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Vigna radiata and *Allmania nodiflora* improves T2DM in rats: Focus on factors affecting metabolism and insulin resistance

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Abstract
Despite an extensive understanding of T2DM, its management is always difficult in terms of both prevention of occurrence and treatment after incidence. The present study aims at determining the preventive and protective effects of bioactivity-guided fractions of *Vigna radiata* and *Allmania nodiflora* in streptozotocin-treated rats fed with a high-fat diet. The bioactive extracts of *Vigna radiata* and *Allmania nodiflora* were administered at both 20 mg/kg and 40 mg/kg doses each for 28 days and the changes in the blood glucose, insulin and leptin levels were measured to determine the effects of the treatment on T2DM. Additionally, serum lipid profile and atherogenic index were evaluated to assess the influence on insulin resistance. The results showed that treatment with *Vigna radiate* and *Allmania nodiflora* ameliorated the elevated blood glucose levels and this effect correlates with the changes in insulin and leptin levels. Moreover, there was a dose-dependent decrease in the total low-density lipoprotein, triglycerides, and very-low-density lipoprotein levels, indicating the protective effect of the bioactive fractions of *Vigna radiate* and *Allmania nodiflora.*

Keywords: Diabetes mellitus, *Vigna radiata*, *Allmania nodiflora*, cholesterol, insulin

Introduction
Diabetes mellitus is a metabolic condition leading to several pathological abnormalities causing severe morbidity and mortality. Globally, estimated populations of 425 million area affected with Type 2 Diabetes Mellitus (T2DM) and this number is expected to increase to 629 million by the year 2045 [1]. Disruption of carbohydrate, protein and lipid metabolism are the main consequences of T2DM and is associated with insulin resistance [2]. Intake of food with high-fat content induces insulin resistance and is one of the underlying pathology of T2DM in majority of the individuals from developed and developing countries [3]. Glucose uptake by the cells is highly dependent on the sensitivity of the insulin receptors on cell surface and the development of cellular resistance to insulin increases the blood glucose levels, which trigger further insulin release from the pancreas, leading to hyperinsulinemia along with hyperglycemia causing this metabolic syndrome [4]. Thus, effective glycemic control can be attained by targeting these physiological changes in the insulin receptors with the treatment that can reverse loss of sensitivity. Existing antidiabetic medications are less effective in reversing the insulin resistance [5], thus reducing the efficiency of insulin mediated glucose uptake by the cells and peripheral utilization of glucose leading to prolonged increase in the blood glucose levels.
With a boom in population and change in the lifestyle, India, often known as the diabetes capital of the world, is seeing significant increase in the incidence of new cases of T2DM [6]. Additionally, due to certain characteristic abnormalities such as high C - reactive protein (CRP) levels, high insulin resistance, waist circumference and adiposity, the Asian Indian phenotype is more prone to T2DM and coronary artery disease.[7]Moreover, the onset of T2DM at an early stage in Indians makes this metabolic disorder a public health challenge to the future generations and requires the development of new, safe and effective treatment alternatives.
The use of medicinal plants for the treatment of various diseases is an integral process of mankind since the dawn of civilization. Developments in molecular biology have enhanced our understanding of the mechanism of action of these herbal sources, which differ in many

aspects from their synthetic counterparts [8]. Therefore, natural products of herbal origin, if identified and used in a systematic way, can play an important role in the treatment of T2DM in the coming days.

Vigna radiata (L.), cultivated for its edible seeds and consumed as sprouts across Asia is one of the common diet-based therapies for weight loss. Xylitol, in the seed hull has been reported to have beneficial effect on serum glucose, triglycerides, and cholesterol [9]. Prodelphinidins, procyanidins, and rhamnosides are some of the previously reported active constituents of *V. Radiata* and are potent antioxidants that inhibit tyrosinase [10]. Flavonoids, phenolic acids, organic acids and lipids have been identified from the seeds and sprouts contribute to its therapeutic effects against paralysis, rheumatism, coughs, fevers and liver ailments [11].

Allmania nodiflora, fruits of which were used by the native people for constipation and dysentery and the leaves were not only consumed as one of the green leafy vegetables but also used as febrifuge, hypolipidemicagent, nutritive and as anappetizer by the native people, they are alsoreported for their antidiabetic and antioxidant effects [12]. In this study, we report the protective effects of extracts of *Vigna radiate* and *Allmania nodiflora* in streptozotocin-induced rat modelsfed with high-fat diet.

Materials and Methods

Plant material and extraction

The *Vigna radiata* and *Allmania nodiflora* plants were collected from fields of Guntur, and hilly areas of Visakhapatnam in Andhra Pradesh, India, respectively. The seeds of *Vigna radiata* and whole plant of *Allmania nodiflora* were sundried and ground into coarse powder. The powders (500g each) were subjected to serial exhaustive extraction with several solvents, such as chloroform, methanol, petroleum ether and water based on their polarities. The solvent was then evaporated and the concentrate was dried off through rotavac with temperature set at 40-50 ^{0}C. The produced marc with considerable blood sugar lowering effect were selected for further fractionation by solvent separation using methanol, chloroform and n-butanol serially. The bioactive fraction is subjected to column separation and partitioned with mixture of two solvents with distant polarity (Chloroform and Methanol) at various ratios. The fraction with good biological activity is subjected to flash chromatography and the resulting bioactive fractions are isolated using preparative TLC, with 20 X 20 cm glass plates (0.5 mm) which are silica gel G coated. The fractions that are biologically active are eluted from the plates and were studied [13, 14].

Animals

Adult female wistar rats were procured from the animal house of Mahaveer Enterprises, Hyderabad, India. The animals were housed individually under standard laboratory conditions with room temperature at 22 ± 2 ^{0}C and 12 hr of light/dark cycle. The animals were adjusted to lab conditions one week prior to drug administration and were given standard pellet chow and water ad libitum. Standard pellet feed and high-fat diet were procured from Rayan's Biotech, Hyderabad, India. Approval of Institutional Ethical Committee was obtained for experimentation from the IAEC [IAEC/X/1/BCOP].

Induction of T2DM using high-fat diet (HFD) with STZ model: Rats were fed with high-fat diet for two weeks. Later, these rats were randomly selected and grouped into 8 groups (n = 8). Group I served as vehicle control, group II and group III served as negative control groups for HFD treatment and HFD + STZ respectively, group IV and group V were treated with *Vigna radiata* Chloroform-Methanol fraction (VRCM) at 20 & 40 mg/kg p. o., group VI and group VII rats were treated with *Allmania nodiflora* Chlorform-methanol fraction (ANCM) at 20 & 40 mg/kg respectively. Group VIII rats served as positive control. Rats in all the groups were administered with respective treatments for 28 days. Streptozotocin (STZ), 55 mg/kg i. p. was administered to all the groups except group I and II. High fat diet was administered to all the groups except group I throughout the study [15].

Fasting blood glucose levels were determined 30 min before drug administration on day 1, 7, 14, 21 and 28 using a Accu-chekActive^{TM} Test glucometer (Roche Diagnostics, Germany) through tail vein method [16]. Retro-orbital puncture method was followed for blood sampling to determine the concentrations of serum insulin and leptin levels, atherogenic index and serum lipid profile.

Serum insulin assay

Serum insulin was determined using the Mercodia rat insulin ELISA kit following manufacturer's protocol. Two monoclonal antibodies against the distinct antigenic determinants (epitopes) on the insulin molecule were selected. Throughout the incubation, insulin undergoes reaction with peroxidase-conjugated anti-insulin antibodies, which bind to the micro titration well. After washing off the unbound enzyme-labeled antibody, bound conjugated insulin was determined by the reaction with 200µL substrate 3, 3', 5, 5'-tetramethylbenzidine, chromogenic agent, in each well. The reaction was ceased on addition of acid to produce a colorimetric endpoint and the optical density was analyzed using microplate reader (Bio-tek Instrument Inc., USA) at a wavelength of 450 nm and was expressed as pmol/l [17].

Serum leptin assay

Leptin levels were detected using a commercial ELISA kit. Optical density was determined with the same micro plate auto reader (BioTek Instruments, Mumbai.) at 450 nm. The serum leptin was expressed as ng/ml [18].

Estimation of lipid profile

Blood samples were collected through retro-orbital puncture and the plasma used for the estimation of total cholesterol [19], triglycerides [20], HDL-cholesterol [21], LDL-cholesterol and the atherogenic index was calculated as specified by Friedewald *et al.* [22].

In vitro alpha-glucosidase inhibitory activity

Inhibition of alpha-glucosidase by the VRCM & ANCM fractions were determined as per the procedure narrated by Kim *et al.*, [23]. The enzymatic reaction was done by taking PNP-glycoside substrate in 0.1 M PIPES buffer (pH 6.8). 1 mL PNP-glycoside (1.0 mM) was pre-combined with 0.1 mL fractions at various concentrations (2 µg/mL–200 µg/mL) in 2.05 mL PIPES buffer and the reaction system was preheated at 37 °C for 30 min. Then, 0.5 mL

enzyme solution (0.11 U/mL) was added into mixture and absorbance was measured at 30-second intervals for 15 min at 415 nm using UV Vis spectrophotometer. The inhibitory activity was obtained by the following equation: % Inhibition = (1-(sample/control)) X 100. Acarbose was also assayed as a positive reference. The number of fractions required to inhibit 50% of the enzymatic activity (IC_{50} values) was identified.

Statistical analysis

All the values were expressed as mean ± standard deviation (SD). All the groups were compared using one-way analysis of variance (ANOVA) and the intergroup significance was determined using Dunnet's test. $p<0.05$ was considered significant.

Results

Table 1: Effect of isolated fractions on blood glucose levels in the high-fat diet - STZ induced diabetes in rats

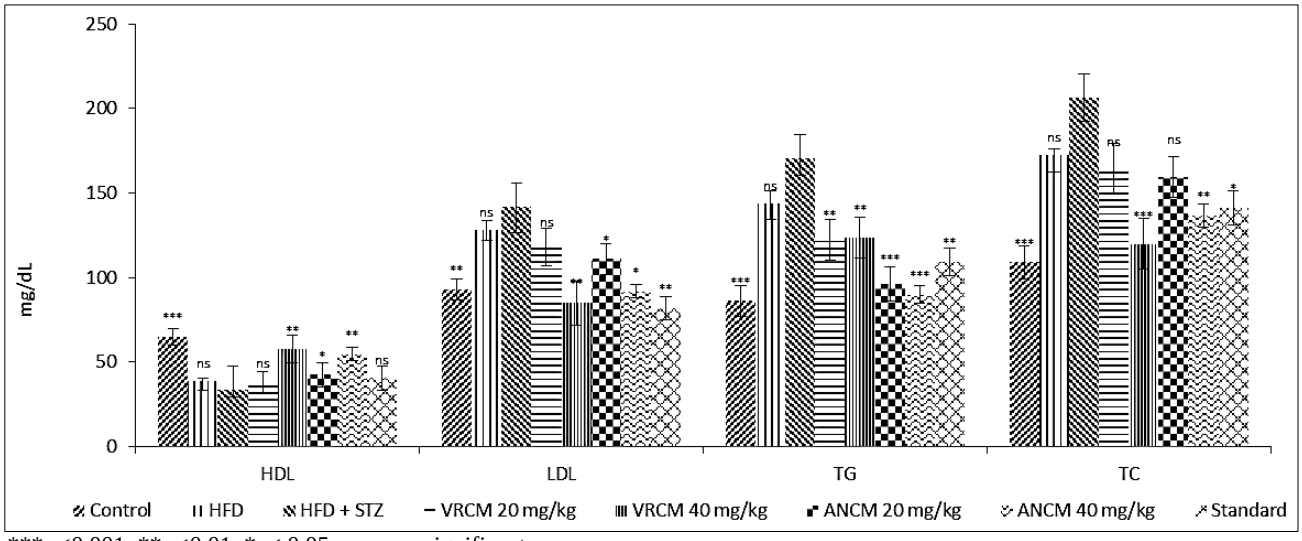
Treatment	Blood glucose levels (mg/dL) at various intervals (days)				
	1	7	14	21	28
Control	91 ± 3.1$^{\#}$	83.5 ± 1.6$^{\#}$	95.8 ± 3.6$^{\#}$	91.33 ± 2.9$^{\#}$	95.50 ± 3.8$^{\#}$
HFD	124.2 ± 4.8**	132 ± 3.7**	139 ± 7.2**	144.2 ± 10.4**	140 ± 8$^{\#}$
HFD + STZ	283.3 ± 7.6	250.8 ± 11.9	285.2 ± 12.6	290.7 ± 11.5	297.8 ± 18.7
VRCM 20 mg/kg	265.3 ± 11.5^{ns}	241.0 ± 10.5^{ns}	212.0 ± 16.4^{ns}	181.3 ± 7.7**	163.3 ± 4.8**
VRCM 40 mg/kg	258.8 ± 18.2^{ns}	220.5 ± 9.6^{ns}	201.2 ± 17.2*	159.8 ± 4.5$^{\#}$	130.5 ± 9.6$^{\#}$
ANCM 20 mg/kg	234.2 ± 10.1^{ns}	211.6 ± 12.4^{ns}	183.2 ± 6.1*	169.4 ± 6.2**	144.7 ± 6.1$^{\#}$
ANCM 40 mg/kg	258.1 ± 12.1^{ns}	207.2 ± 8.4^{ns}	176.3 ± 7.2**	153.7 ± 8.3$^{\#}$	124.2 ± 7.2$^{\#}$
Glibenclamide 0.5 mg/kg	262.8 ± 13.3^{ns}	180.8 ± 5.8*	161.0 ± 9.5**	120.0 ± 3.6$^{\#}$	109.8 ± 4.2$^{\#}$

Data are mean ± SD of blood glucose levels in rats treated with the indicated compounds for 28 days after the onset of diabetes. Blood glucose levels of all the groups (n = 6) were compared with that of negative control group (HFD + STZ) using one way ANOVA followed by Dunnett's test. #$p<0.001$, **$p< 0.01$, *$p< 0.05$, ns - non significant.

Table 2: Effect of isolated fractions on serum insulin and leptin levels

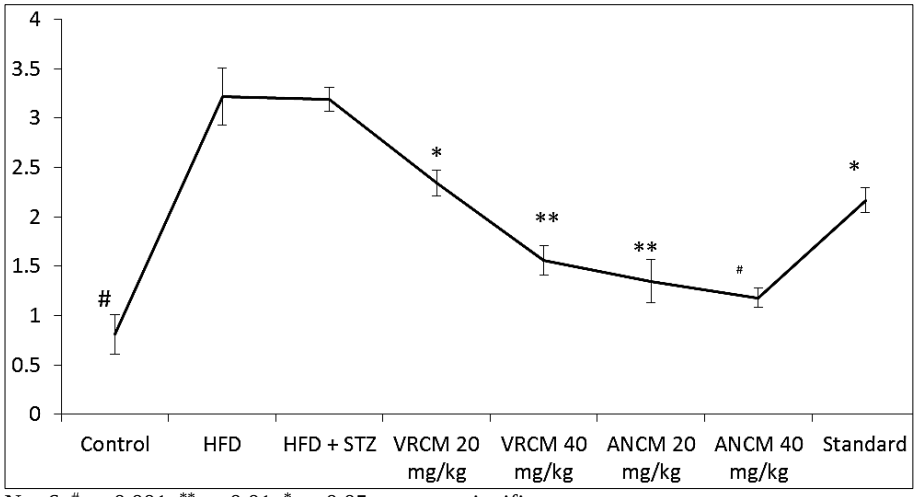
Treatment	Serum insulin (pm/l)	Serum leptin (µg/l)
Control	192.2 ± 7.2$^{\#}$	1.3 ± 0.2$^{\#}$
HFD	415.6 ± 19.5^{ns}	4 ± 0.14^{ns}
HFD + STZ	433 ± 11.3	4.3 ± 0.19
VRCM, 20 mg/kg	266.2 ± 16.6*	2.4 ± 0.21*
VRCM, 40 mg/kg	217.5 ± 9.2**	1.7 ± 0.11$^{\#}$
ANCM, 20 mg/kg	228.4 ± 8.8**	1.8 ± 0.15$^{\#}$
ANCM, 40 mg/kg	209.3 ± 7.4$^{\#}$	1.4 ± 0.2$^{\#}$
Glibenclamide, 0.5 mg/kg	277.8 ± 10.1*	1.2 ± 0.12$^{\#}$

Values are mean ± SD of serum insulin and leptin. Observations of all the treatments are compared with negative control group (HFD + STZ) using one way ANOVA followed by Dunnett's test. #$p< 0.001$, **$p< 0.01$, *$p< 0.05$, ns - non significant.



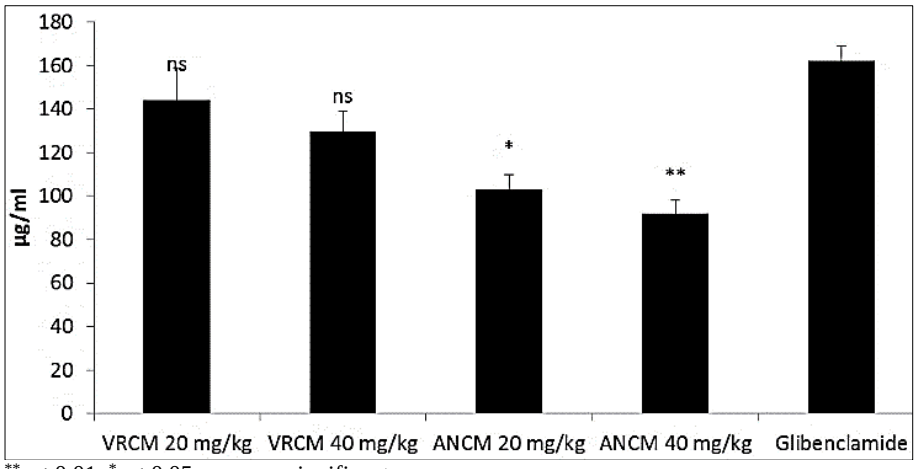
***$p<0.001$, **$p<0.01$, *$p< 0.05$, ns - non significant

Fig 1: Effect of VRCM and ANCM on the serum lipid profile (mg/dL). Observations of all the treatments are compared with negative control group (HFD + STZ) using one way ANOVA followed by Dunnett's test



N = 6, $^{\#}p< 0.001$, $^{**}p< 0.01$, $^{*}p< 0.05$, ns - non significant

Fig 2: Graph showing the effect of various treatments on atherogenic index in comparison to negative control (HFD + STZ) using one-way ANOVA followed by Dunnett's post hoc test



$^{**}p< 0.01$, $^{*}p< 0.05$, ns - non significant

Fig 3: Graph showing the IC_{50} (µg/ml) of α-glucosidase inhibition by various treatments. The inhibitory effects of isolated fractions are compared with that of Glibenclamide using one-way ANOVA followed by Dunnett's post hoc test

Discussion

Vigna radiata and *Allmania nodiflora* are traditionally used in the treatment of disorders related to blood glucose levels and obesity, though they are used for their antidiabetic effects, the therapeutic outcome varies widely inter individually. Moreover, the specific reason for this and the mechanism underlying these effects are not clear. Using the animal studies, it is demonstrated by us that the isolated bioactive fractions, VRCM and ANCM, ameliorates T2DM by regulating multiple factors such as insulin resistance, carbohydrate metabolism and endocrine function, which are the key factors underlying the pathophysiology of T2DM [24, 25]. In this study the anti-diabetic activity of the isolated fractions of the VRCM and ANCM were studied in STZ-induced diabetic rats under high-fat diet. A significant rise in the blood glucose was seen in rats with HFD than control group and is highly significant when rats were administered with STZ, indicating an additive effect of high-fat diet to the STZ in inducing diabetes. Treatment with VRCM 20 mg/kg exhibited a marked difference $p< 0.01$ in blood glucose only on the 21st and 28th day of initiation of therapy collated to negative control.

(HFD + STZ), whereas, VRCM 40 mg/kg exhibited a marked reduction $p< 0.05$, $p< 0.001$ and $p< 0.001$ on 14th, 21st and 28th days of treatment respectively. ANCM 20 mg/kg exhibited a marked reduction $p< 0.05$, $p< 0.01$ and $p< 0.001$ on the 14th, 21st and 28th days of treatment respectively. Whereas ANCM 40 mg/kg exhibited a marked reduction $p< 0.01$, $p< 0.001$ and $p< 0.001$ on 14th, 21st and 28th days of the treatment respectively as shown in Table-1.

A marked rise in the insulin was seen in rats under high-fat diet and HFD + STZ ($P< 0.001$), Treatment with VRCM 20 and 40 mg/kg had shown a marked reduction $p< 0.05$ and $p< 0.01$ respectively in the insulin levels compared to HFD + STZ treated group. ANCM 20 and 40 mg/kg had also shown a marked decrease $p< 0.01$ and $p< 0.001$ compared to that of HFD + STZ treated group. The observed results specifies development of insulin resistance in rats fed with HFD and gives out a novel proof for the use of *Vigna radiata* and *Allmania nodiflora* as a treatment for their weight loss and hypolipidemic effects. Blood glucose & Insulin levels are directly linked to the insulin resistance and weight gain. Abnormalities in glucose and (or) insulin tolerance, insulin, and other appetite-regulating hormones are widely reported in diabetic patients. The present study shows that treatment with VRCM and ANCM could ameliorate these abnormal conditions in rats. Extensive studies on diabetes confirmed that it is not solely insulin resistance or carbohydrate metabolism but also altered lipid metabolism plays a major role in its aetiology [26]. Increased

lipid levels are accompanied with T2DM, a well-known fact.

A marked increase $p< 0.001$ in the serum leptin was observed in both the negative control groups (HFD and HFD + STZ), when compared with that of control groups. VRCM 20 and 40 mg/kg exhibited a marked reduction $P< 0.05$ and $P< 0.001$ respectively in the leptin concentrations when compared to HFD + STZ treated groups. ANCM 20 and 40 mg/kg exhibited a marked reduction $p< 0.001$ when compared with that of negative control group. Table-2. leptin has an important role in regulating serum insulin levels and insulin resistance.

STZ induces, not only the apoptosis of β cells of the pancreas but also reduces the expression of pancreatic proteins such as Pancreatic and Duodenal homeobox 1 (PDX – 1) [27] which regulates the overall development of pancreas and function of β cells [28]. The observed increase in the insulin levels with VRCM and ANCM and decreased blood glucose levels can be attributed to these proteins as they are involved in regulating the improved function of β cells. Impaired glucose utilization by the body cells, especially in the skeletal muscle, liver, and adipose tissue is much higher than the rest and is the prime underlying cause in the pathogenesis of T2DM [29, 30]. This study had shown that HFD + STZ had increased the blood glucose levels, which can be explained either to be due to reduced insulin receptors or increase in number of non-functional ones. Several studies on simultaneous use of HFD and STZ reported that such treatment-induced T2DM affects the cascade of signaling in the insulin receptor, proteins such as Insulin receptor substrate-1 are inhibited, affecting the activation of the receptor's enzymatic activity in the cascade, which in turn prevents the translocation of GLUT4 in the plasma membrane, affecting the rate of glucose uptake with insulin [31].

In the effect on total cholesterol, a marked increase $p< 0.001$ was observed in the negative control group (HFD + STZ) compared with that of control rats, treatment with VRCM and ANCM 20 mg/kg doesn't show a significant alteration. Whereas treatment with VRCM and ANCM 40 mg/kg exhibited a marked $p< 0.001$ and $p< 0.01$ decrease respectively. In the effect on LDL, a significant decrease $p< 0.001$ and $p< 0.05$ was observed in rats treated with VRCM 40 mg/kg and ANCM 20, 40 mg/kg respectively. Whereas the increase in HDL was significant $p< 0.001$, $p< 0.05$ and $p< 0.001$ with VRCM 40 mg/kg, ANCM 20 and 40 mg/kg respectively. Triglyceride (TG) levels reduced significantly $p< 0.01$ and $p< 0.001$ on treatment with VRCM & ANCM respectively on treatment with both the doses Figure - 1. Increased LDL increases the risk of atherosclerosis, whereas increased HDL decreases the same [32]. Flavonoids and other phytochemicals in both *Allmania nodiflora* and *Vigna radiata* may be the underlying cause that are involved in increasing the uptake of LDL and enhancing its degradation, either by increasing the LDL receptor encoding mRNA or inhibition of hepatic cholesterol biosynthesis [33]. HDL levels and coronary artery diseases are inversely correlated, hence their increase attributes them a protective role against cardiovascular complications in obese T2DM, who are at a greater risk.

Atherogenic index (AI) specifies the extent of accumulation of foam cells and plaque or fatty infiltration of lipids in heart, coronaries, aorta, liver and kidneys. The more the AI, more is the risk of all the above organs for oxidative damage [34]. A marked rise in the AI was seen in rats under high fat diet and HFD with STZ ($p< 0.001$) compared to that of control, VRCM 20 & 40 mg/kg exhibited a marked reduction $p< 0.05$ and $p< 0.01$ respectively in the atherogenic index, whereas treatment with ANCM 20 and 40 mg/kg exhibited a marked reduction $p< 0.01$ and $p< 0.001$ respectively, compared to the negative control (HFD + STZ) group. Standard also had shown a marked reduction $p< 0.05$ Fig 2. The observed reduction in the AI in test drug-treated rats confirms their role in reducing the risk of cardiovascular disease and further the insulin resistance [35].

In the effect on α-glucosidase inhibitory effect, VRCM 20 and 40 mg/kg had not shown any significant inhibition, whereas treatment with ANCM 20 and 40 mg/kg exhibited a marked inhibitory effect $p< 0.05$ and $p< 0.01$ respectively when compared with that of standard treatment Fig 3.

In T2DM, impaired activation of skeletal muscle glycogen synthase occurs with the insulin deficiency leading to the expression of GLUT-4 transporters, which decreases with the reduced activation of the enzyme PI-3K (Phosphatidylinositol 3 Kinase) and the possible cause might be due to defective phosphorylation of Insulin Receptor Substrate-1 that occurs with the deficient activation of glycogen synthase normally observed with insulin deficiency [36].

Conclusion

Our study demonstrated that *Vigna radiata* and *Allmania nodiflora* have significant anti-hyperglycemic effect in HFD-STZ induced T2DM in rats, their chemical constituent's bioactivity could be due to enhancement of the beta-cell function and further increasing the insulin levels in the rats with an additional decrease in the elevated leptin levels. Further exploration to report the active molecules from these bioactive fractions is required to get a lead molecule for the development of a new and potent antidiabetic drug.

Conflict of interest

The authors declare no conflict of interest.

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