Final Report

Title of the Project

To Study Effect of Vasant Kusumakar Rasa in Animal Model of Type 2 Diabetes

Sponsored by

Shree Dhootapapeshwar Limited
135, Nanubhai Desai Road, Khetwadi
Mumbai - 400 004.

Report Submitted by

Dr. Yogesh A. Kulkarni
Associate Professor

SVKM'S
NMIMS
Deemed to be UNIVERSITY

Shobhaben Pratapbhai Patel
School of Pharmacy and Technology Management
V.L. Mehta Road, Vile Parle (West)
Mumbai - 400056

1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder developed due to defect in insulin secretion, insulin action or may be because of both (Laddha and Kulkarni, 2019). Type 2 diabetes mellitus is an outcome of combined effect of insulin resistance and insufficient insulin secretion from pancreatic β-cells (Holman et al., 2008). Epidemiological study revealed that, type 2 diabetes has been widely spread as compared to type 1 diabetes. Among diabetic population in the world which was around 463 million in 2019, 90% of people suffered from type 2 diabetes and remaining 10% people suffered from type 1 and other specific type of diabetes (Duke, L., Fereira de Moura, A., de Lapertosa, S., 2019). Between the year 2000 to 2016, globally 1.6 million deaths were directly reported due to diabetes (World Health Organization [WHO], 2020). India has reported an increase in burden of type 2 diabetes in the last few years. Prevalence of type 2 diabetes is reported to be 2.4% in rural and 11.6% in urban population.

Prolonged and uncontrolled increase in levels of glucose in the blood affects vascular system which results into development of vascular complications involving damage to the various organs like kidney, eye, nerve and heart. It is reported that, type 2 diabetes is a leading cause of kidney failure and coronary artery damage (Alicic et al., 2017).

Various risk factors such as obesity, unhealthy diet, inadequate physical activity are some common factors responsible for the progression of type 2 diabetes (Al-Goblan et al., 2014). Presently a wide range of anti-diabetic drugs are being used for treatment of type II diabetes and various new drugs are being tested for their efficacy. The currently available drugs are associated with several side effects. Therefore, there is a need for effective and safe anti-diabetic medications.

Ayurved medicines (herbs, minerals, and their formulations) have the potential to prevent, effectively manage type 2 diabetes mellitus and reduce the risk of diabetic complications. **Vasant Kusumakar Rasa** is a traditional herbomineral formulation reported in Ayurvedic system of medicine for management of type 2 diabetes and associated complications. Vasant Kusumakar Rasa offers the benefits of multiple ingredients like Suvarna Bhasma, Rajata Bhasma, Vanga Bhasma, Naga Bhasma, Kantaloha Bhasma, Abhraka Bhasma, Pravala Bhasma, Mouktik Bhasma and Rasasindoor processed in Godugdha (cow milk), Ikshu Swarasa (juice of *Saccharum officinarum*), Vasa Swarasa (extract of *Adhatoda vasica*), Chandan Kwath (decoction of *Santalum* album), Usheer Kwath (decoction of *Vetiveria zizanioides*), Rheebera

Kwath (decoction of *Pavonia odorata*), Haridra Kwath (decoction of *Curcuma longa*), Kadali Kanda Swarasa (extract of *Musa paradisiaca*), Kamal Pushpa Swarasa (extract of Nelumbium speciosum) and Jatipushpa Swarasa (extract of Jasminum *officinale*).

Ayurved text Yoga Ratnakar mentions that **Vasant Kusumakar Rasa** is beneficial in the management of Prameha, which can be corelated with Diabetes Mellitus and is an excellent Rasayana (tissue rejuvenator). **Vasant Kusumakar Rasa** has been documented for its anti-hyperglycemic action (Gandhi S et. Al., 2013). It is also reported to prevent the progression and development of Diabetic retinopathy and restore altered serum lipid profile which is a hallmark of diabetes induced metabolic dysfunction (Tamoli, Sanjay Motilal et al; 2020).

In the present study, **Vasant Kusumakar Rasa** was evaluated for its effect in streptozotocin induced type 2 diabetes in Male *Sprague Dawley* rats.

2. PRODUCT DESCRIPTION

Product Name: Vasant Kusumakar Rasa (VKR) Tablets

Manufactured and Marketed by: Shree Dhootapapeshwar Limited, Mumbai.

Batch Number: P200700217

Date of Manufacturing: 07/2020

Date of Expiry: 06/2025

Composition of VKR tablets

#	Ingredients	Botanical/Common Name	Reference	Qty/Tab (62.5 mg)
1	Suvarna Bhasma	Processed Gold	B.B.R. 5/8357	4.310 mg
2	Rajata Bhasma	Processed Silver	R.T. 16/26-28	4.310 mg
3	Vanga Bhasma	Processed Tin	R.T. 18/15-18	6.465 mg
4	Naga Bhasma	Processed Lead	R.T. 19/29-33	6.465 mg
5	Kantaloha Bhasma	Processed Iron	B.B.R. 4/6416	6.465 mg
6	Rasasindoor	Generic Ayurvedic Formulation	R.Y.S. 2/115	8.620 mg
7	Abhraka Bhasma	Processed Mica	R.T. 10/39-42	8.620 mg
8	Pravala Bhasma	Processed Coral	R.T. 23/134-135	8.620 mg
9	Mouktik Bhasma	Processed Pearl	R.T. 23/71	8.620 mg
	Processed in:			q.s.
10	Godugdha	Cow milk	B.P., Dugdha Varga	q.s.
11	Ikshu Swarasa	*Saccharum officinarum*	B.P., Ikshu Varga	q.s.
12	Vasa Swarasa	*Adhatoda vasica*	B.P., Guduchyadi Varga, Sr. No. 34	q.s.
13	Chandan (Shweta Kwath)	*Santalum album*	B.P., Karpooradi Varga, Sr. No. 6	q.s.
14	Usheer Kwath	*Vetiveria zizanioides*	B.P., Karpooradi Varga, Sr. No. 33	q.s.
15	Rheebera Kwath	*Pavonia odorata*	B.P., Karpooradi Varga, Sr. No. 32	q.s.
16	Haridra Kwath	*Curcuma longa*	B.P., Haritakyadi Varga, Sr. No. 67	q.s.
17	Kadali Kwath	*Musa paradisiaca*	A.P.I., Vol. III, Pg. No. 73-74	q.s.
18	Kamal Pushpa Swarasa	*Nelumbium speciosum*	B.P., Pushpa Varga, Sr. No. 1	q.s.
19	Jati Pushpa Swarasa	*Jasminum officinale*	A.P.I., Vol. III, Pg. No. 71-72	q.s.

RT - Rasatarangini
BBR - Bharat Bhaishajya Ratnakar
RYS - Rasayogsagar
BP - Bhavaprakash Nighantu
API - Ayurvedic Pharmacopoeia of India

3. MATERIALS AND METHODS:

Male *Sprague Dawley* rats (180-220 g) were procured from the National Institute of Biosciences, Pune, Maharashtra, India and housed in the animal facility. The temperature of 22 ± 2°C, relative humidity 75 ± 5%, and a 12 h light/dark cycle was maintained in the animal facility throughout the study. Animals received a basal multi-nutritional diet and purified water, *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee.

Chemicals and Kits:

Streptozotocin (STZ) was procured from MP Biomedicals, LLC (USA). 2-Thiobarbituric acid, Reduced glutathione, 1,1,3,3-tetramethoxypropane, and Nitrobluetetrazolium were procured from Sigma (St. Louis, MO, USA). Diagnostic kits like Total Cholesterol (TC), Triglyceride (TG), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) were purchased from Transasia Biomedicals Ltd., India. Rat insulin ELISA kit was procured from Abbkine, USA. SIRT1 primary antibody was procured from Santa Cruz Biotech, USA.

Dose Calculation:

Formulation	Weight per Tablet	Human Recommended dose	Animal dose calculated from human dose
Vasant Kusumakar Rasa (VKR)	156 mg	**Dose 1 (Low Dose):** 2 Tablets/day	**Dose 1 (Low Dose):** 28.08 mg/kg/day
		Dose 2 (High Dose): 4 Tablets/day	**Dose 2 (High Dose):** 56.16 mg/kg/day

Stock Solution Preparation:

Stock solution of VKR Tablets for Low Dose treatment was prepared by suspending 28.08 mg of powdered VKR Tablets in 10 mL of 0.5% Sodium carboxymethyl Cellulose (CMC).
Stock solution of VKR Tablets for High Dose treatment was prepared by suspending 56.16 mg of powdered VKR Tablets in 10 mL of 0.5% Sodium carboxymethyl Cellulose (CMC).
Stock solution of Glipizide was prepared by suspending 5 mg of powder in 10 mL of 0.5% Sodium carboxymethyl Cellulose (CMC).
Streptozotocin solution was freshly prepared each time in citrate buffer (pH 4.4) 1 to 2 minutes before administration.

Experimental Design:

Induction of type-2 diabetes and treatment

Type 2 diabetes was induced using low dose of STZ (35 mg/kg, i.p.) after 2 weeks of dietary modification using High Fat Diet.

High Fat Diet

High Fat Diet was prepared in laboratory consisting of 58% fat, 17% carbohydrate, and 25% protein as a percentage of total kcal as per the procedure described by Srinivasan et al (Srinivasan et al. 2005). The composition and method of preparation of High Fat Diet is as follows:

Ingredients	Quantity (g/kg)
Powdered normal pellet diet	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and mineral mix	60
dl-Methionine	03
Yeast powder	01
Sodium chloride	01

Required quantity of powdered normal pellet diet was mixed with specified quantity of casein, cholesterol, vitamin and mineral mix, dl-methionine, yeast powder and sodium chloride. Liquid form of lard was poured in the mixture and mixed well and "cakes" of mixture (100 gm) were prepared manually.

The animals with plasma glucose level greater than 300 mg/dL were considered diabetic and selected for the further study (Srinivasan et al. 2005). Treatment with Glipizide (Standard Drug) and Vasant Kusumakar Rasa (VKR) was started after confirmation of diabetes and continued for 4 weeks. The treatment was administered in the form of suspension of VKR and Glipizide prepared using 0.5% Sodium carboxymethyl cellulose (CMC).

Grouping of experimental animals:

Group I	Normal Control
Group II	Diabetic Control [STZ (35 mg/kg, i.p.)]
Group III	Diabetic + VKR (Low Dose; 28.08 mg/kg/day)
Group IV	Diabetic + VKR (High Dose; 56.16 mg/kg/day)
Group V	Diabetic + Glipizide (5 mg/kg)

Parameters Assessed:

Assessment of body weight

Body weight of the animals from different treatment group were recorded after 28 days of treatment.

Determination of plasma glucose and lipid marker enzymes

Blood was collected from retro-orbital plexus in micro centrifuge tube containing anti-coagulant. Plasma was separated by centrifugation at 4500 rpm for 20 minutes. Plasma glucose levels and lipid markers like Total Cholesterol (TC), Triglyceride (TG), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL) were determined using commercial diagnostic kits supplied by Transasia Biomedicals Ltd., India and estimated using ERBA Chem 7 Biochemical Analyser, Germany.

Determination of liver marker enzymes

Liver marker enzyme such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using diagnostic kits supplied by Transasia Biomedicals Ltd., India and estimated using ERBA Chem 7 Biochemical Analyser, Germany.

Measurement of Glycohaemoglobin

Glycohaemoglobin was determined in freshly collected whole blood sample at the end of study using ion exchange resin method as per manufactures protocol (Transasia Biomedicals Ltd., India)

Oral Glucose Tolerance Test (OGTT)

Oral Glucose Tolerance Test was performed after completion of 28 days treatment period. Animals were kept fasting for 8 hours prior to the blood withdrawal. Blood was collected from retro-orbital plexus to obtain baseline blood glucose level (0 minute). Subsequently, all the animals were dosed with 40% D-glucose (2g/kg body weight) solution orally. Blood was withdrawn at predetermined time interval of 0, 30, 60, 90 and 120 minutes from all groups and plasma glucose levels were determined. Remaining plasma sample of time 0 minute was stored in -80 °C deep freezer for measurement of plasma insulin levels.

Estimation of Plasma Insulin, Homeostatic Model Assessment- Insulin Resistance (HOMA-IR) and Insulin Sensitivity Index (ISI)

Insulin levels in plasma sample was determined using rat insulin enzyme linked immunosorbent assay (ELISA) kits supplied by Abbkine, USA. Homeostatic Model Assessment - Insulin Resistance (HOMA-IR) and Insulin Sensitivity Index (ISI) were calculated using the following formula:

$$\text{HOMA-IR} = (\text{Insulin} \times \text{Glucose}) / 22.5$$

$$\text{ISI} = \text{Ln}\ (1/\text{Fasting Insulin}*\text{Fasting Blood Glucose})$$

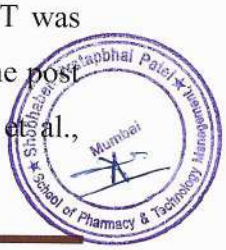
Where, concentration of insulin was expressed in mIU/L and glucose in mmol/L.

Pancreatic tissue collection and processing

At the end, animals from different treatment groups were humanly sacrificed and pancreatic tissue was isolated out for determination of oxidative stress parameters, histopathological studies and immunohistochemistry analysis.

Determination of oxidative stress parameters

For oxidative stress parameters, 10% (w/v) tissue homogenate was prepared in phosphate buffer (50mM, pH 7.4). Oxidative stress parameters like malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) were estimated in pancreatic tissue. GSH and MDA levels were determined in pancreatic tissue homogenate. CAT was estimated in the post-nuclear fraction of homogenate whereas SOD was estimated in the post mitochondrial fraction of homogenate (Ellman, 1959; Ohkawa et al., 1979; Paoletti et al., 1990).



Histopathology of pancreatic tissue:

For histopathology, pancreatic tissue was fixed in 10% buffered formalin. Formalin fixed tissues of pancreas were trimmed longitudinally and routinely processed. Tissue processing was done to dehydrate in ascending grades of alcohol, clearing in xylene and embedded in paraffin wax. Paraffin wax embedded tissue blocks were sectioned at 3 mm thickness with the Rotary Microtome. All the slides of pancreas were stained with Hematoxylin & Eosin (H & E) stain as per previously reported method for determination of tissue necrosis under a photomicroscope (Oza and Kulkarni, 2018a).

Immunohistochemistry

The sections of pancreatic tissue embedded in paraffin block were cut at 3 mm size, placed on poly-L-Lysine coated slide and incubated for 1 hour. Later the sections were deparaffinised and rehydrated. The rehydrated sections were incubated in citrate buffer (pH 6) using decloaking chamber. To block the endogenous peroxidase the sections were incubated in 3% hydrogen peroxide solution for 20 minutes. The sections were treated with the primary monoclonal antibody, i.e., Mouse anti-rat SIRT1 (B-7) (Santa Cruz Biotechnology, Inc., USA). The visual observation was performed using diaminobenzidine colour reagent and counter staining with Hematoxylin. Lastly the sections were washed with Tris-buffered saline (TBS) and dehydrated using alcohol. The dehydrated sections were cleared in xylene and mounted using DPX. The sections treated with antibodies were examined using light microscope to observe the intensity of antigen antibody reaction.

Statistical analysis:

All the data were expressed in Mean ± SEM. Statistical analysis was carried out using Graph pad prism 5 software. One way ANOVA followed by Bonferroni's multiple comparison tests was carried out to determine the level of significance and $p<0.05$ were kept as the level of significance.

4. RESULTS

4A. Effect of Vasant Kusumakar Rasa (VKR) on Body Weight

No significant change in body weight was observed in Normal Control, Diabetic Control and any of the treatment groups. (Refer Table No. 4A and Figure No. 4A)

Figure No. 4A:

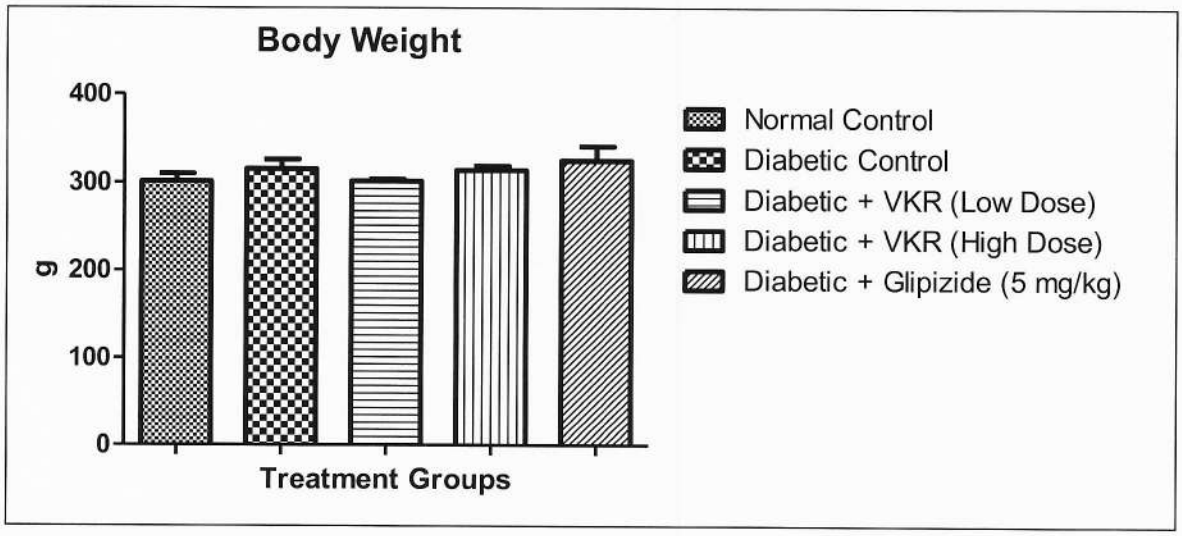


Table No. 4A:

Normal Control	Diabetic Control	Diabetic + VKR (Low Dose)	Diabetic + VKR (High Dose)	Diabetic + Glipizide (5 mg/kg)
301.2 ± 8.50	315.1 ±10.41	301.4 ± 2.19	313.7 ± 4.64	324.5 ± 16.30

All values are expressed as Mean ± S.E.M. (n=6).

4B. Effect of Vasant Kusumakar Rasa (VKR) on Plasma Glucose

Diabetic Control Group animals exhibited significant increase in plasma glucose levels as compared to the Normal Control Group animals ($^{###}p<0.001$). Treatment with **VKR** at low and high dose significantly reduced the levels of elevated plasma glucose levels as compared to the Diabetic Control Group animals ($^{*}p<0.05$). Glipizide treatment also exhibited significant reduction ($^{***}p<0.001$) in plasma glucose levels. No significant difference was observed in the **VKR** treated groups and Glipizide treated group. (Refer Table No. 4B and Figure No. 4B)

Figure No. 4B:

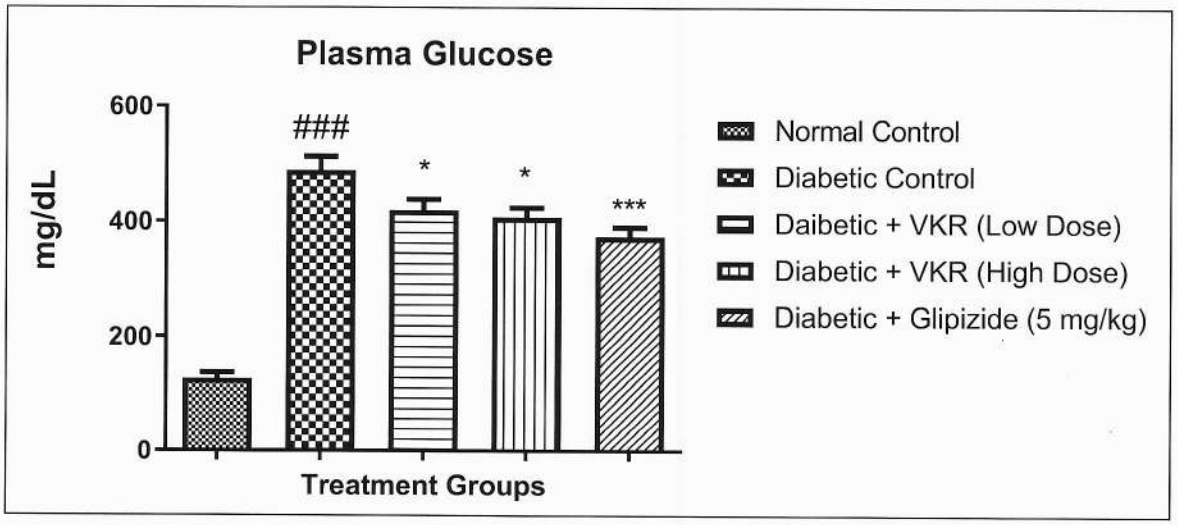


Table No. 4B:

Normal Control	Diabetic Control	Diabetic + VKR (Low Dose)	Diabetic + VKR (High Dose)	Diabetic + Glipizide (5 mg/kg)
126.7 ± 11.02	488.1 ± 24.34$^{###}$	419.8 ± 19.08	407.7 ± 16.39*	341.4 ± 11.25***

All values are expressed as Mean ± S.E.M. (n=6). $^{###}p<0.001$ vs. Normal Control Group. $^{*}p<0.05$, $^{***}p<0.001$ vs. Diabetic Control Group.

4C. Effect of Vasant Kusumakar Rasa (VKR) on Lipid Parameters

Diabetic Control Group animals exhibited significant increase in plasma levels of total cholesterol and triglycerides as compared to the Normal Control Group animals ($^{###}p<0.001$). Treatment with **VKR** at low and high dose significantly reduced the elevated plasma levels of total cholesterol and triglycerides as compared to the Diabetic Control Group animals ($^{*}p<0.05$ and $^{*}p<0.01$).

No significant reduction in the elevated plasma levels of total cholesterol and triglycerides was observed in the Glipizide treated group. (Refer Table No. 4C and Figure No. 4C)

Significant elevation was observed in the levels of Low-Density Lipoproteins in the Diabetic Control Group animals as compared to the Normal Control Group animals ($^{###}p<0.001$). Treatment with **VKR** at low and high dose significantly reduced the elevated levels of Low-Density Lipoproteins as compared to the Diabetic Control Group animals ($^{**}p<0.01$).

Glipizide treatment also significantly reduced the elevated levels of Low-Density Lipoproteins ($^{*}p<0.05$). (Refer Table No. 4C and Figure No. 4C)

Diabetic Control Group animals exhibited significant reduction in plasma levels of High-Density Lipoproteins as compared to the Normal Control Group animals ($^{###}p<0.001$). **VKR** at low and high dose significantly improved the plasma levels of High-Density Lipoproteins as compared to the Diabetic Control Group animals ($^{*}p<0.05$ and $^{***}p<0.001$).

No significant improvement in the plasma levels of High-Density Lipoproteins was observed in Glipizide treated group. (Refer Table No. 4C and Figure No. 4C)

Figure No. 4C:

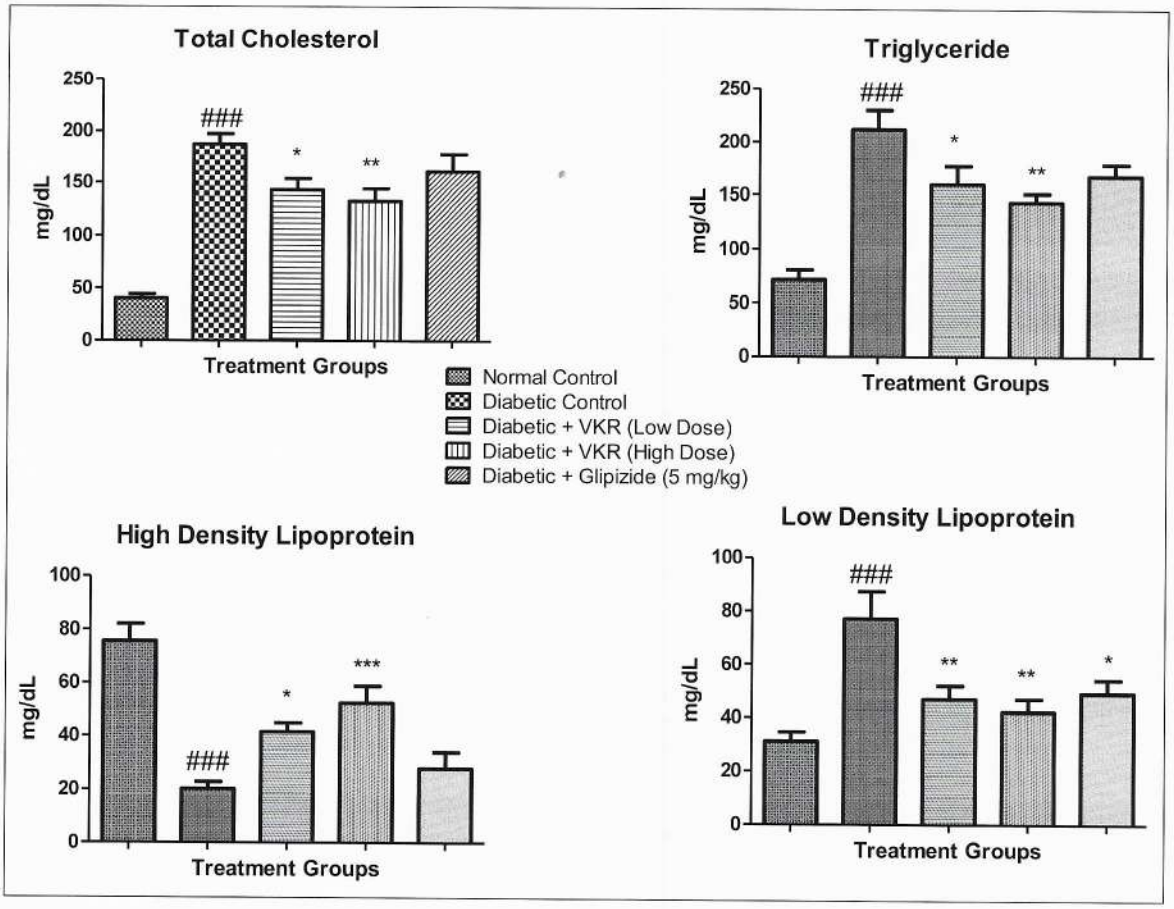


Table No. 4C:

	Normal Control	Diabetic Control	Diabetic + VKR (Low Dose)	Diabetic + VKR (High Dose)	Diabetic + Glipizide (5 mg/kg)
Total Cholesterol (mg/dL)	40.24± 10.68	186.7± 28.05###	142.7 ± 11.16*	132.4 ± 11.53**	160.9± 44.36
Triglyceride (mg/dL)	71.68± 9.055	211.8± 18.05###	159.7 ± 17.71*	142.9 ± 7.95**	167.4± 11.49
High density lipoprotein (mg/dL)	75.60 ± 6.474	20.22 ± 2.538###	41.54 ± 3.37*	52.35 ± 6.42***	27.84 ± 6.05
Low density lipoprotein (mg/dL)	31.02± 3.408	77.16± 10.15###	46.97 ± 4.97**	42.1 ± 4.83**	49.01± 5.152*

All values are expressed as Mean ± S.E.M. (n=6). ###p<0.001 vs. Normal Control Group. *p<0.05, **p<0.01, ***p<0.001 vs. Diabetic Control Group.

4D. Effect of Vasant Kusumakar Rasa (VKR) on Liver Marker Enzymes

Diabetic Control Group animals exhibited significant increase in the levels of AST and ALT as compared to the Normal Control Group animals ($^{###}p<0.001$). Treatment with **VKR** at low and high dose significantly reduced the elevated levels of AST (*p<0.05 and **p<0.01) and ALT (*p<0.05 and **p<0.01) as compared to the Diabetic Control Group animals.

Glipizide treatment also exhibited reduction in the levels of AST and ALT, but the reduction was significant only in the levels of AST (*p<0.05). (Refer Table No. 4D and Figure No. 4D)

Figure No. 4D:

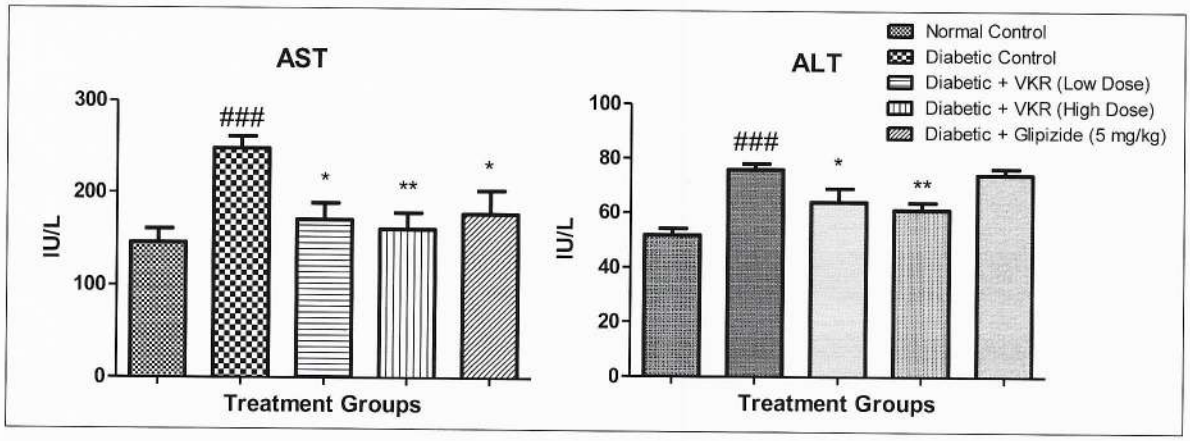


Figure No. 4D:

	Normal Control	Diabetic Control	Diabetic + VKR (Low Dose)	Diabetic + VKR (High Dose)	Diabetic + Glipizide (5 mg/kg)
AST (IU/L)	145.9 ± 14.92	248.4 ± 13.07$^{###}$	170.9.3 ± 18.31*	161 ± 17.47**	177.3 ± 25.23*
ALT (IU/L)	51.78± 2.46	75.87 ± 2.20$^{###}$	63.98 ± 4.95*	61.08 ± 2.73**	73.86 ± 2.40

All values are expressed as Mean ± S.E.M. (n=6). $^{###}p<0.001$ vs. Normal Control Group. *p<0.05, **p<0.01 vs. Diabetic Control Group.

4E. Effect of Vasant Kusumakar Rasa (VKR) on Glycohaemoglobin (HbA1c) levels

Diabetic Control Group animals exhibited significant increase in the levels of HbA1c as compared to the Normal Control Group animals ($^{###}p<0.001$). Although all the treatment groups exhibited reduction in HbA1c levels as compared to the Diabetic Control Group animals, the reduction was significant only in **VKR** high dose group ($*p<0.05$) and Glipizide treated group ($***p<0.01$). No significant difference was observed in the **VKR** high dose treated group and Glipizide treated group. (Refer Table No. 4E and Figure No. 4E)

Figure No. 4E:

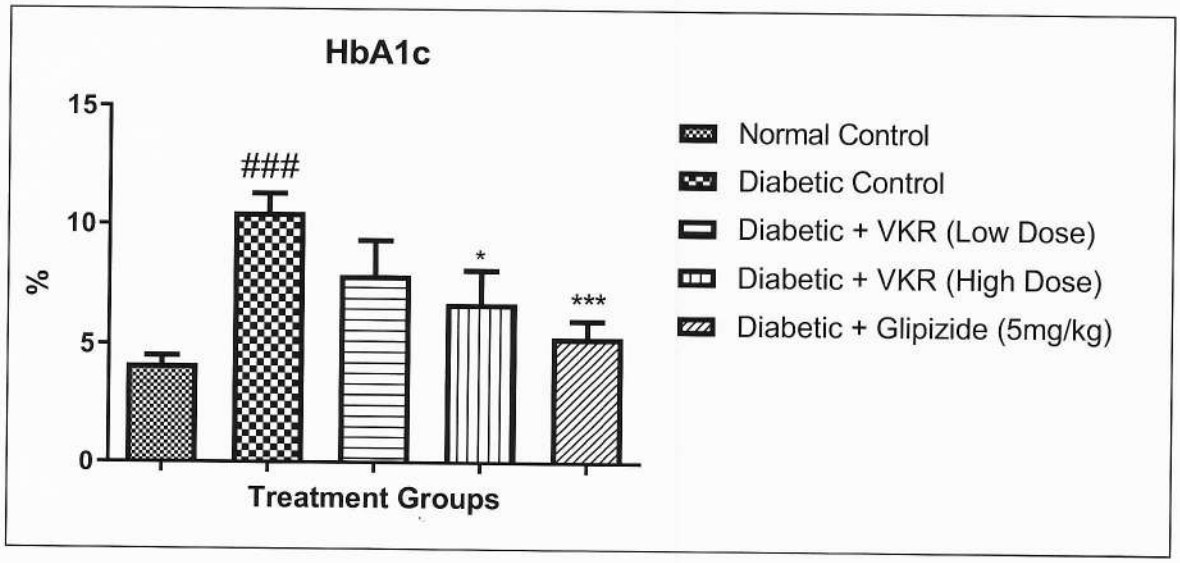


Table No. 4E:

	Normal Control	Diabetic Control	Diabetic + VKR (Low Dose)	Diabetic + VKR (High Dose)	Diabetic + Glipizide (5 mg/kg)
HbA1c (%)	4.16± 0.35	10.50 ± 0.79$^{###}$	7.90 ± 1.42	6.75 ± 0.89*	5.34 ± 0.66***

All values are expressed as Mean ± S.E.M. (n=6). $^{###}p<0.001$ vs. Normal Control Group. $*p<0.05$, $**p<0.01$ vs. Diabetic Control Group.

4F. Effect of Vasant Kusumakar Rasa (VKR) on Oral Glucose Tolerance Test (OGTT)

Diabetic Control Group animals exhibited significant increase plasma glucose levels at 30, 60, 90, and 120 minutes as compared to the Normal Control Group animals ($^{###}p<0.001$). Although, all the treatment groups exhibited a gradual decline in OGTT curve, it was better at all time points in **VKR** high dose treated group. A significant improvement in OGTT curve after 120 minutes was observed in **VKR** low and high ($^{***}p<0.001$) and Glipizide treated ($^{***}p<0.001$) groups as compared to the Diabetic Control Group animals. (Refer Table No. 4F and Figure No. 4F)

Figure No. 4F:

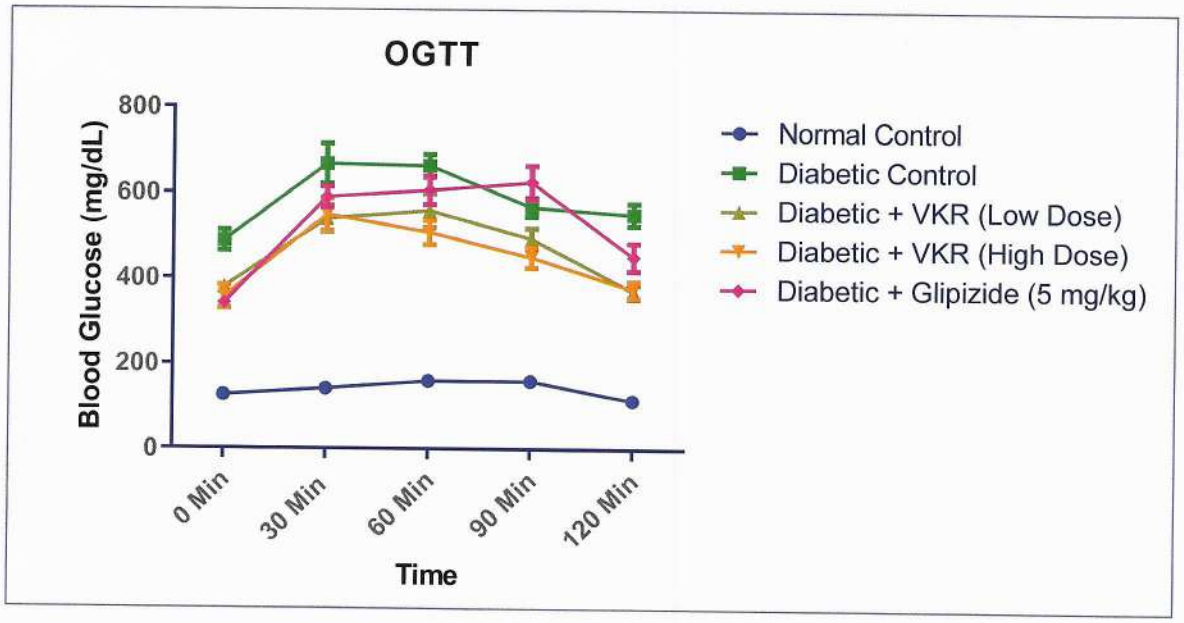


Table No. 4F:

Time	Normal Control	Diabetic Control	Diabetic + VKR (Low Dose)	Diabetic + VKR (High Dose)	Diabetic + Glipizide (5mg /kg)
0 min.	126.7 ± 11.02	488.1 ± 24.34$^{###}$	419.75 ± 19.08	407.7 ± 16.39	341.4 ± 11.25***
30 min.	142.6 ± 4.21	668.4 ± 46.50$^{###}$	539.86 ± 30.93*	548.75 ± 38.12*	590.4 ± 24.05
60 min.	160.6 ± 6.48	664.1 ± 26.99$^{###}$	558.75 ± 40.09	507.94 ± 28.99***	606.33 ± 33.34
90 min.	160.3 ± 8.40	567.40 ± 22.31$^{###}$	493.51 ± 25.12	449.81 ± 24.12*	625.90 ± 38.54
120 min.	115.0 ± 5.22	550.90 ± 26.26$^{###}$	370.36 ± 16.14***	374.88 ± 19.77***	368.9 ± 21.40***

All values are expressed as Mean ± S.E.M. (n=6). $^{###}p<0.001$ vs. Normal Control Group. *p<0.05, **p<0.01, ***p<0.001 vs. Diabetic Control Group (at respective time points).

4G. Effect of Vasant Kusumakar Rasa (VKR) on Plasma Insulin, Homeostatic Model Assessment- Insulin Resistance (HOMA-IR) and Insulin Sensitivity Index (ISI)

Diabetic Control Group animals exhibited significant increase in the plasma insulin levels as compared to the Normal Control Group animals ($^{###}p<0.001$). Treatment with **VKR** at high dose significantly reduced the elevated levels of plasma insulin (***$p<0.001$) as compared to the Diabetic Control Group animals. Glipizide treatment also exhibited significant reduction in the plasma insulin levels (***$p<0.001$). (Refer Table No. 4G and Figure No. 4G)

HOMA-IR was also significantly elevated in the Diabetic Control Group animals as compared to the Normal Control Group animals ($^{###}p<0.001$). Treatment with VKR at low and high dose significantly reduced the elevated HOMA-IR levels as compared to the Diabetic Control Group animals (***$p<0.001$). Glipizide treatment group also exhibited a significant reduction in HOMA-IR (***$p<0.001$) levels. (Refer Table No. 4G and Figure No. 4G)

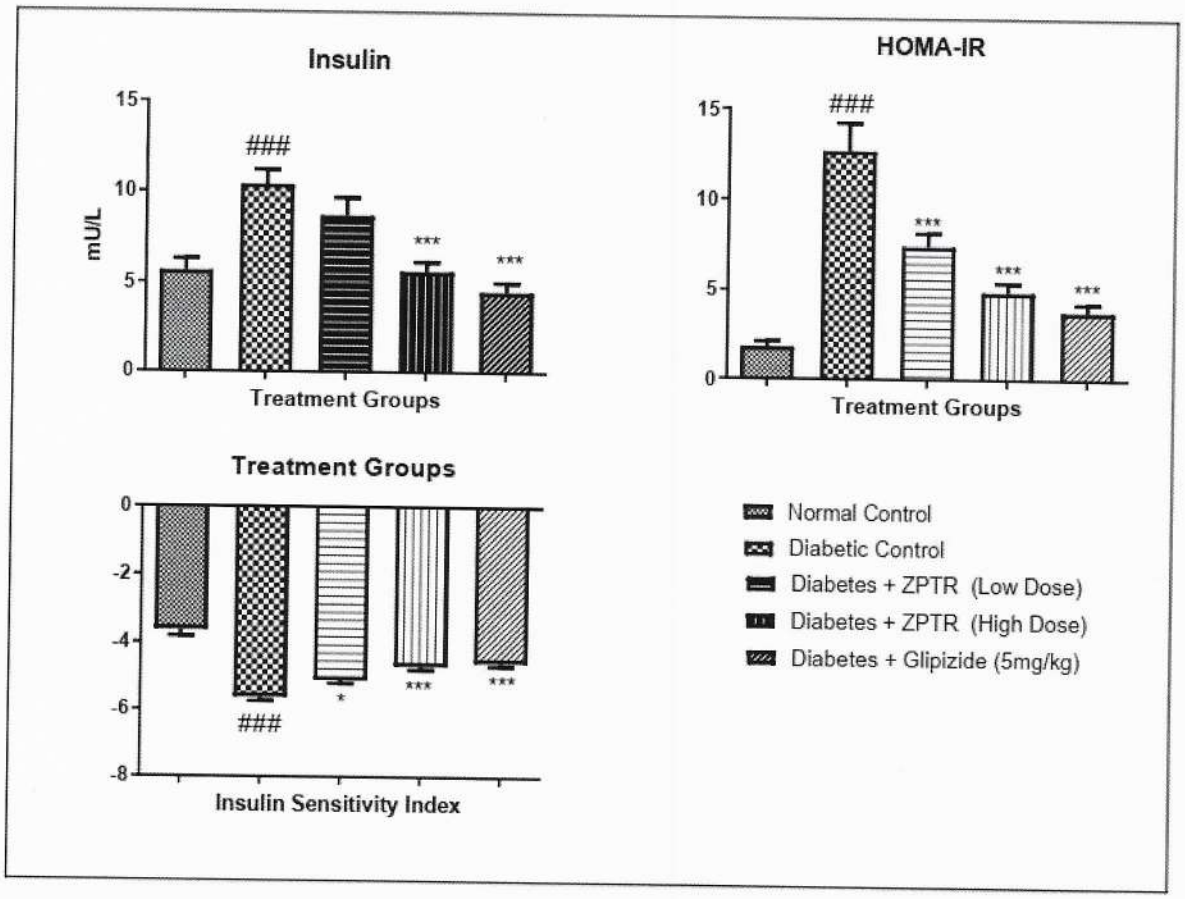
ISI was significantly impaired / reduced in the Diabetic Control Group animals as compared to the Normal Control Group animals ($^{###}p<0.001$). Treatment with **VKR** at low and high dose significantly improved the impaired / reduced ISI levels as compared to the Diabetic Control Group animals (*$p<0.05$ and ***$p<0.001$). Glipizide treatment group also exhibited improvement in ISI (***$p<0.001$). (Refer Table No. 4G and Figure No. 4G)

Table No. 4G:

	Normal Control	Diabetic Control	Diabetic + VKR (Low Dose)	Diabetic + VKR (High Dose)	Diabetic + Glipizide (5 mg/kg)
Plasma Insulin (mU/L)	5.644 ± 0.645	10.39 ± 0.816$^{###}$	7.300 ± 0.866	4.978 ± 0.611***	4.534 ± 0.505***
HOMA-IR	1.805 ± 0.288	12.69 ± 1.55$^{###}$	7.453 ± 0.727***	4.917 ± 0.510***	3.819 ± 0.443***
ISI	-3.625 ± 0.188	-5.619 ± 0.114$^{###}$	-5.097 ± 0.103*	-4.678 ± 0.109***	-4.418 ± 0.121***

All values are expressed as Mean ± S.E.M. (n=6). $^{###}p<0.001$ vs. Normal Control Group. *$p<0.05$, ***$p<0.001$ vs. Diabetic Control Group.

Figure No. 4G:



###p<0.001 vs. Normal Control Group. *p<0.05, **p<0.01, ***p<0.001 vs. Diabetic Control Group. (n=6)

4H. Effect of Vasant Kusumakar Rasa (VKR) on Oxidative Stress Parameters

Diabetic Control Group animals exhibited significant reduction in the levels of GSH, SOD and CAT ($^{###}p<0.001$) and a significant increase in the levels of MDA ($^{##}p<0.01$) as compared to the Normal Control Group animals. Treatment with **VKR** at low and high dose significantly prevented the loss of GSH (*p<0.05 and **p<0.01) as compared to the Diabetic Control Group animals. Only, **VKR** treatment at a high dose significantly prevented the loss of SOD (*p<0.05) and CAT (**p<0.01) as compared to the Diabetic Control Group animals. Glipizide treatment did not exhibit significant prevention in the loss of GSH, SOD and CAT. (Refer Table No. 4H and Figure No. 4H)

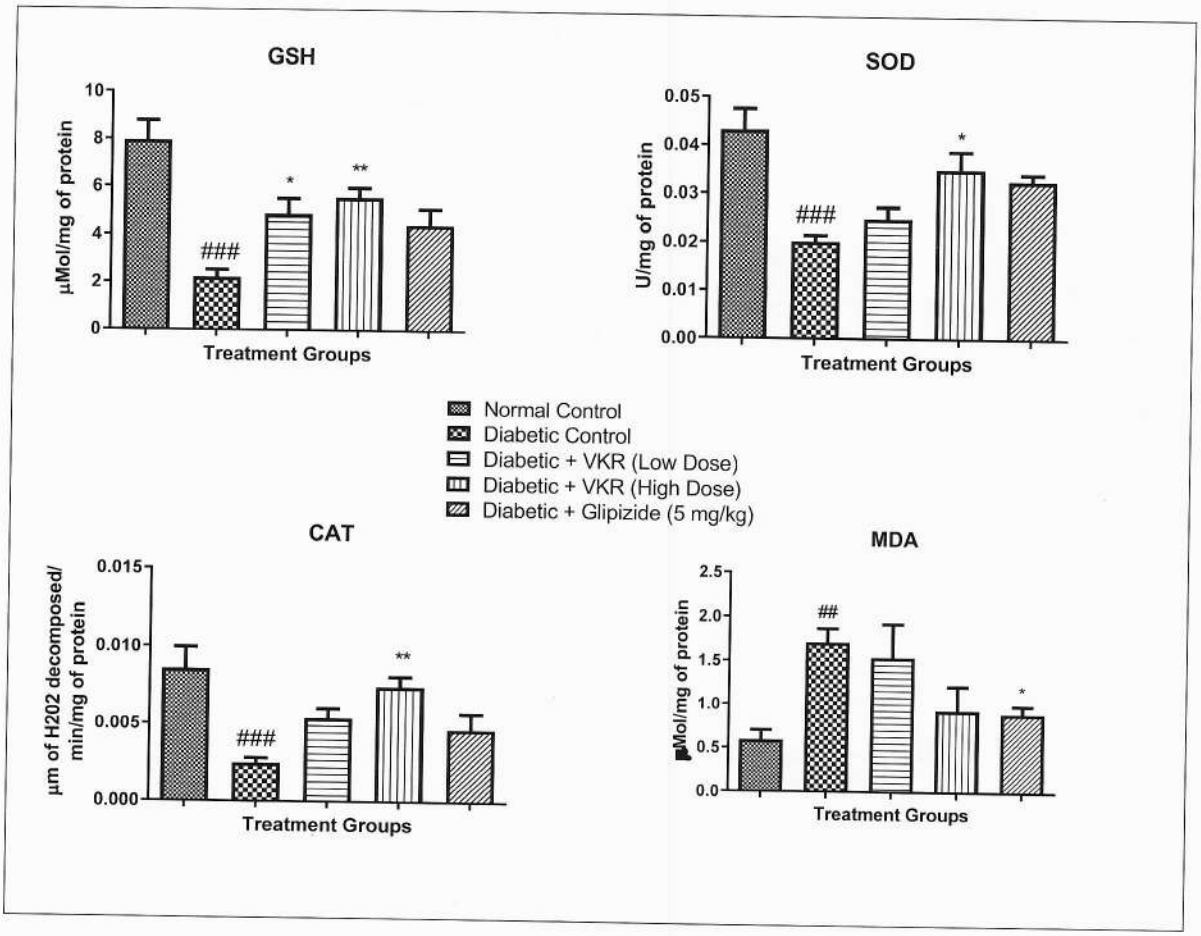
Although all the treatment groups exhibited a positive trend in prevention of MDA prevention in the pancreatic tissue homogenate, this was significant only in Glipizide treated group (*p<0.05). (Refer Table No. 4H and Figure No. 4H)

Table No. 4H:

	Normal Control	**Diabetic Control**	**Diabetic + VKR (Low Dose)**	**Diabetic + VKR (High Dose)**	**Diabetic + Glipizide (5 mg/kg)**
GSH (µMol/mg of protein)	7.949±0.82	2.214±0.31$^{###}$	4.883 ± 0.638*	5.583 ± 0.383**	4.46±0.65
SOD (U/mg of protein)	0.04314± 0.004319	0.02018± 0.001209$^{###}$	0.0249 ± 0.002	0.0350 ± 0.0037*	0.03269± 0.0013
CAT (µm of H_2O_2 decomposed/ min/mg of protein	0.008554± 0.001395	0.002448± 0.0003196$^{###}$	0.0054 ± 0.0006	0.0074 ± 0.0007**	0.004693± 0.0010
MDA µMol/mg of protein	0.5922± 0.1140	1.712± 0.1530$^{##}$	1.543 ± 0.377	0.9396 ± 0.27	0.9056± 0.08478*

All values are expressed as Mean ± S.E.M. (n=6). $^{##}p<0.01$, $^{###}p<0.001$ vs. Normal Control Group. *p<0.05, **p<0.01 vs. Diabetic Control Group.

Figure No. 4H:



$^{##}p<0.01$, $^{###}p<0.001$ vs. Normal Control Group. *p<0.05, **p<0.01, ***p<0.001 vs. Diabetic Control Group. (n=6)

4I. Effect of Vasant Kusumakar Rasa on pancreatic tissue histology

H & E staining (400X) of pancreatic tissue of Normal Control Group animals exhibited normal pancreatic tissue histology i.e., acinus (A), interlobular duct (IL), intercalated duct (IC) and Islet of Langerhans (L). Diabetic Control Group animals exhibited a significant increase in adipose deposition at acini lobule (AD), degeneration of endocrine pancreas (D), lymphocytic infiltration (LI) as compared to the Normal Control Group animals. Treatment with **VKR** at a low and high dose reduced the severity of damage in pancreatic tissue. The degree of severity of damage in Disease Control Group animals and treatment groups are as depicted in Figure No. 4Ii, 4Iii, 4Iiii, 4Iiv & 4Iv and Table No. 4I.

Figure No. 4Ii:

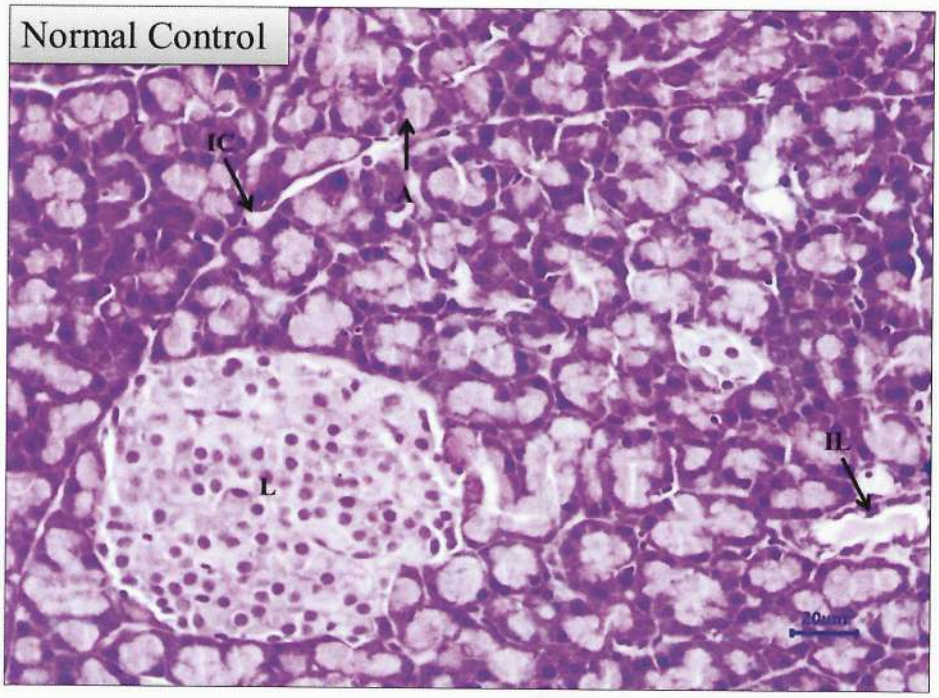


Figure No. 4Iii:

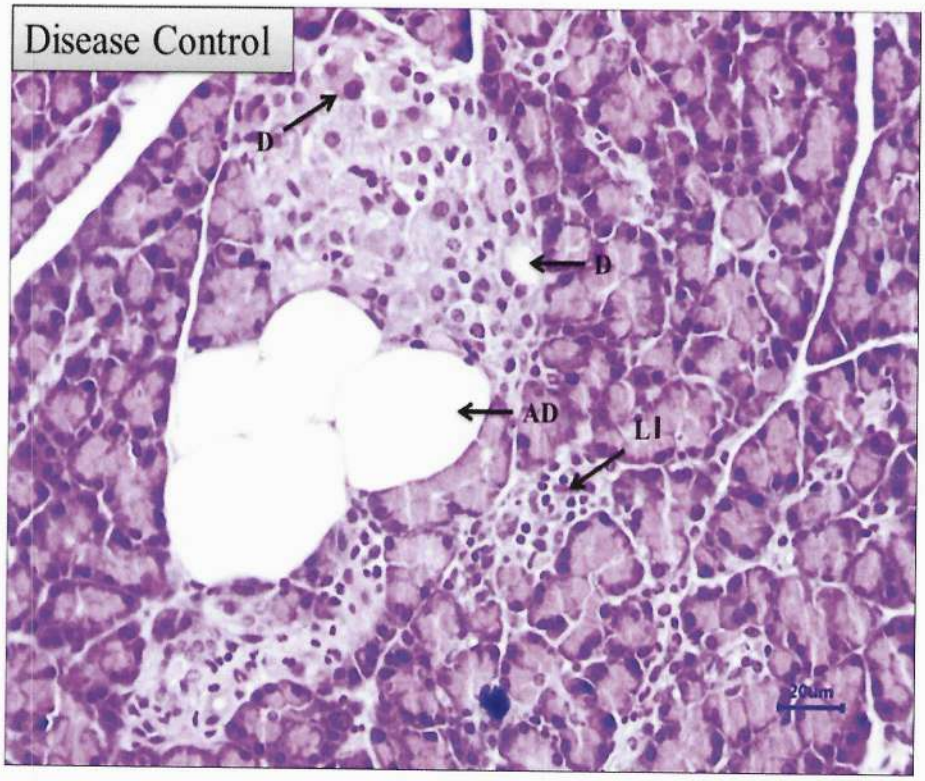


Figure No. 4Iiii:

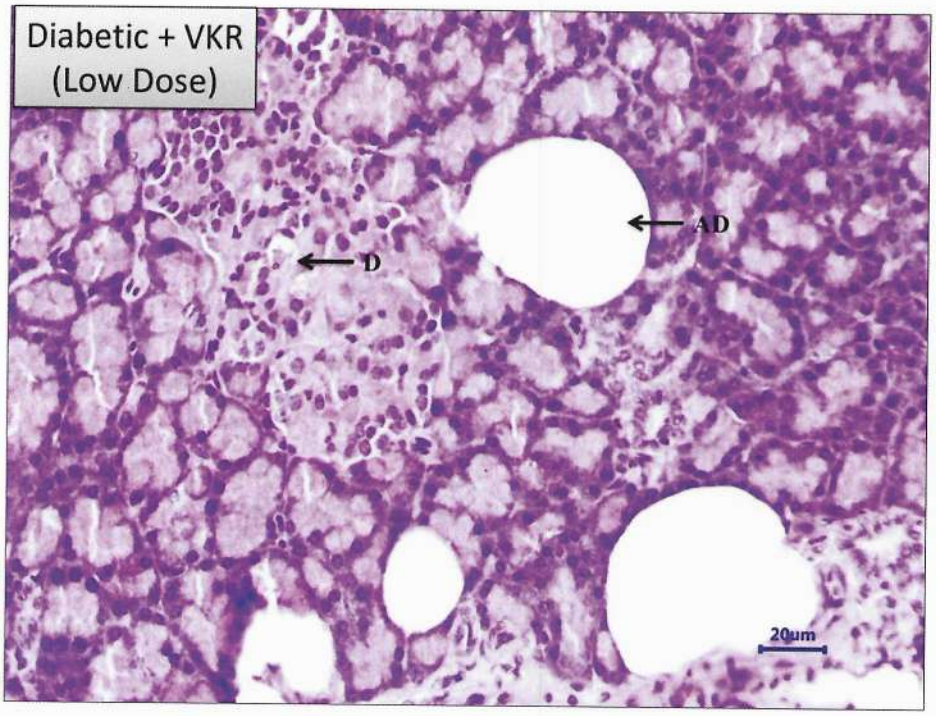


Figure No. 4Iiv:

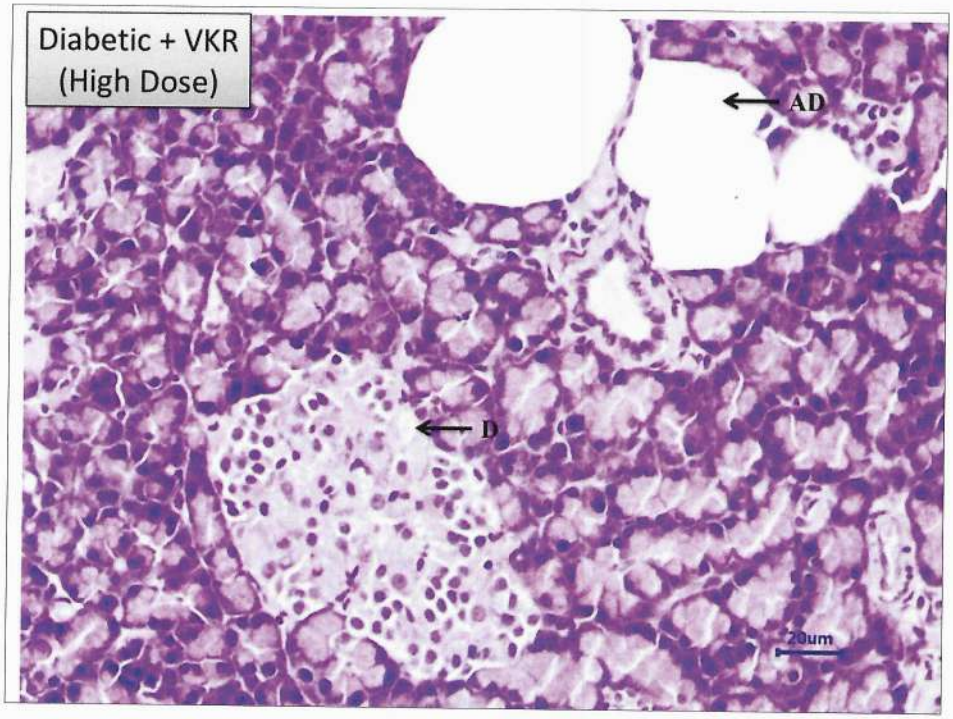
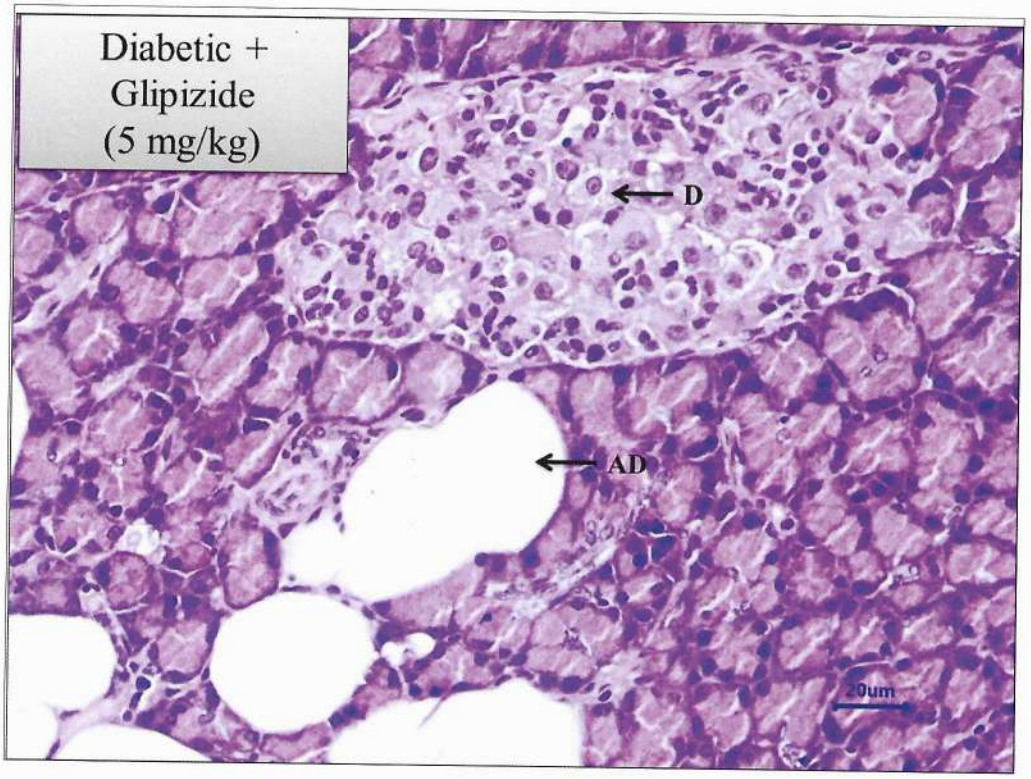


Figure No. 4Iv:



A- Acinus, IL- Interlobular duct, IC- Intercalated duct, L- Islet of Langerhans, AD- Adipose deposition at acini lobule, D- Degeneration of endocrine pancreas, LI- Lymphocytic infiltration

Table No. 4I:

	Normal Control	Diabetic Control	Diabetic + VKR (Low Dose)	Diabetic + VKR (High Dose)	Diabetic + Glipizide (5 mg/kg)
Focal mild to multifocal moderate adipose deposition at acini lobule	0	3	2	1	2
Focal minimal to multifocal mild degeneration of endocrine pancreas	0	3	1	1	1
Focal mild lymphocytic infiltration	0	2	0	0	0
Focal mild neutrophilic infiltration	0	2	0	0	0

0= Not Present, 1= Minimal (<1%), 2= Mild (1-25%), 3= Severe (26-60%)

4J. Effect of Vasant Kusumakar Rasa (VKR) on immunohistochemistry

Light microscopic examination of immunohistochemical stained slides of pancreas (400 X) revealed SIRT1 expression, which was evident in the form of positive brown particles in the Islet of Langerhans and acini of exocrine pancreas. Pancreas of Normal Control Group animals exhibited moderate to marked SIRT1 expression in Islet of Langerhans and acini of exocrine pancreas. Expression of SIRT1 in pancreatic tissue of Diabetic Control Group animals was significantly reduced as compared to the Normal Control Group animals. **VKR** treatment at low and high dose exhibited increase in SIRT1 expression in pancreatic tissue as compared to the Glipizide treated group and Diabetic Control Group animals. (Arrow shows expression of SIRT1 in Islet of Langerhans in Figure No. 4Ji, 4Jii, 4Jiii, 4Jiv & 4Jv)

Figure No. 4Ji:

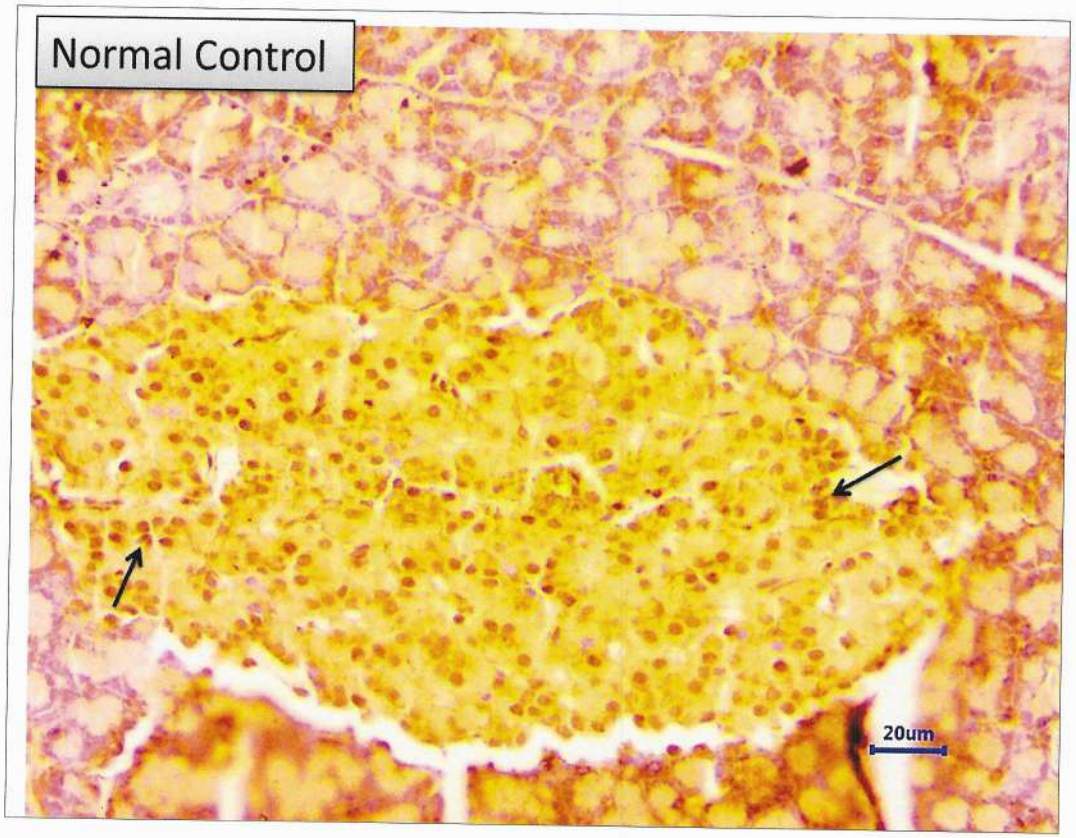


Figure No. 4Jii:

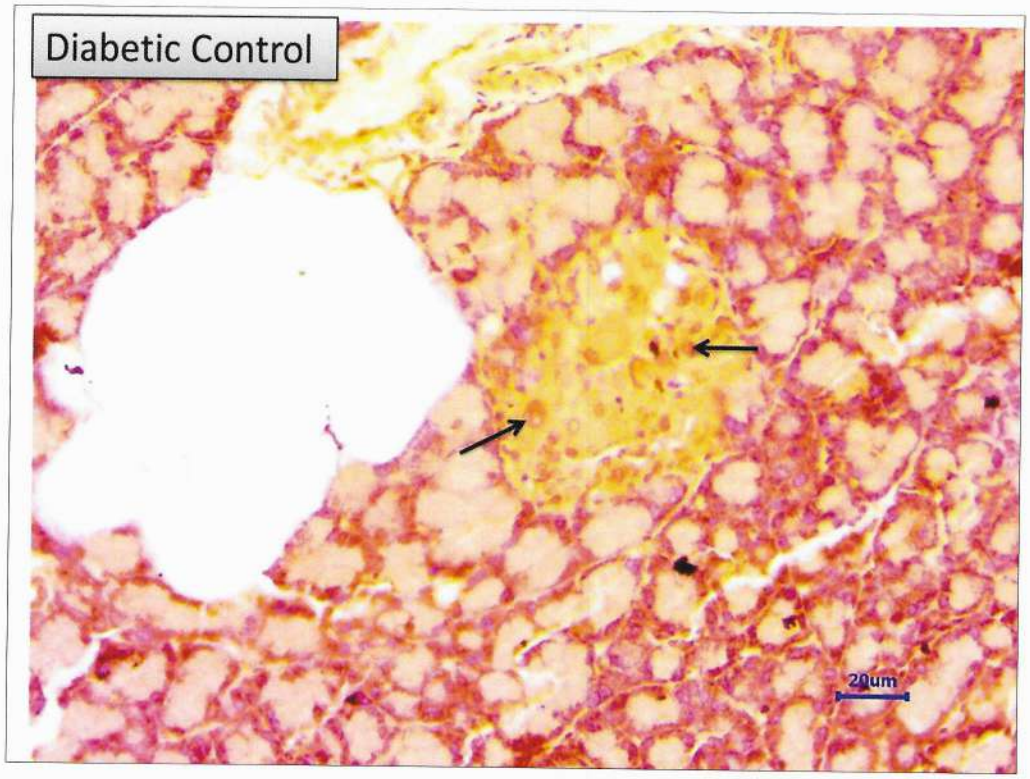


Figure No. 4Jiii:

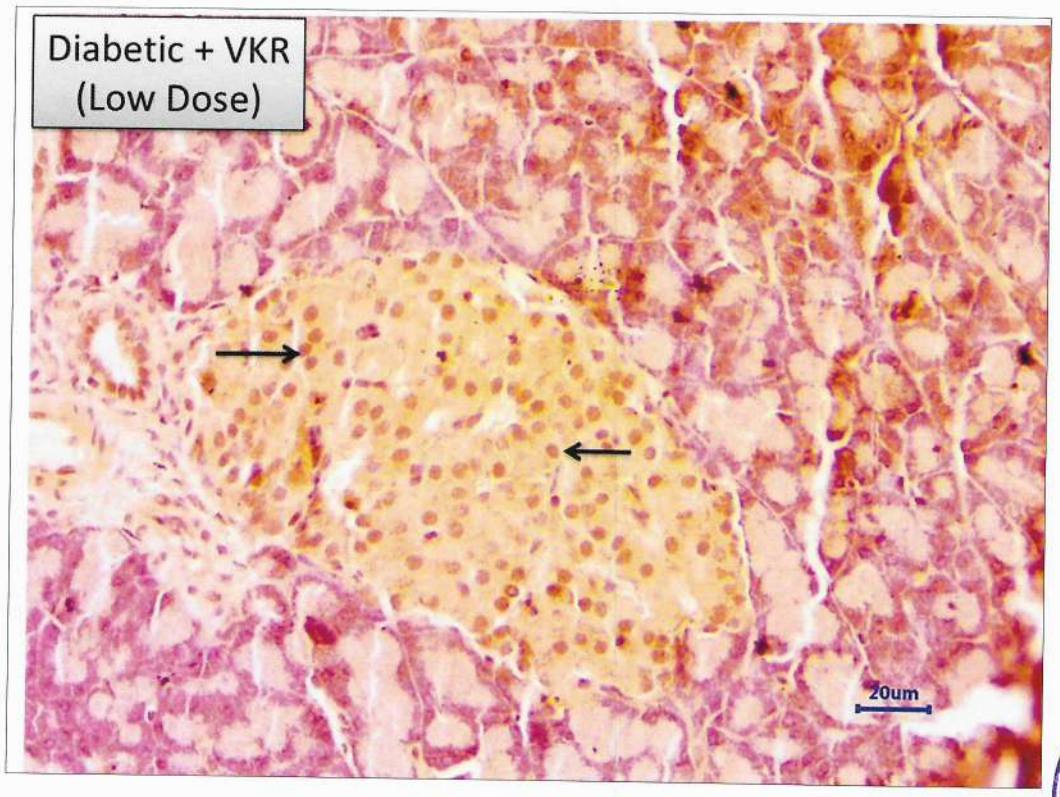


Figure No. 4Jiv:

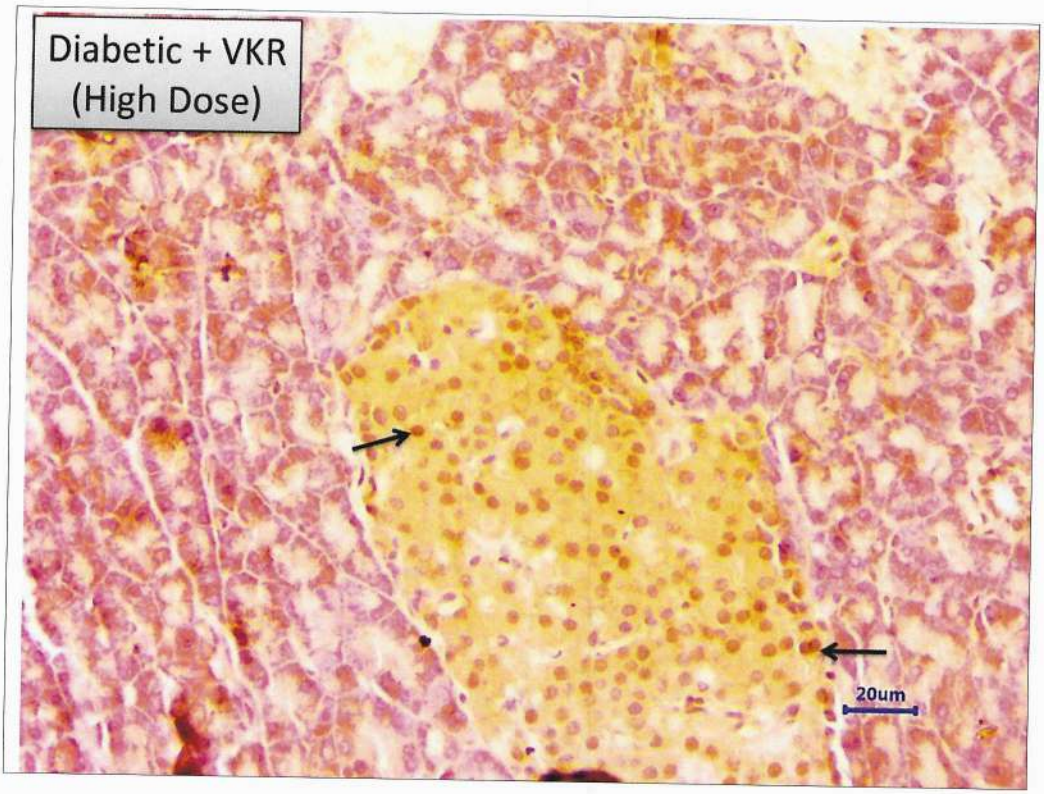
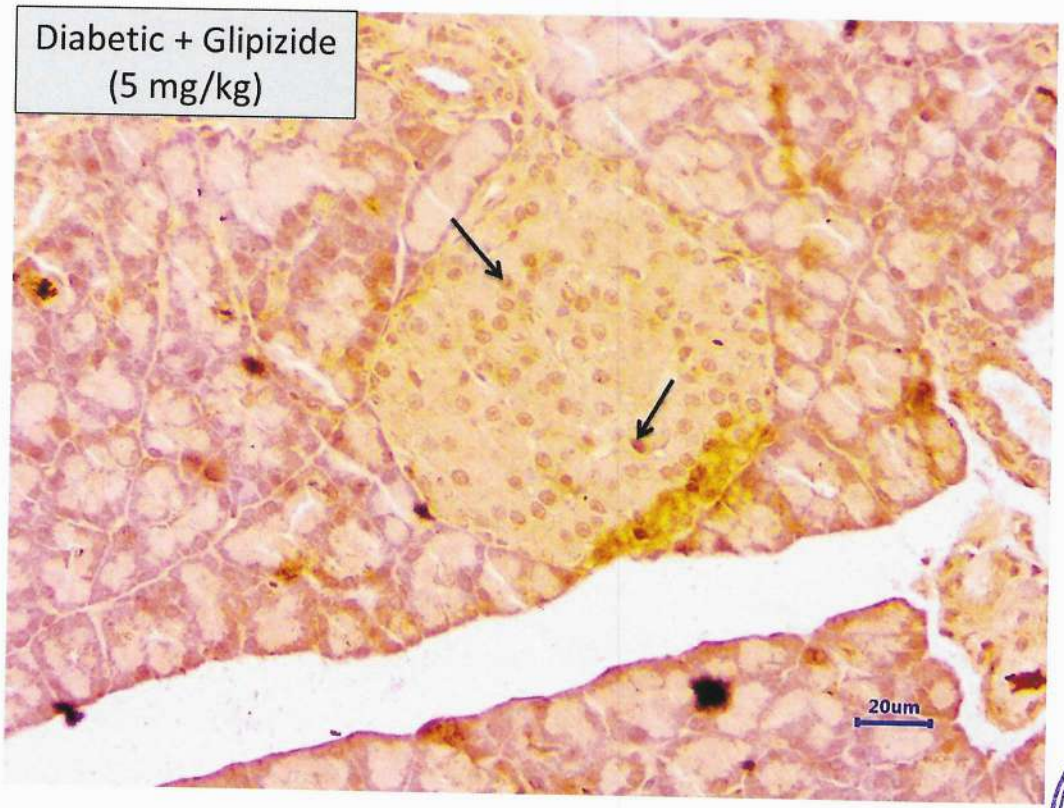


Figure No. 4Jv:



5. DISCUSSION

Various animal models are established to mimic type 2 diabetes in animals. In present study *Sprague Dawley* rats were selected to induce pathological features mimicking type 2 diabetes mellitus in humans (Båvenholm et al., 2001). Type 2 diabetes model development / establishment in rats, is characterized by 2 important features viz. reduction in insulin sensitivity and pancreatic β cell dysfunction. Reduction in insulin sensitivity was induced by means of dietary modification in the form of High Fat Diet (Ohtsubo et al., 2011). Pancreatic β-cell damage was triggered by streptozotocin (STZ) intraperitoneal injection. Dose of STZ plays an important role in induction of diabetes. Low dose of STZ is used to induce type 2 diabetes in rats (Srinivasan et al., 2005). Thus, to develop insulin resistance and partial β-cell dysfunction in this experimental study, the animals were fed High Fat Diet along with a single low dose of STZ. In the present study, all the animals except normal control animals were fed with High Fat Diet for 2 weeks before STZ administration to induce insulin resistance. High Fat Diet comprised of 58% fat, 25% protein and 17% of carbohydrate. STZ at dose 35 mg/kg was selected for partial pancreatic β cell destruction. Induction of type 2 diabetes is characterized by hyperglycemia, hypertriglyceridemia, hypercholesterolemia, and hyperinsulinemia, which is comparable to that of prediabetic, insulin-resistant state in humans (Reaven, 1991).

In Diabetic Control Group animals significant increase in plasma glucose levels was observed due to pancreatic β-cell damage and insulin resistance. Results of the **Oral Glucose Tolerance Test (OGTT)** also support these findings. **Vasant Kusumakar Rasa (VKR)** treatment in a low and high dose exhibited a significant reduction in elevated plasma glucose levels and gradual decline in **OGTT** curve as compared to the Diabetic Control Group animals. The reduction in plasma glucose in **VKR** treated groups was comparable to the Glipizide treated group.

The animals in **VKR** high dose treated groups exhibited a significant reduction in the elevated plasma insulin concentration as compared to the animals in Diabetic Control Group. This indicates its role in improving of insulin sensitivity or reducing insulin resistance. **VKR** treatment in both the doses improved the Insulin Sensitivity Index (ISI) and reduced the elevated HOMA-IR levels which indicate improvement in glucose metabolism and proper utilization of insulin by the tissues.

Glucose homeostasis in the body mainly depends on proper insulin secretion from pancreatic β cells and tissue sensitivity towards insulin to increase glucose uptake. In normal states insulin suppresses hepatic glucose production and promotes glucose disposal in the peripheral tissues. However, in type 2 diabetes these mechanisms are disturbed resulting into insulin resistance and hyperglycemia (Shanik et al., 2008). **VKR** treatment prevented pancreatic β cell damage and also improved insulin resistance.

Insulin resistance also plays an important role in development of dyslipidemia in type 2 diabetes. Alteration in triglyceride-rich lipoprotein metabolism and increase hepatic lipase activity results into increased level of triglycerides, cholesterol, and LDL with reduction in HDL cholesterol in diabetics (Hirano, 2018). Plasma lipid profile was found to be significantly dysregulated in the Diabetic Control Group. Treatment with **VKR** significantly improved lipid parameters. Increased levels of liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are indicators of hepatocellular injury. Increased levels of these markers are also associated with insulin resistance, metabolic syndrome, and type 2 diabetes. (Mohamed et al., 2016). **VKR** treatment prevented hyperglycaemia and prevented liver tissue damage which was evident from a significant reduction in the elevated levels of AST and ALT as compared to the Diabetic Control Group.

In type 2 diabetes, protein glycation is a natural process including formation of Glycohaemoglobin where glucose reacts with haemoglobin at N-terminal end of β -chain which is responsible for formation of Glycohaemoglobin (Sherwani et al., 2016). HbA1c does not give an idea of blood glucose on daily basis, but it indicates an average of blood glucose over past few days (Makris and Spanou, 2011). In the present study, **VKR** treatment steadily controlled blood glucose levels and thus prevented formation of Glycohaemoglobin as compared to Diabetic Control animals indicating stable control of blood glucose levels.

Oxidative damage to the pancreatic tissue due to prolonged hyperglycaemia also affects the antioxidant enzyme status in the pancreas. GSH is most abundant antioxidant present in the body. Together with SOD and CAT, GSH protects pancreas from superoxide, alkoxy radicals and H_2O_2 damage. Hyperglycaemia also causes polyunsaturated lipid peroxidation and forms MDA which is a reactive aldehyde and considered as electrophile species that develops toxic stress to the pancreatic tissue (Tsai et al., 1998). **VKR** treatment prevented oxidative damage

in the pancreatic tissue as evidenced by significant improvement in the levels of GSH, SOD and CAT in pancreatic tissue via its antioxidant mechanism.

Histopathological examinations of the pancreatic tissue in Diabetic Control Group revealed presence of adipose deposition at acini lobule, degeneration of endocrine pancreas and lymphocytic infiltration. Treatment with **VKR** reduced the severity of pancreatic tissue damage caused due to STZ administration. This indicates that, **VKR** has a protective effect on the pancreatic tissue.

Sirtuin 1 (SIRT1) protein, is a member of Silent Information Regulator 2 (Sir2) protein family and has a role in regulating cellular health. Evidence suggests that SIRT1 is involved in the initiation and progression of several diseases, particularly fibrotic diseases such as liver fibrosis, cardiac fibrosis, and renal fibrosis (Chou et al., 2017). Oza et al. have reported that upregulated SIRT1 expression inhibits the formation and development of pancreatic tissue fibrosis in type 2 diabetes in rats. Also, SIRT1 participates in regulation of glucose homeostasis by regulating hepatic glucose production and lipid metabolism along with regulation of insulin production and sensitivity (Oza and Kulkarni, 2018b). In our study, reduction in the expression of SIRT1 were observed in pancreatic tissue of diabetic control group. Treatment with **VKR** improved SIRT1 expression indicating protection against pancreatic tissue damage.

6. CONCLUSION

Treatment with **Vasant Kusumakar Rasa** significantly:

a. Prevented hyperglycaemia and reduced the elevated Glycohaemoglobin (%) in animal model of type 2 diabetes.
b. Improved the lipid parameters and reduced the elevated levels of liver marker enzymes.
c. Tackled insulin resistance by reducing plasma insulin levels, HOMA-IR and improving ISI.
d. Prevented the loss of antioxidant enzymes (GSH, SOD and CAT).

Vasant Kusumakar Rasa improved SIRT1 expression in pancreatic tissue. Histopathological findings also provide evidence for protective effect of **Vasant Kusumakar Rasa** treatment against pancreatic tissue damage.

From all the results, it can be concluded that **Vasant Kusumakar Rasa** possesses potent anti-diabetic activity in experimental model of High Fat Diet and low dose streptozotocin induced type 2 diabetes.

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Dr. Yogesh A. Kulkarni
Associate Professor
Study In-charge

Shobhaben Pratapbhai Patel School of Pharmacy & Technology Management Mumbai