibenclamide treatment (Fig.7A). Similar observations were also recorded by other investigators⁴⁵. These observations imply that entry of glucose into the cells is facilitated by the EHN and glibenclamide treatment, which in turn would stimulate the activity of this enzyme. The activity of glucose-6-phosphate and Fructose- 1, 6-bisphosphatase were increased in the liver of diabetic rats due to regulation of the gluconeogenesis. These results are comparable to others where several plant extracts decreased the activity of this enzyme in diabetic condition⁴⁶. In our study, oral administration of different doses of EHN and Glibenclamide reversed the glucose-6- phosphatase and fructose- 1, 6-bisphosphatase activities in STZ-NC induced diabetic rats which are

responsible for the improved glycemic control (Fig.7B-C). The decrease in hepatic glycogen may be observed due to insufficient insulin and inactivation of glycogen synthetase system in diabetic state⁴⁷. However, after the treatment with different doses of EHN and glibenclamide increase in liver glycogen level in diabetic rats may be due to utilization of insulin and activation of glycogen synthetase (Fig.7D).

The histological analysis of pancreas tissues showed destruction of β -cells was observed in diabetic control rats when compared to normal control rats due to DNA alkylation (Fig.8A), nitric oxide production and free radical generation, leading to a total lack or deprived insulin production and chronic hyperglycaemia³⁸. STZ-NC induced diabetic rats results in degenerative changes in the islets of langerhans of the pancreas (Fig.8B). The islet is considerably reduced and shrunken, there is the destruction of some β -cells with central hyalinization with pyknotic nuclei and the number of cells is lower⁴⁸. Treatment with different doses of EHN and glibenclamide restored the activity of the islets of langerhans (Fig.8C-F). These suggested that one of the possible mechanisms of the hypoglycemic effects may be acted by protecting the pancreatic β -cells and stimulating insulin secretion from the remaining pancreatic β -cells.

CONCLUSION

In conclusion, the present findings clearly demonstrated at first time that the EHN exhibited excellent antihyperglycemic, antihyperlipidemic and antioxidant activities in STZ-NC induced type 2 diabetes model. Indeed, this study has undoubtedly provided scientific confirmation and evidence for the safety and use of the root of HN by traditional healers in the treatment of diabetes. However, further studies are necessary for the isolation and purification of bioactive compounds present in EHN and for elucidation of their molecular mechanisms can be carried out in diabetes and diabetic complications.

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