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A study on phytochemical screening and antidiabetic potential of *Vitex negundo* leaf extract in rats

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Abstract
Objective: The main objective of this study was to perform the phytochemical analysis of the hydroethanolic extract of leaves of *Vitex negundo* (HEVN) and to assess its antidiabetic potential following oral administration @ 100 mg/kg and 200 mg/kg B.WT for 28 days in Streptozotocin induced diabetic rats. For the *in-vivo* study, forty-two rats of 2-2.5 months of age were divided randomly into seven groups with six rats in each group. Group I served as normal control group. Group II, III, VI and VII, received a single intraperitoneal injection of Streptozotocin @ 45 mg/kg B.WT to induce diabetes. Rats showing blood glucose level greater than 200 mg/dl on 7th day were considered hyperglycemic and chosen for the experiment. Group II represented as diabetic control. Group III (diabetic) was treated with glibenclamide @ 1 mg/kg B.WT. Orally for 28 days. Group IV and V (non-diabetic) were given HEVN orally @100 mg/kg B.WT and 200 mg/kg B.WT, respectively, for 28days. Group VI and VII (diabetic) were given HEVN orally @100 mg/kg B.WT and 200 mg/kg B.WT, respectively, for 28 days. The phytochemical screening of hen was also performed to detect the presence of phytoconstituents. The qualitative phytochemical analysis of HEVN revealed the presence of alkaloids, flavonoids, tannins, terpenoids, saponins, glycosides, phenols and fixed oils and fats. The quantitative analysis of HEVN revealed total phenolic content to be 390.74±3.60 mg Gallic acid equivalent (GAE) per gm. of the dry weight of extract and total flavonoid content to be 291.00±3.42 mg rutin per gm. of the dry weight of extract. The results of the *in-vivo* study showed that Streptozotocin administration caused significant ($p < 0.05$) elevation in blood glucose and HbA1c level in rats which was restored back towards normal in HEVN treated groups in a dose-dependent manner. On histopathological examination, group II showed histopathological disintegrate in pancreas which was reverted back towards normal in HEVN treated rats @ 200 mg/kg B.WT. Thus, the hydroethanolic extract of leaves of *Vitex negundo* (HEVN) @ 100 mg/kg and 200 mg/kg has ameliorative potential against Streptozotocin (@ 45 mg/kg) induced diabetes in rats in a dose-dependent manner.

Keywords: *Vitex negundo*, Hydroethanolic extract of *Vitex negundo* (HEVN), Streptozotocin, Hypoglycemia, Antidiabetic, Blood glucose

Introduction
Diabetes, a rapidly growing metabolic disorder is considered to be one of the top 10 causes of adult death worldwide. It is associated with abnormally elevated blood sugar levels as a result of insufficient or no pancreatic insulin secretion or inability of the body to mobilize glucose properly into cells called insulin resistance or both (Kharroubi and Darwish, 2015) [1]. Although conventional medicines for the treatment of diabetes are very much popular in the market, they show side effects and withdrawal symptoms after discontinuation. Also, the patient develops a lifelong dependency on regular or frequent consumption of these medicines. In such circumstances, Ayurveda can offer a lot. The herbal drugs stimulate the natural healing of the body strengthening the immune system and also stabilizing hormones and metabolism. Over 400 traditional herbal therapies have been reported but only a few of them were studied and evaluated scientifically to be potentially effective in the treatment of diabetes. The main pathogenesis of hyperglycemia is mainly attributed to the production of reactive oxygen species leading to oxidative stress in body cells and degeneration of pancreatic beta cells. Antihyperglycaemic plants contain such phytoconstituents that can heal and regenerate damaged pancreatic cells eventually resulting in increased insulin secretion or show insulin-mimetic action, reduced glycogenolysis or reduced glucose absorption from the intestine and increased glucose utilization peripherally with additional antioxidant effects (Patel *et al*., 2012) [2].

Vitex negundo, a large aromatic deciduous shrub classified under the family Verbenaceae, has been described as an important plant with immense potential in the field of modern medicine development possessing various bioactive phytoconstituents (Nyeem *et al*., 2017) [3]. Almost every part of the plant has been traditionally used in the treatment of various ailments especially leaves in headache, common cold, flu, sore throat, dysmenorrhea, gonorrhea, cancer, gout, catarrhal fever, whooping cough, as an analgesic in backache, chest pain and body ache, as anti-inflammatory (Dharmasiri *et al*., 2003) [4], antihistaminic, hepatoprotective, tonic, sedative, vermifuge, anti-rheumatic, antifungal and antihyperglycemic (Rani and Sharma, 2013) [5]. The whole plant has been described to possess number of important phytochemical secondary metabolites including the iridoid glycosides, negundoside and agnuside, which are considered to be the main bioactive constituents responsible for the multiple therapeutic actions of the plant (Tasduq *et al*., 2008) [6].

Plants serve as a source of medicines long before the prehistoric period with the belief that herbs are the only source for curing numerous health ailments. However, there is still a significant lack of research data for the efficacious use of these traditional approaches in such a way so as to squeeze out maximum benefit from them avoiding minor side effects, if any persists. Keeping this view, the current investigation aims to study the qualitative and quantitative phytochemical constituents of hydroethanolic extracts of leaves of *Vitex negundo* (HEVN) *in-vitro* and antidiabetic activity of hydroethanolic extract of leaves of *Vitex negundo* (HEVN) following oral administration for 28 days in Wistar rats.

Materials and methods

Phytochemical Analysis

The plant leaves were procured from Medicinal Plants Research and Development Center, Pantnagar (MRDC). The leaves were washed with water and shade dried for 2 months to remove excess moisture and then ground to a fine powder. The hydroethanolic extract of *Vitex negundo* (HEVN) was prepared using the cold extraction technique [7] after soaking 500g of the fine powder in 5000ml of hydroethanolic solution (50:50) for 24hrs. The harvested extract was then transferred into air tight glass container and stored at 4°C for further use.

Qualitative phytochemical analysis of Hydroethanolic extract of *Vitex negundo* (HEVN) was done using the standard procedure (Harborne, 1973) [8] for the presence of various phytoconstituents like alkaloids, saponins, flavonoids, tannins, phenols, terpenoids, carbohydrates, glycosides, proteins, amino acid, fixed oils and fats. Total phenol content in the plant was determined by the spectrophotometric method of Kamtekar *et al*., 2014 [9]. The total flavonoid content of the crude extract was determined following the method given by Zhishen *et al*. 1999 [10].

Experimental Design

42 adult Wistar rats (male and female) weighing 150-180 g were procured from IVRI, Izzatnagar, Bareilly. The rats were randomly and equally segregated into 7 groups consisting of 6 animals including three male and three female rats separately in each group. The rats were housed in plastic cages and acclimatized for a period of two weeks to the laboratory conditions prior to the commencement of the experiment under standard manage mental conditions. The rats were ensured *ad libitum* fresh water and standard feed throughout the experimental period. All experimental procedures were in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and were approved by Institutional Animal Ethics Committee (IAEC/CVASC/VPT/405).

Group I served as normal control and was injected with 0.1M citrate buffer (pH 4.5) once. Diabetes mellitus was induced by a single intraperitoneal injection of freshly prepared Streptozotocin (Sigma-Aldrich, US) solution in 0.1M citrate buffer (pH 4.5) @ 45 mg/kg b wt. in overnight fasted rats of group II, III, VI and VII, 7 days prior to the start of the experiment. They were given free access to water during fasting. After 7 days of Streptozotocin, Group I received distilled water orally for 28 days. Group II served as diabetic control (Streptozotocin, I.P., once) and received distilled water orally for 28 days. In rats of group III, Streptozotocin (I.P., once) along with glibenclamide (Sigma-Aldrich, US) @ 1 mg/kg b wt. orally, Q.D. was given for 28 days. In group IV, rats were given HEVN @ 100 mg/kg b wt. orally, Q.D. for 28 days. Group V rats were given HEVN @ 200 mg/kg b wt. orally, Q.D. for 28 days. Group VI rats were injected with Streptozotocin (I.P., once) along with HEVN @100 mg/kg b wt. orally, Q.D. for 28 days. In Group VII, Streptozotocin (once, I.P.) and HEVN @ 200 mg/kg b wt. orally, Q.D. for 28 days was given. The rats were monitored daily for the presence of any apparent clinical signs or symptoms. The body weights were recorded on weekly basis.

Blood Glucose Estimation

Blood glucose estimations were done periodically (days 0, 7, 14, 21 and 28) by tail puncture method using a glucometer (Accu-Chek active, India) in overnight fasted rats. On the 28th day, an oral glucose tolerance test (OGTT) was performed (Chaimum-aom *et al*., 2017) [11]. Blood glucose of overnight fasted rats was taken. Then the rats of all groups were given the respective treatment as per the experimental design. After 30 minutes of respective treatment as per the experimental design, oral glucose tolerance test was conducted. Rats of all group were given 20% oral glucose solution @ 2 g/kg B.WT. The blood glucose level was measured at 0, 30, 60, 90 and 120 minutes using a glucometer. A hemolysate was prepared from fresh whole blood collected on the 28th day from retro-orbital sinus and was used for assessing glycosylated hemoglobin (HbA1C) by ion exchange resin method using glycosylated hemoglobin kit (Excel Diagnostics Pvt. Ltd, India).

Histopathological Examination

On the 28th day, rats were anaesthetized by diethyl ether and sacrificed humanely by cervical dislocation. The pancreas of different groups were collected and absolute and relative organ weight was observed. Tissue samples of pancreas were collected in 10% neutral buffered formalin and histopathological examination was done using hematoxylin and eosin (H&E) stain (Lillie, 1965) [12].

Statistical analysis

The data was analyzed for statistical significance by employing ANOVA. Statistical difference between respective means for various parameters was evaluated using Duncan's multiple range test at 5% level of significance (Snedecor and Cochran, 1967) [13].

Results and discussion

The qualitative phytochemical analysis revealed the presence

of various phytochemical groups like alkaloids, flavonoids, tannins, terpenoids, saponins, glycosides, carbohydrates, phenols, fixed oils and fats. The quantitative phytochemical analysis showed the total phenolic content in HEVN to be 390.74±3.60 mg Gallic acid equivalent (GAE) per gm. of the dry weight of extract and total flavonoid content in HEVN to be 291.00±3.42 mg rutin per gm. of the dry weight of extract (Table 1). The phenols and flavonoids are considered important bioactive constituents which contribute to the pharmacological activity of the plant extract as they possess free radical scavenging activity due to their antioxidant property.

Table 1: Qualitative and quantitative phytochemical analysis of HEVN

Qualitative analysis			Quantitative analysis	
S. No.	**Phytochemicals**	**Inference**	**Compound**	**Amount**
1	Alkaloids	+	Total phenolic content	390.74±3.60 mg Gallic acid equivalent (GAE) per gm. of dry weight of extract
2	Flavonoids	+		
3	Tannins	+		
4	Terpenoids	+		
5	Saponins	+		
6	Glycosides	+	Total flavonoid content	291.00±3.42 mg rutin per gm. of dry weight of extract
7	Proteins	-		
8	Carbohydrates	+		
9	Phenols	+		
10	Fixed oils and fats	+		

On clinical observation, rats in group II were found dull and depressed with apparent clinical signs and symptoms like polydipsia and polyuria. In group VI and VII (HEVN treated diabetic rat groups) mild polydipsia and polyuria was observed, which improved following treatment with HEVN in a time-dependent manner. A significant ($p < 0.05$) decrease in body weight was seen in group II as compared to the normal control group whereas STZ induced body weight reduction was significantly ($p < 0.05$) reversed in a dose-dependent manner by HEVN and the results were comparable to that of glibenclamide treatment (Fig 1). The induction of diabetes is associated with insufficient secretion or lack of insulin that results in hyperglycemia. The body stops the uptake of glucose from the blood into cells for utilizing it as an energy source. Hence as an alternative, an extensive breakdown of proteins, muscle wasting and mobilization of fats from stored tissues occur which eventually results in decreased body weight in diabetes (Ramakrishnan *et al*., 2017) [14] as also observed in the present study. The administration of HEVN restored the decreased body weight towards normal in a dose-dependent manner which can be attributed to its hypoglycemic action thus preventing the breakdown of proteins and reduction in body weight.

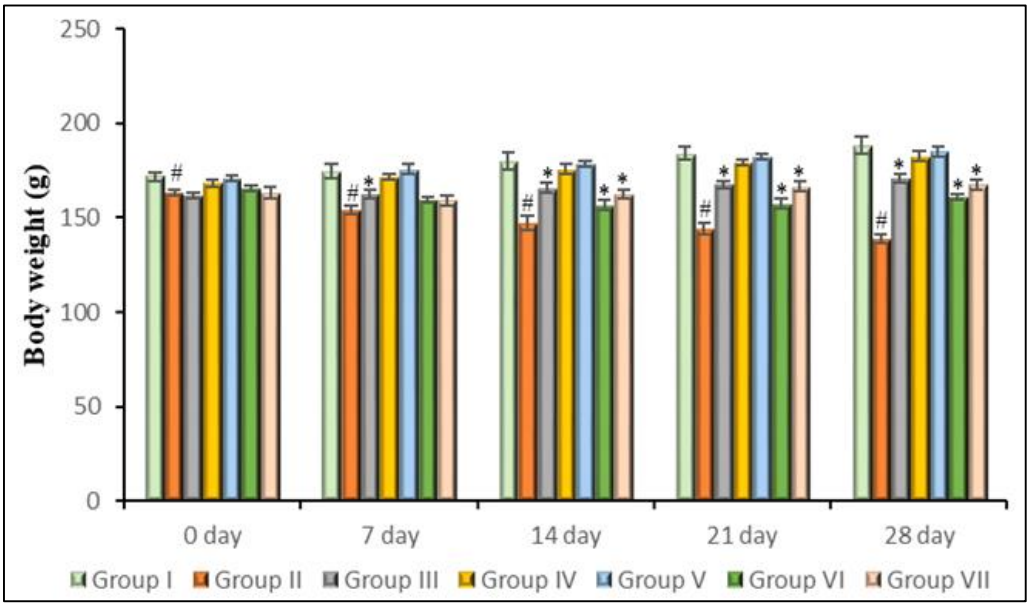

Fig 1: Effect on body weight (g) following oral administration of HEVN (N=5-6); #, $p < 0.05$ vs group I; *, $p < 0.05$ vs group II

A significant ($p < 0.05$) elevation in blood glucose level was seen in group II following STZ administration. Treatment with HEVN @ 100 mg/kg B.WT and 200 mg/kg B.WT. produced significant ($p < 0.05$) and dose-dependent decrease in blood glucose levels as compared to group II (Table 2). Streptozotocin administration results in hyperglycemia because Streptozotocin acts as a selective toxic agent for pancreatic β-cells in rat models thus resulting in impaired function of pancreatic β-cells to secrete insulin leading to disturbed metabolism of carbohydrates and eventually leads to the development of persistent hyperglycemia by reduction of biosynthesis and secretion of insulin (Schnedl *et al*., 1994; Al Nahdi *et al*., 2017) [15, 16]. The significant ($p < 0.05$) reduction in abnormally elevated blood glucose following oral administration of HEVN @ 100 mg/kg B.WT. and 200 mg/kg B.WT in a dose-dependent manner suggests the hypoglycemic effect of HEVN. The findings of this study are corroborated by the similar findings of Falguni *et al*. 2017 [17] in

Streptozotocin induced diabetic mice. The hypoglycemic activity of plant extract may be attributed to its phytoconstituents action on β-cells of islets of pancreas which might have acted by following mechanisms: enhancing insulin production, peripheral increase in glucose uptake by cells, stimulation of glycogenesis and inhibition of gluconeogenesis or α-amylase and α-glucosidase inhibition.

Table 2: Effect on blood glucose and HbA1c following oral administration of HEVN

Groups	Treatment	Dose and Route	Blood Glucose (mg/dl)					HbA1c (%)
			0 day	7th day	14th day	21st day	28th day	28th day
I	Control	0.1M citrate buffer; I.P, once + DW; P.O, Q.D. for 28 days	107.5±3.83^{a}	103.66±6.99^{a}	110.16±6.11^{a}	104.50±5.34^{a}	109.16±4.79^{a}	5.66±0.10^{a}
II	STZ* (Diabetic control)	@ 45 mg/kg; I.P. once + DW; P.O, Q.D. for 28 days	277.16±10.53^{bc}	293.33±8.36^{d}	313.00±7.38^{e}	299.60±5.25^{e}	333.60±10.98^{e}	14.32±0.22^{g}
III	STZ + Glibenclamide	@ 45 mg/kg; I.P once + @ 1 mg/kg; P.O, Q.D. for 28 days	268.66±8.74^{b}	185.00±6.23^{b}	162.66±3.54^{b}	134.16±4.20^{b}	135.33±6.71^{b}	7.26±0.14^{d}
IV	HEVN-1	@ 100 mg/kg; P.O, Q.D. for 28 days	104.83±4.14^{a}	108.00±6.12^{a}	107.83±4.55^{a}	111.5±6.31^{a}	108.66±5.44^{a}	6.33±0.12^{b}
V	HEVN-2	@ 200 mg/kg; P.O., Q.D. for 28 days	110.16±6.58^{a}	111.16±3.33^{a}	109.16±6.41^{a}	106.16±2.70^{a}	107.16±4.59^{a}	6.80±0.14^{c}
VI	STZ + HEVN-1	@ 45 mg/kg; I.P once + @ 100 mg/kg; P.O., Q.D. for 28 days	280.50±5.72^{bc}	242.33±5.61^{c}	199.16±5.21^{d}	232.50±18.68^{d}	221.60±14.86^{d}	9.78±0.16^{f}
VII	STZ + HEVN-2	@ 45 mg/kg; I.P once + @ 200 mg/kg; P.O., Q.D. for 28 days	292.50±3.93^{c}	236.50±6.79^{c}	181.16±8.97^{c}	172.50±6.46^{c}	184.33±7.57^{c}	9.25±0.15^{e}

DW: Distilled water; P.O.: *per OS*; Q.D.: *Quaque die*.
Values in the table are mean ±S.E. (n=5-6); STZ= Streptozotocin; HEVN=Hydroethanolic extract of leaves of *Vitex negundo*.
Values bearing different superscripts (a, b, c, d, e) differed significantly at 5% level ($p < 0.05$) within a column.
* STZ administered 7 days before conduct of experiment in groups II-VII.

Oral glucose loading in normal rats resulted in an increase in blood glucose level with the maximum level at 30 min which returned to normal at 90 min. However, the blood glucose level of group II did not return to normal at 120 min of oral glucose loading whereas in glibenclamide and HEVN treated groups the blood glucose level returned to baseline at 90 min of oral glucose loading (Fig 2). The HEVN treated rats showed better glucose reduction potential and clearance in a dose-dependent manner as compared to glibenclamide treated rats. The decrease in postprandial glucose in HEVN treated group may be attributed to reduced glucose absorption by inhibition of α- amylase or α-glucosidase enzymes or by an increase in insulin secretion in response to increasing glucose load or by enhancing glucose utilization peripherally or by inhibition of hepatic gluconeogenesis (Laishram *et al*., 2016) [18].

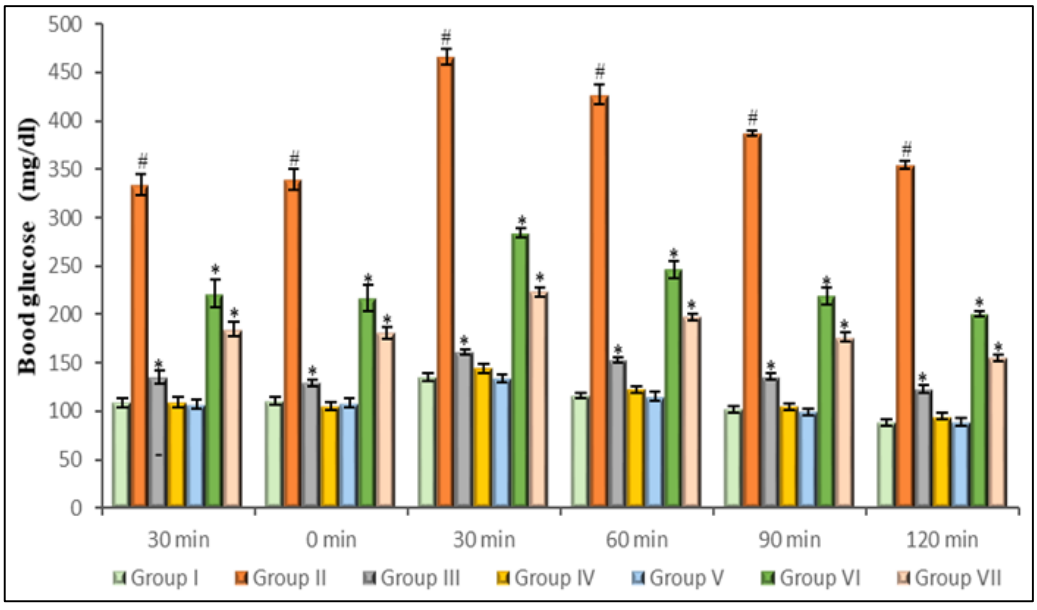

Fig 2: Effect on Oral glucose tolerance test (OGTT) following administration of HEVN (N=5-6); #, $p < 0.05$ vs group I; *, $p < 0.05$ vs group II

A significantly ($p < 0.05$) higher level of HbA1c was seen in Group II on the 28th day as compared to normal rats (Table 2). HEVN treated groups showed a significant ($p < 0.05$) decrease in HbA1c level in a dose-dependent manner as compared to group II. Glycosylated hemoglobin (HbA1c) measurement is considered a standard biomarker for assessment of impaired glycemic control in diabetes and is a measure of blood glucose level over a period of about 2-3 months. The excess blood glucose binds with amino acid residues of hemoglobin to generate HbA1c molecules (Kumaresan *et al*., 2014) [19]. High HbA1c levels increase free radical production in blood cells which alters their membrane and leads to blood cells aggregation, impaired blood flow, inflammatory changes and atherosclerotic plaque formation

(Nwaogwugwu *et al*., 2020) [20]. More the blood glucose level, the higher is the binding of glucose to hemoglobin and hence high is the level of HbA1c in blood (Malini *et al*., 2011) [21]. In the present study, HEVN treatment lowered the HbA1c values as compared to the diabetic control group which is indicative of its anti-hyperglycemic activity.

The absolute weight of pancreas was found to decrease significantly ($p < 0.05$) in group II as compared to the normal control group which was restored towards normal in HEVN treated and glibenclamide treated groups (Fig 3). This may be attributed to the hypoglycemic activity of HEVN thus preventing further destruction of pancreas due to hyperglycemia. However, the relative weights of pancreas of all groups were not found to differ significantly as compared to group I. On gross examination, pancreas of the diabetic control group appeared small in size. On histopathological examination, pancreas of the diabetic control group revealed a reduced number of islets of Langerhans with deteriorated architectural details, vacillation, mononuclear cell infiltration in exocrine acini and amyloid deposition around islets (Fig 4). Rats of HEVN treated group @ 200 mg/kg B.WT showed relatively normal islet morphology with mild lymphocytic infiltration in pancreas. Thus, HEVN treatment prevented STZ induced pancreatic β-cell damage and restored the histopathological disintegrate towards normal at the dose of 200 mg/kg body weight.

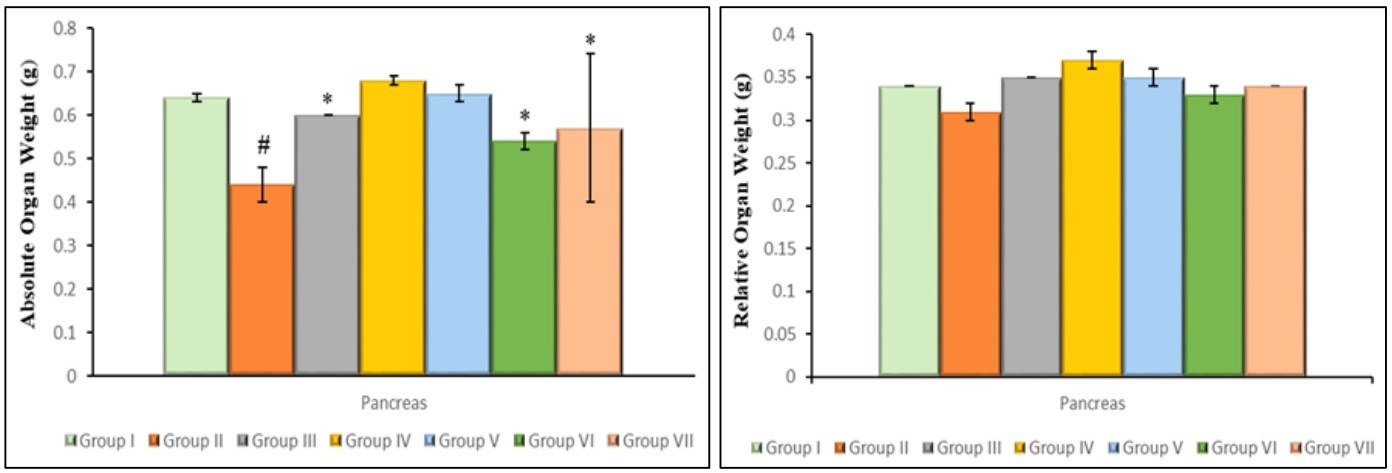

Fig 3: Effect on absolute and relative weight (g) of pancreas following oral administration of HEVN (n=5-6); #, $p < 0.05$ vs group I; *, $p < 0.05$ vs group II.

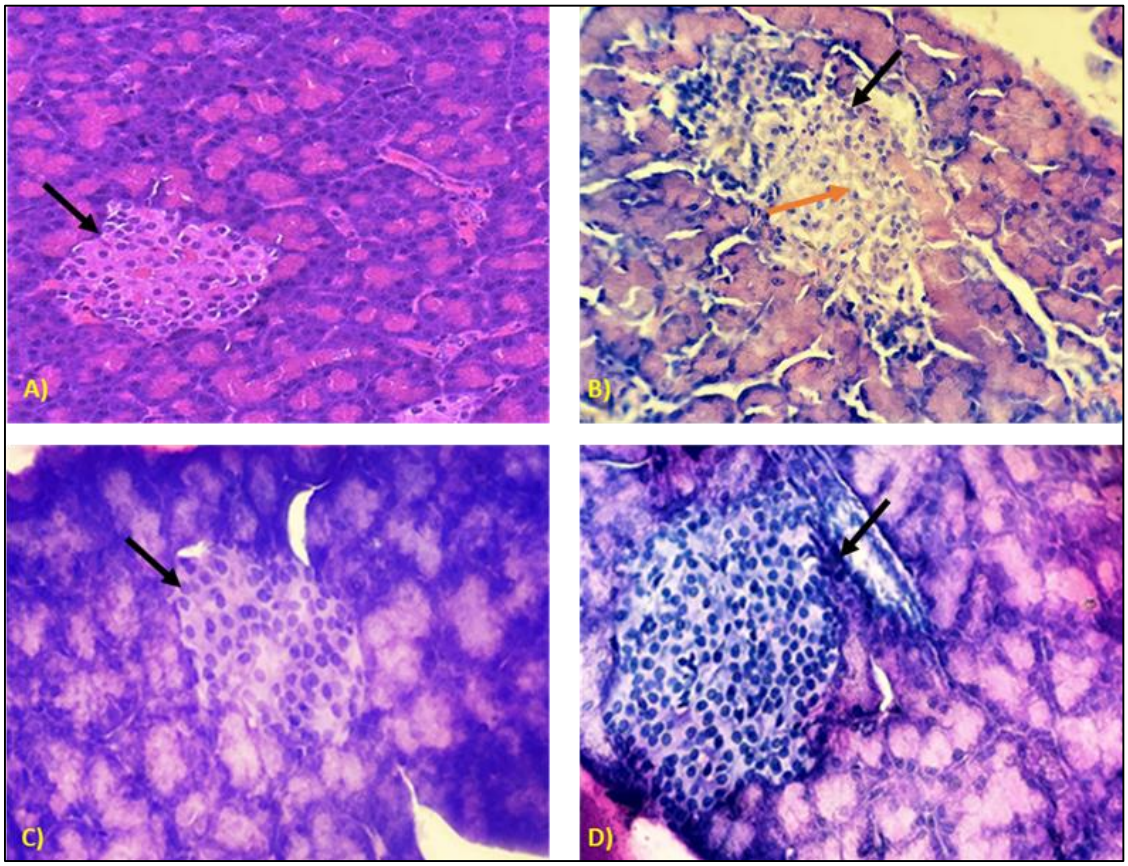

Fig 4: Representative photomicrographs of pancreas (H&E, 400X) (a) Group I showing normal architecture of islet of Langerhans (black arrow) surrounded by exocrine acini cells (b) Group II showing distorted architecture of islet of Langerhans (black arrow) and vacillation (orange arrow) (c) Group III showing normal islet of Langerhans (black arrow) and pancreatic acini (d) Group VII showing relatively normal islet morphology with occasional lymphocytic infiltration (black arrow) and normal exocrine acini.

Conclusion

From this study, it can be concluded that hydroethanolic extract of leaves of *Vitex negundo* (HEVN) possess various phytoconstituents with a relatively very high amount of phenols and flavonoids in it and the oral administration of hydroethanolic extract of leaves of *Vitex negundo* (HEVN) @ 100 mg/kg B.WT and 200 mg/kg B.WT for 28 days showed ameliorative potential against Streptozotocin induced hyperglycemia in a dose-dependent manner revealing hypoglycemic activity of plant extract. The study scientifically corroborates the ethno medically claimed antidiabetic action of *Vitex negundo.* Further detailed studies are warranted to isolate the bioactive components and determine the exact mechanism of antidiabetic activity of *Vitex negundo* to use it as a potential agent against diabetes.

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