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Isolation and Characterization of Anti-Diabetic Components (Bioactivity Guided Fractionation) from *Verbascum chinense* (Scrophulariaceae) Leaves.

Virender Kaur[1]*, Milind Pande[2], and Kumud Upadhyaya[3].

[1]NIMS Institute of Pharmacy, NIMS University, Shobha Nagar, Jaipur-Delhi Highway, Jaipur, Rajasthan, India, 303121.
[2]Department of Pharmaceutical Sciences, Kumaun University, Bhimtal Campus, Nainital, Uttarakhand, India, 263136.

ABSTRACT

To isolate and characterize anti-diabetic components (bioactivity guided fractionation) from the ethanolic extract of Verbascum chinense leaves. Fraction F3-F5 and F11- F12 were isolated from an ethanolic extract of *Verbascum chinense* leaves by column chromatography. Fraction F3-F5 and F11- F12 were screened for anti diabetic activity in streptozotocin induced diabetic rats by estimating serum glucose levels and other physiological parameters. The isolated bioactive components were elucidated on the basis of extensive spectroscopic (IR, Mass, 1H NMR) data analysis. The bioactive fractions F3-F5 and F11- F12 were found to be potent anti-diabetic by ameliorating glucose and other physiological parameters (body weight, food intake, water intake and urine output). The extensive spectroscopic data analysis reveals that, the isolated bioactive components elucidated as triterpenoids (lupenone) and phenolic acids (caffeic acid). Our present study concluded that lupenone and caffeic acid isolated from leaves of *Verbascum chinense* has great anti-diabetic potential.

Keywords: *Verbascum chinense,* bioactivity guided fractionation, triterpenoids, phenolic acids, anti-diabetic activity

Corresponding author

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INTRODUCTION

Diabetes mellitus (DM) is an endocrine metabolic disorder of carbohydrate, fat, and protein that results from the defective insulin action or secretion and leads to hyperglycemia, abnormalities of lipoprotein, a defect in enzymes, damage to pancreatic beta cells induced through oxidative stress [1]. The micro and macro vascular complications, cerebrovascular diseases [2, 3] neuropathy, nephropathy and cardiovascular degeneration are the major complications associated with diabetes [4, 5]. World Health Organization reported that over 150 million people all around the world were suffering from DM by the year 2000, and in the year 2010 the number had risen to 221 million and by the year 2025 around 300 million people will be suffering from diabetes. In urban areas, about 172 million people are diabetic, while in rural areas 119 million people are suffering from diabetes. By the year 2030, the expected difference is still to rise to 314 million people living in urban areas and 143 million in rural areas [6].

DM is characterized by elevated blood glucose levels. Type I and II are generally two types of DM. Type I diabetes is insulin-dependent that resulted from less amount of insulin production due to a defect in insulin gene[7]. In type II diabetes there is an imbalance between absorption of blood sugar and secretion of insulin. Type II DM is also developed due to postprandial hyperglycemia. The postprandial rise in blood glucose level can be decreased by inhibiting the enzymes that hydrolyze dietary carbohydrates. α-Amylase is secreted by the pancreas and the salivary glands. Starch gets converted into alpha-limit dextrins, maltose and maltotriose by cleavage of α-D-1, 4 glucosidic linkages by α-amylase action [8-10]. The enzyme α-glucosidase present in the epithelial mucosa of the small intestine digests carbohydrates by hydrolyzing polysaccharide to monosaccharide which is absorbed through the intestinal tract [11, 12]. Inhibition of α-amylase and α-glucosidase enzymes can slow uptake of dietary carbohydrates and suppress postprandial hyperglycemia. Gastrointestinal disorders such as bloating, visceral discomfort, diarrhoea, and flatulence are the common side effects caused by synthetic enzyme inhibitors, therefore, are less efficacious [13].

Medicinal plants with antidiabetic potential are serving as an alternative for the treatment of diabetes [14, 15]. Plants possessing hypoglycemic action, act by different mechanisms [16, 17]. Some of the medicinal plants inhibit endogenous glucose production [18] or interfere with gastrointestinal glucose absorption [19] or some may have insulin-like substances [20].

Verbascum chinense (*Celsia coromandeliana*) (Scrophulariaceae) is commonly known as “Gidar Tambaku”. It is annual, sparsely pubescent simple or branched herb with a height of about 30-90 cm [21]. *Verbascum chinense* is traditionally used as a sedative, astringent, febrifuge, and for skin eruptions. *Verbascum chinense* is also found effective in the treatment of diarrhoea and dysentery, cuts, wound healing, jaundice, skin disorders, as an anti-inflammatory, emetic [22, 23]. From the literature search, it was found that the plant *V. chinense* is used traditionally for the treatment of DM [24]. The present study was planned to scientifically validate antidiabetic activity of *V. chinense* leaves.

Collection and authentication of plant material

The leaves of *Verbascum chinense* were collected from Nainital region (Uttarakhand) in the month of April 2015, was identified and authenticated by Principal Scientist, National Bureau of Plant Genetic Resources Regional Station, (N.B.P.G.R) Niglat, Bhowali, Uttarakhand. The specimen has been deposited in Herbarium at N.B.P.G.R. Niglat, Bhowali, Uttarakhand with herbarium specimen number VK-03.

Extraction: The leaves of *Verbascum chinense* were shade dried, crushed and a coarse powder was made separately using a mechanical grinder. The dried powdered leaves of *V. chinense* (2.5 kg) each after defatting with petroleum ether was subjected separately to hot continuous extraction with ethanol using soxhlet apparatus for 72 h till solvent became colourless. The ethanol extract was further fractionated with chloroform, ethyl acetate, n-butanol. The liquids were concentrated in a rotary evaporator.

Pharmacological screening

***In vitro* antidiabetic activity**

Alpha-amylase inhibition assay

Plant extracts (500µl) and a (100-1000 µg/ml) of standard drug were separately added to 500 µl of 0.20 mM phosphate buffer (pH 6.9) containing enzyme alpha amylase (0.5mg/ml) solution and was incubated for 10 min at 25 ºC. Further 1% starch solution 500 µl in 0.02M sodium phosphate buffer (pH 6.9) was added to test tube. The reaction mixtures were incubated at 25 ºC for 10 min. By addition 1.0 ml of 3, 5 dinitro salicylic acid the reaction was stopped. Boiling of test tubes was done on water bath for few minutes and then reaction mixture was cooled at room temperature. The reaction mixture was diluted and absorbance was noted at 540 nm. Similar test for control was also performed. Acarbose was used as a standard drug [25]. The experiments were repeated three times and the % inhibition of the test sample or acarbose and IC_{50} [26] was calculated by the following equation:

$$\% \, inhibition = [100 - ((Abs \, sample] - Abs \, blank) \times 100)] \, Abs \, blank$$

Alpha-glucosidase inhibition assay

α- glucosidase inhibitory activity was performed as per the method adopted by Pistia Brueggeman and Hollingsworth, (2001) [27]. Phosphate buffer (500 µl) (50 mM; pH 6.8), enzyme (100 µl) (1 U/ml) and plant extract (10 µM) comprised the assay mixture. Pre-incubation of assay mixture for 10 min at 37°C was done and then glutathione 100 µl (1 mg/ml) and mM p-nitrophenyl-α-D-glucopyranoside 100 µl was incorporated to assay mixture followed by incubation at 37°C for 20 min. By adding (500 µl of Na_2CO_3 (0.1M)) the reaction was stopped. The formation of p-nitrophenol (yellow colour) was recorded at 405 nm. Same buffer solution was prepared for purified enzyme and substrate solutions. Acarbose was used as a positive control. The experiments were repeated three times and the % inhibition of the test sample or acarbose and IC_{50} [26] was calculated by the following equation:

$$\% \, inhibition = [100 - ((Abs \, sample] - Abs \, blank) \times 100)] \, Abs \, blank$$

Separation and isolations of chemical constituents from leaves of *V. chinense*

Thin layer chromatography of ethanol extract

Thin layer chromatography using silica gel G 60 F precoated plate was performed with suitable mobile phases; the examination of chromatograms was done under UV light **at short and long wavelengths.**

Column chromatography

Chromatography was carried out using silica gel (60-120 mesh) as an adsorbent. The column was taken and packed with cotton. The slurry of silica gel was prepared by using n-hexane. Slurry was poured gradually with continuous tapping of column for uniform packing up of the column. The ethanol extract was adsorbed separately on solid, dried silica, was added on top of the column and gradient elution was performed. The fractions were collected in a conical flask and marked. The marked fractions were subjected to thin layer chromatography. The fractions having similar R_f values were pooled and concentrated.

Table1: Fractions (F1-F12) extracted by different solvent systems Isolation of compound VC-1, VC -2 from F3-F5, and F11-F12

Fractions Collected	Solvent	Fraction
1-4	N-Hexane	F1
5-7	N-Hexane	F2
8-10	N-Hexane: Dichloromethane (9:1)	F3
11-13	N-Hexane: Dichloromethane(8:2)	F4

14-16	N-Hexane: Dichloromethane(7: 3)	F5
17-19	Dichloromethane	F6
20-22	Dichloromethane: Ethyl Acetate(9:1)	F7
23-24	Dichloromethane: Ethyl Acetate(8:2)	F8
25-27	Dichloromethane: Ethyl Acetate(7:3)	F9
28-29	Ethyl Acetate	F10
30-31	Ethyl Acetate: Methanol (9:1)	F11
32-34	Methanol	F12

All the extracts such as chloroform, ethyl acetate, n-butanol, and ethanol were evaluated for in vitro anti-diabetic activity. Only ethanol extract showed significant *in vitro* anti-diabetic activity and hence it was subjected to column chromatography.

As the Rf value of fraction F3-F5 were same, the fractions were mixed together and allowed to crystallize. It furnished yellowish amorphous powder and marked as VC-1, m.p. 170 - 171°C, a triterpenoid. It gave a positive Salkowski test and Libermann- Burchard's test. Similarly, fraction F11- F12 were mixed together and crystallized. It furnished white amorphous powder and was marked as VC-2, m.p. 137-140ºC, which gave a positive test for phenolic acids.

IR, Mass, and NMR spectroscopy elucidated the structure of compounds VC-1 and VC-2. Both VC-1 and VC-2 were subjected to acute toxicity and other pharmacological studies.

Acute toxicity study

The toxicity parameters of compounds VC-1, VC-2 were studied. Wistar rats (150 – 250 gm body weight each) of good physical health (n=6) of both the sex were randomly selected. The rats were fasted overnight and provided only with water, after that the extract was administered orally at 5 mg/kg b.w. to 2000 mg/kg b.w. by gastric intubation and observed for 14 days and no mortality was observed at any above-mentioned dose level . The compounds VC-1, VC-2 were regarded as non-toxic.

***In vivo* anti-diabetic activity**

Experimental animals

Wistar rats in good physical health (150 – 250 gm body weight each) 28 maintained on standard laboratory diet and water ad libitum kept under 12 hours light and 12 hours dark cycle were taken. The animals were rehabilitated to the laboratory environment for one week before the start of the experiment. The study was approved by IAEC before the experiment (Regd. No. KUDOPS/23).

Dosage

The compounds of V. chinense (VC-1 and VC-2) at a dose of 50 mg/kg (each) were suspended in distilled water using 2% gum acacia as the suspending agent. Similarly, the standard drug glibenclamide (30mg/kg) was also prepared. The vehicle, test samples and standard drug were administered by orally.

Experimental protocol

Wistar rats in good physical health were selected and divided randomly into 7 Groups with (n=6) as:

GROUP I Normal received 1 ml of (0.9%) normal saline orally
GROUP II Diabetic control, received 1 ml of 2% gum acacia suspension orally
GROUP III Diabetic group, received 50mg/kg b.wt of VC-1 orally
GROUP IV Diabetic group, received 50mg/kg b.wt of VC-2 orally
GROUP V Standard drug group, received glibenclamide, 30 mg/kg b. wt. orally

These animals received their doses by oral route using an oral gastric tube. All the groups were given respective doses daily for 21 days.

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Induction of diabetes

Diabetes was induced in each group using streptozotocin (STZ). Using fresh ice- cold citrate buffer (0.1 M, pH 4.5) STZ solution was prepared. Diabetic rats were fasted for 18 hours and diabetes was induced by giving a single i. p. injection of streptozotocin (STZ) (30 mg/kg b. w.) following the standard methodology of intraperitoneal route of drug administration. After six hours of STZ injection, the rats were supplied with 20% glucose solution in water bottles for next 24 hours. The FBGL of the rats was measured after 72 hours. The rats having FBGL above 200mg/dl were selected for further experimentation [29].

Collection of blood and determination of blood glucose levels

Collection of blood was done on the 1st day, 7th day, 14th day and 21st day through punctured tail vein and collected blood samples analysis was performed using glucose oxidase-peroxidase reactive strips and Accu-check Glucometer. Blood glucose levels were expressed in terms of mg/dl. [28]

Estimation of other physiologic specifications

Other physiologic specifications like food and water intake, urine excretion and body mass were keenly monitored throughout the study. Body mass of rats was recorded prior to the administration of the compounds VC-1, VC-2 and on 1st, 7th, 14th day 21st day. The food intake value was calculated by taking initial and final weight of feed provided to rats. Similarly the water intake value was calculated by recording initial and final volume of water provided to rats [29].

Statistical analysis

The results of the study such as fasting blood glucose level, body weight and other physiological parameters were mentioned as mean±standard error of means (S.E.M.) and analyzed for Newman Keuls test significantly different at (*p<0.05,**p<0.01, ***p< **0.001) when compared with diabetic control group.**

Compounds isolated from *Verbascum chinense*

Compound VC-1

Fraction F3-F5 furnished as yellowish amorphous powder m.p 170 - 171°C (VC-1), giving a positive test to a triterpenoids. Yield = 390mg M.P. =170 - 171°C. TLC was performed and a R_f value of 0.19 was observed when *n*-hexane: dichloromethane in the ratio of 3:2 was taken as mobile phase and Vanillin sulphuric acid was used as detecting agent. IR spectrum of compound VC-1 have shown ν max at 2918 cm-1 for C-H stretching, peak at 1706.4 cm^{-1} C=O stretching, peak at 1641.7 cm^{-1} for C=C stretching and peak at 882.4 cm-1 shows alkene out of plane C-H bending. NMR ($CDCl_3$) δ value at 1.107-1.134(12H)-CH3, 1.164-1.284(12H)-CH3, 1.33-1.39(10H)-CH2, 1.598(2H)-CH2, 1.976(2H)-CH2, 1.256(4H)-CH2, 1.39(1H)-CH, 1.43(1H)-CH, 2.000-2.107(2H)-CH, 4.569(1H)-CH, 4.688(1H)-CH.

In mass spectra, the [M+H] ion peak at 425 confirms the presence of the compound VC-1 as Lupenone.

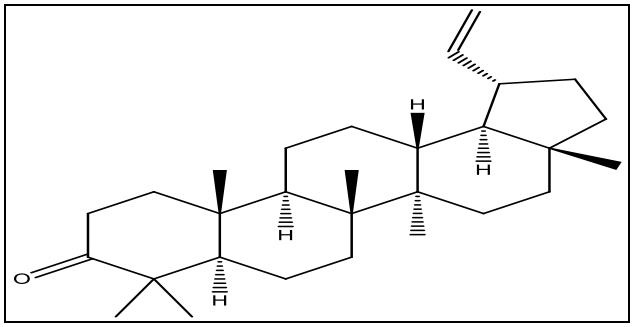

Fig 1: Lupenone

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Compound VC-2

Fraction F11- F12 furnished yellowish amorphous powder m.p =137-140ºC (VC-2), which after re-crystallization gave positive test for tannins and phenols. Yield = 310mg M.P = 137-140ºC. TLC was performed and an R_f value of 0.72 was observed when *n*-chloroform: methanol in the ratio of 7:3 was taken as mobile phase. IR spectrum of compound VC-2 have shown v max at 3368.34 cm^{-1} OH- stretching vibration of phenol-OH, peak at 3020.34 cm^{-1} shows the OH- stretching vibration of COOH , peak at 1710.79 cm^{-1} shows the C=O stretching vibration, peak at 1616.76 cm^{-1} shows the C=C stretching vibration -OC=O-, peak at 1538.36 cm^{-1} shows the C=C stretching vibration for benzene ring, peak at 1435.42 cm^{-1} shows the OH- bending vibration for phenol-OH, peak at 1215.37 cm^{-1} shows the C-OH stretching vibration for phenol-OH), peak at 665-900 cm^{-1} shows the aromatic C-H out of plane bending vibration.

NMR ($CDCl_3$) δ value at 7.357 [1H] ethylene, 7.09 [1H] CH-benzene, 7.058 [1H] CH-benzene, 6.818 [1H] CH-benzene, 6.4 [1H] ethylene, 5.301 Aromatic (C-OH), 5.301 Aromatic (C-OH), 11 [1H] Carboxylic acid. In the mass spectra, the [M+1] ion peak at 181 clearly confirms that the compound VC-2 as Caffeic acid.

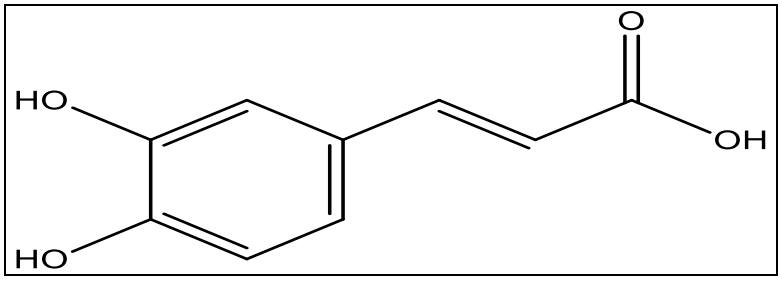

Fig 2: Caffeic acid

Pharmacological screening

Screening for *in vitro* antidiabetic activity

Crude chloroform, ethyl acetate, n-butanol and ethanol extracts of *Verbascum chinense* leaves were prepared by means of a sequential solvent extraction procedure and screened for inhibitory activities against α-glucosidase, α-amylase using standard procedures for assaying the activities of these enzymes. All extracts illustrated α-amylase and α-glucosidase inhibitory activity in dose-dependent manner. The % α-glucosidase and α-amylase inhibition at varying concentration are shown in Fig. 3, Fig.4. Inhibitory effect and IC_{50} values of extracts signifying substantial inhibitory activity against α-glucosidase and α-amylase were calculated and comparison was done with standard drug Acarbose. The significant α-glucosidase and α-amylase inhibitory activity were observed with ethanol extract of the plants *Verbascum chinense*. The ethanolic extract from leaves of the plant *Verbascum chinense* showed notable alpha-amylase (IC_{50} = 3.42µg /ml) and alpha-glucosidase inhibitory activity (IC_{50} =3.3µg/ml) as compared to standard acarbose (IC_{50} = 3.175µg/ml).

Table 2: *IC_{50}* Value of α--Amylase and α--Glucosidase inhibitory activity of various extracts of *Verbascum chinense*

Plant Extract	Inhibitory concentration IC_{50} (µg/ml)	
	α-Amylase	α-Glucosidase
Acarbose	3.175	3.175
Ethanolic	3.42	3.3
Chloroform	4.216	4.5
Ethyl Acetate	3.73	4.8
N-Butanol	3.92	3.7

The values are expressed as mean ±SEM (n=3)

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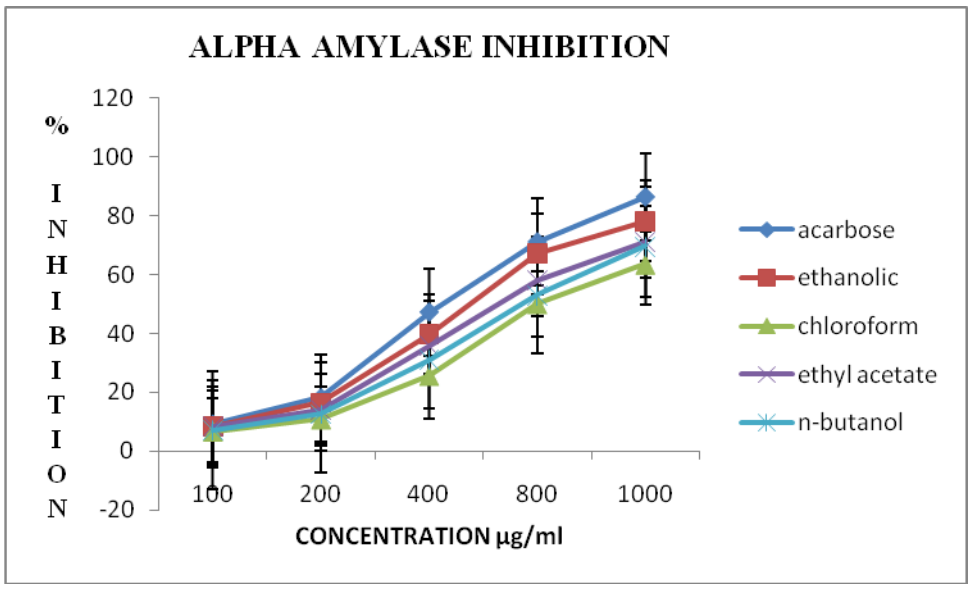

Fig 3: *In vitro* inhibition of α -amylase enzyme by various extracts of leaves of *Verbascum chinense.* The results represents the mean±SEM (n=3)

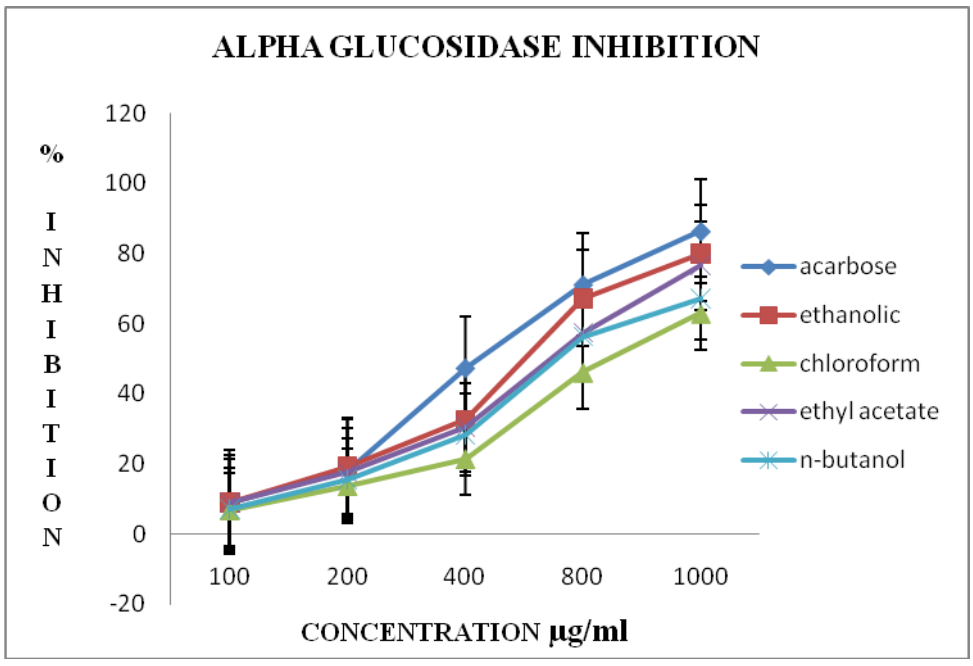

Fig 4: *In vitro* inhibition of α - glucosidase enzyme by various extracts of leaves of *Verbascum chinense.* The results represents the mean±SEM (n=3)

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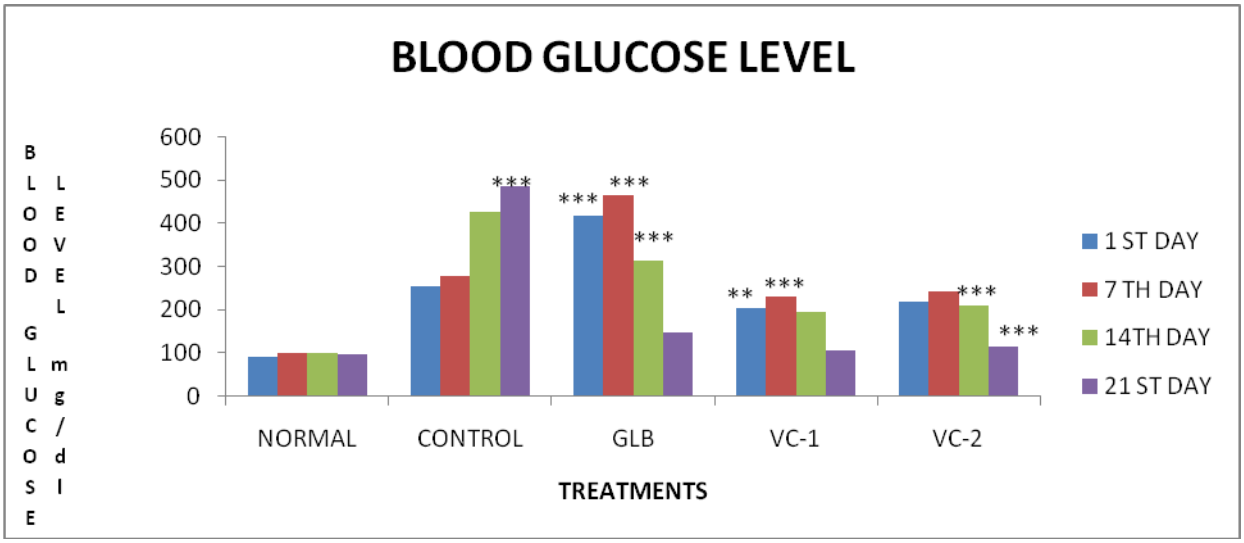

Fig 5: Effect of isolated fractions from VC on fasting blood glucose (mg/dl) levels on STZ Induced Rats Each bar represents the as mean ± S.E.M values of 6 animals each. * **p<0.001, **p<0.05, *p<0.01 (Newman Keuls test); diabetic control was compared with the bioactive compounds and GLB treated groups were compared with the diabetic control GLB-Glibenclamide

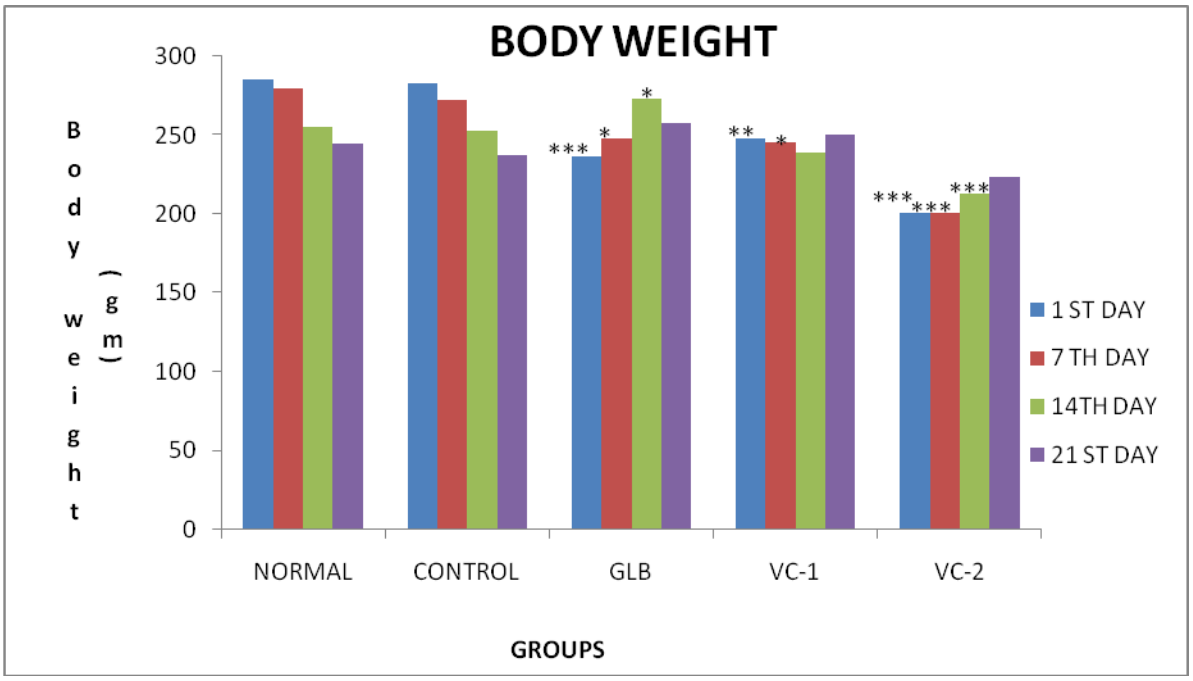

Fig 6: Effect of isolated fractions from VC on body weight (gm) on STZ induced rats Each bar represents the mean ± S.E.M values of 6 animals each. * **p<0.001, **p<0.05, *p<0.01 (Newman Keuls test); diabetic control was compared with the bioactive compounds and GLB treated groups were compared with the diabetic control GLB-Glibenclamide

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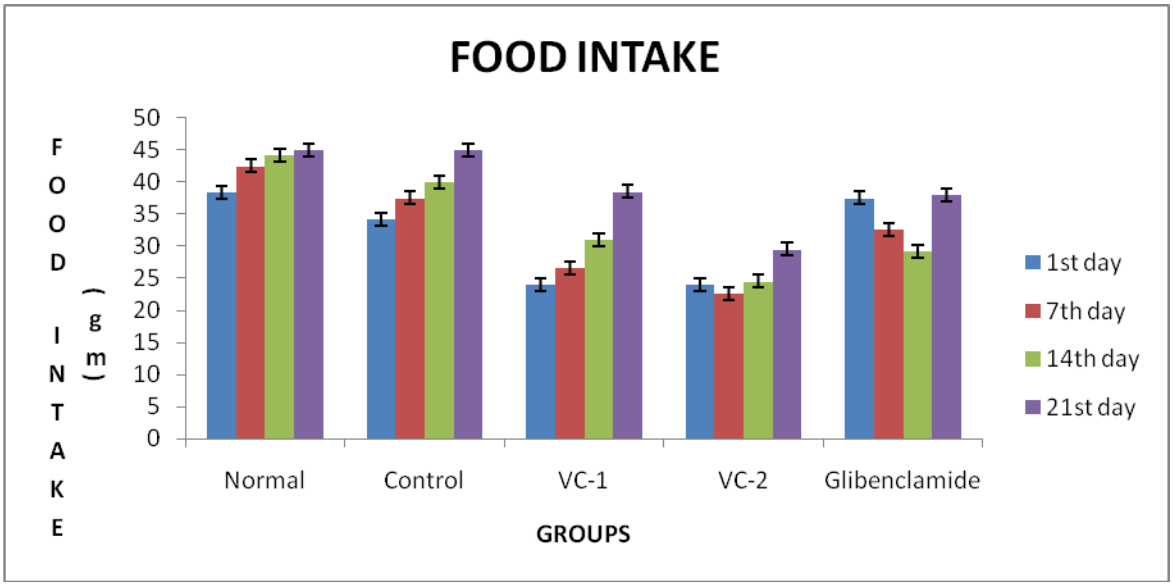

Fig 7: Effect of Isolated Fractions from VC on food intake (gm) on STZ Induced Rats. Each bar represents the mean± S.E.M (n=6)

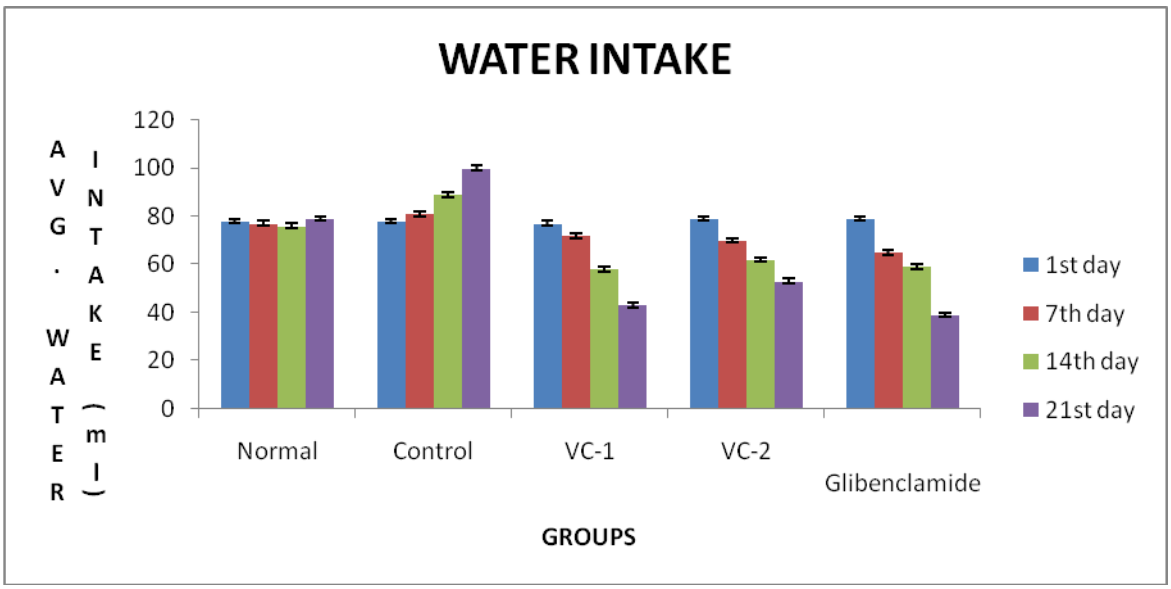

Fig 8: Effect of Isolated Fractions from VC on water intake (ml/rat/day) on STZ Induced Rats. Each bar represents the mean± S.E.M (n=6)

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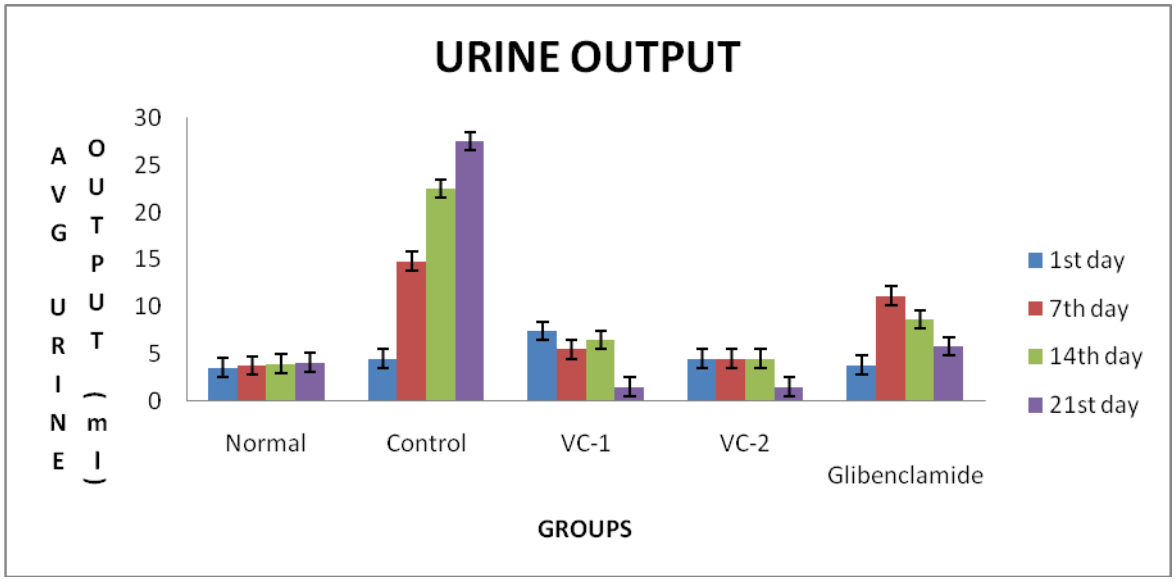

Fig 9: Effect of Isolated Fractions from VC on Urine Output on STZ Induced Rats. Each bar represents the mean± S.E.M (n=6)

DISCUSSION

Ethnomedicinal plants and naturopathic treatments are being used by people from all walks of life for living a healthy living all over the world. Nearly 50% drugs available are derivatives of medicinal plants.

Globally the ratio of diabetic patients has increased considerably. New synthetic OHA agents, drugs derived from plants and moreover effective changes in diet plan are being designed by physicians all over the world for the treatment of DM. Several medicinal plants have the ability to reduce FBG level and in managing other complications related to diabetes [30-32]. Many phytoconstituents viz. carbohydrates, alkaloids, flavonoids, saponins, amino acids, steroids, peptides, terpenoids are plants derivatives. These phytoconstituents possess hypoglycemic, anti-hyperglycemic and glucose suppressive activities 32. The specific biochemical interaction is either by facilitating the release of insulin from pancreatic β-cells, hindering the absorption of glucose in the gut, invigorating glycogenesis in the liver and/ or increasing the utilization of glucose in the body [33-36].

Postprandial hyperglycemia is a major problem in diabetes mellitus and it can be controlled by inhibiting the enzymes α-amylase and alpha-glucosidase that hydrolyze dietary carbohydrates [37-40].

The potential enzyme inhibitory effects of *V. chinense* were carried out in the present study. Different concentrations of the plant extracts of *V. chinense* showed an obvious enzyme inhibition effect was observed at all the tested concentrations. Acarbose was used as a standard drug. The results indicate that the various extracts of *V. chinense* have enzyme inhibitory activities [Figure 3, 4]. Ethanolic extract of *V. chinense* showed strong inhibition against enzymes α-glucosidase and α-amylase with IC_{50} value of 3.3 µg/ml and 3.42µg/ml respectively.

The ethanol extract of the leaves of *V. chinense* have shown high *in vitro* antidiabetic activity through the investigations. Thus the plants have been proven to have anti-hyperglycemic properties. The *in vivo* antidiabetic activity of the phytoconstituents obtained by column chromatography of the ethanol extracts of leaves of *V. chinense* was also evaluated. Compounds VC-1, VC-2 at doses of 50 mg/kg (each) isolated from *V. chinense* displayed a significant reduction in BGL of rats, thus revealing the antidiabetic nature of the phytocompounds. The results of the phyto compounds were compared to that of diabetic control and standard drug glibenclamide and were statistically significant ($p<0.05$, 0.001) as shown in Figure 5.

Streptozotocin is considered a good experimental model for inducing hyperglycemia in rats it is not as much toxic as other chemical agents. It results in selective destruction of pancreatic insulin secreting beta cell, which makes the cell less active and leads to poor utilization of glucose by tissues [40].

The outcome of STZ induced diabetes is a loss of body mass which is due to increased muscle wasting [41-43] and loss of tissue proteins. Diabetic rats treated with compounds isolated from *V. chinense* showed significantly ($p<0.05$, 0.001) increase in body weight as compared to diabetic control, which may be due to its effect in controlling muscle wasting (i.e. by reversal of gluconeogenesis and glycogenolysis) and may also be due to improvement in insulin secretion and glycemic control [41-43] (Figure 6) . The other parameters like food intake and water intake, urine output affected due to hyperglycemia were significantly reversed to normal by drug treatment as shown in Fig.7, 8, 9.

It is evident from the present work that triterpenoid and phenolic acid isolated from the leaves of *V. chinense* has a potential *in vitro and in vivo* antidiabetic activity, which is bioactivity of great relevance to diabetes mellitus complication therapy.

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