

INSULIN SECRETAGOGUE FRACTION OF *ARGYROLOBIUM ROSEUM*

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Key words: *Argyrobium roseum*, butanolic fraction, insulin secretagogue, vitexin, RINm5F cells

SUMMARY

Argyrobium roseum Cambers Jaub & Spauch; Papilionaceae, is a sexually reproducing, rare, annual herb that grows in tropical and sub-temperate tracts of the north-western Himalayan region of the Indian sub-continent; it has neither been documented for treating diabetes mellitus nor any other biological activity is attributed to this herb. Its ethanolic extract (KA-030) exhibited antihyperglycemic effect in oral glucose tolerance test and streptozotocin treated Wistar rats. This extract was further fractionated into petroleum ether (KA-131), chloroform (KA-132), butanolic (KA-133) and aqueous (KA-134) fractions. Of these fractions, KA-133 evoked a dose dependent stimulation of insulin secretion in the in vitro (RINm5F cells) and in vivo models when compared with glibenclamide. These results demonstrate the presence of natural antidiabetic and insulin secreting product(s) in *Argyrobium roseum*. A pure principle

characterized as vitexin has been isolated from KA-133 fraction and when evaluated for in vitro insulin secretion showed a dose dependent insulin secretagogue activity. Chemical data depict vitexin as a major compound in KA-133. However, further isolation of more pure molecules, their chemical characterization and bioevaluation have been carried out separately. Subacute toxicity and preclinical general pharmacological studies conducted on ethanolic extract proved it as safe and free from any adverse effect on gross behavior and general body system up to a dose of 2000 mg/kg p.o.

INTRODUCTION

Diabetes mellitus has been recognized as a growing world-wide epidemic by many health advocacy groups including the World Health Organization (WHO) (1). Approximately 5% of the world's population suffer from diabetes. Independent forecasters have suggested that the global prevalence of the disease will increase from 150 million in 2000 to 220 million in 2010 and to 300 million by 2025 (2). The global burden of diabetes mellitus would rise from 135.3 million in 1995 to 300 million in 2025 (3). The most pervasive and costly chronic disease is the leading cause of adult blindness and end stage renal disease. Additionally, diabetics are two to four times more likely to have heart disease or

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suffer a stroke. The WHO has estimated that diabetes will be one of the leading causes of death and disability within the next quarter century (4).

Insulin discovery in 1921 was the major breakthrough in the treatment of diabetes mellitus. Also initially, insulin was considered the only remedy for diabetes. Before the introduction of insulin, the treatment of diabetes mellitus mainly relied on dietary measures, which included the use of traditional plant therapies. Many traditional plant treatments for diabetes exist (5-7). With the advent of modern science, various pharmacological approaches are used to improve diabetes *via* different modes of action such as stimulation of insulin release, increase in the number of glucose transporters, inhibition of gluconeogenesis, and reduction of absorption of glucose from the intestine (8). Although all these contribute to the alleviation of diabetes, several complications still persist. Recently, the importance of biologically active substances in natural form and from complementary medicine has received much attention for various reasons (9-12). The nature has provided abundant plant wealth for all living creatures, which possess medicinal virtues. The essential values of some plants have long been published but a large number of them have remained unexplored as yet. So there is a necessity to explore their uses and to conduct pharmacognostic and pharmacological studies to ascertain their therapeutic properties (13). Therefore, despite considerable progress in the management of diabetes mellitus by conventional synthetic drugs, the search for natural anti-diabetic plant products for controlling diabetes is going on. There are, though, many hypoglycemic plants known through the folklore but their introduction into modern therapy awaits the discovery of animal test system that closely parallels the pathological course of diabetes in human. Hypoglycemic activity has been reported in many plants during the last twenty years (14).

In search for new compounds, within the exploration of natural resources, the insulin-like and insulin releasing effects of the medicinal plants such as *Agaricus campestris* (15), *Viscum album* (16), *Sambucus nigra* (17), *Averrhoa bilimbi* (18), *Ocimum canum* (19), *Nigella sativa* (20), *Urtica dioica* (21) and

Scoparia dulcis (22) have been evaluated. Some insulin secreting compounds have also been isolated from Indian *Scoparia dulcis* (23-26) and *Blighia sapida* (27). But such potential oral hypoglycemic agents like the cyclopropanoid amino acids and hypoglycins A and B are too toxic for use as insulin substitutes. Moreover, their action differs from that of insulin in that they appear to act as antimetabolites, capable of blocking the pathway of oxidation of fatty acids. This depletion of liver glycogen subsequently induces hypoglycemia (27).

The search is on globally to identify and isolate natural products to be used for the treatment of diabetes mellitus and its secondary complications that could exert a beneficial effect in the diabetic situation either by enhancing insulin secretion and/or by improving/mimicking insulin action. *Argyrobium roseum* (28-30), hitherto unreported for any biological activity, has been investigated for insulin secretagogue activity. Administration of ethanolic extract of *A. roseum* to glucose loaded rats and streptozotocin treated rats was found to possess this type of clinical indication as it reduced the increased level of blood glucose. In order to understand and elucidate the mechanism by which *A. roseum* ameliorates hyperglycemia condition, the activity-guided fractionation of the ethanolic extract was done and the effect of polar and non-polar fractions and a pure compound (vitexin) isolated from butanolic fraction on insulin secretion from RINm5F cells was investigated.

MATERIALS AND METHODS

In vivo studies

Plant material

A. roseum was collected from a wild source at foothills of the Jammu (J&K) region. The plant was identified and authenticated at the Herbarium of Regional Research Laboratory (CSIR), Jammu. A voucher specimen was deposited in the Herbarium of RRL, Jammu under Accession No. 18013. Whole plant

material was shade dried, ground to fine powder and sieved through mesh and then stored at room temperature in screw top jars until used.

Preparation of ethanolic extract (KA-030)

Ethanolic extract of *A. roseum* was prepared by percolation with 95% ethyl alcohol. In brief, 0.5 kg powdered material was successively percolated at ambient temperature for 4x15 h times with 2 L 95% ethanol (Fig. 1). Then it was desolventized at 55±5 °C under diminished pressure to obtain the ethanolic extract (KA-030).

Fractionation of ethanolic extract (KA-030)

The ethanolic extract KA-030 was further fractionated into petroleum ether, chloroform, butanolic and aqueous fractions. In brief, 50 g ethanolic extract was successively triturated with n-hexane (4x500 mL) to get non-polar fraction, KA-131, and then with chloroform to obtain chloroform fraction, KA-132. The residual portion thus left over was dissolved in distilled water (1 L) and partitioned between n-butanol (4x500 mL) and water to get n-butanol and aqueous fraction, KA-133 and KA-134, respectively (Fig. 1).

Isolation of vitexin from butanolic fraction (KA-133)

A 20-g butanolic fraction (KA-133) was subjected to column chromatography on silica gel (60-120 mesh). Elution was carried out using increasing proportions of methanol in ethyl acetate. Homogeneous fractions were pooled together. Fractions resulting from ETOAC:MeOH (1:1) were crystallized from MeOH to yield vitexin (1.1 g), M.P. 255-57 °C (Fig. 2a).

Standardization of KA-133 by HPLC

KA-133 was standardized on the basis of vitexin using HPLC Shimadzu system consisting of HPLC column RP-18 (250 nm×4.0, i.d. 5 µm) Merck Column which was operated at 30 °C to provide efficiency to the peaks. Binary gradient consisting of 2% acetic acid in water (A) and acetonitrile (B) was used to analyze

Figure 1. Preparation of ethanolic extract and four fractions (petroleum ether, chloroform, butanolic and aqueous residual) of the whole plant *Argyrobium roseum*.

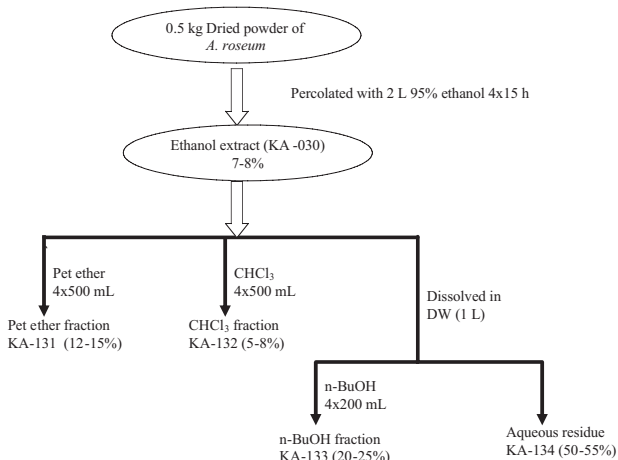
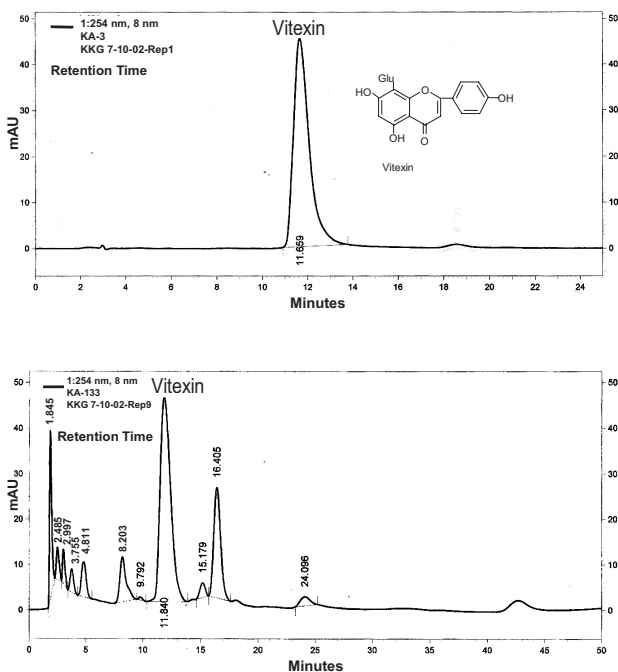


Figure 2. (a) HPLC of vitexin; (b) HPLC profile of butanolic fraction (KA-133) of *Argyrobium roseum* showing vitexin at RT 11.840.



vitexin at a flow rate of 1 mL/min. The gradient used was 8 to 90% acetonitrile in 60 minutes at a wavelength of 254 nm vitexin eluted at a retention time of 11.584 min (Fig. 2b).

Animals

Adult male/female Wistar rats (8 weeks), weighing 180-200 g, bred in the Animal House, Regional Research Laboratory (CSIR), Jammu, were used. All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC), Regional Research Laboratory (CSIR), Jammu. The animals were housed in polycarbonate cages in a room with a 12-h day-night cycle, temperature of 22 ± 2 °C, humidity of 45-64%. During the whole experimental period, animals were fed a balanced commercial pellet diet (Ashirwad Industries, Chandigarh, India) and water *ad libitum*.

Oral glucose tolerance test (OGTT)

Wistar rats (male/female), 6 animals in each group, were fasted overnight. The animals were divided into normal untreated control, negative control (glucose primed), test (glucose primed + test extract/fraction) treated and reference (glucose primed + glibenclamide) treated group. The test and reference drug treatment was done at 0 h. Glucose (1.5 g/kg; 10% sol) was administered to all groups except normal untreated at 1.5 h. Blood glucose determination was done at 0 h (prior to any treatment), 0.5 and 1 h (post-glucose administration) (31).

Induction of diabetes in rats

Four groups of 6 animals (Wistar rats) each received a freshly prepared solution of streptozotocin (STZ) (40 mg/kg) in 0.1 M sodium citrate buffer, pH 4.5, injected intraperitoneally in a volume of 1 mL/100 g (Siddique *et al.* 1987). Normal rats (6 rats) received 1 mL citrate buffer as a vehicle. Five days after STZ administration, the rats with glycosuria and hyperglycemia (i.e. blood glucose levels of 250–350 mg/dL) were used for the experiment. These diabetic animals were divided into 4 groups, and an additional group of 6 animals that received no STZ served as normal control. The test treatment of 5 groups was done as: group 1: normal (untreated); group 2: STZ control; group 3: STZ+100 mg/kg p.o. KA-030; group 4: STZ+200 mg/kg p.o. KA-030; and group 5: STZ+400 mg/kg p.o. KA-030. The treatment period was three weeks starting on day 7.

Measurement of serum glucose and plasma insulin

For oral glucose tolerance test (OGTT) and streptozotocin diabetic studies, the quantitative determination of serum glucose was done by glucose oxidase-peroxidase-GOD/POD method (32). Insulin was measured by the ELISA using the kits supplied by Mercodia AB (Rat Insulin ELISA kit, Mercodia, Uppsala, Sweden).

In vitro studies

Cell line, chemicals and kits

A rat insulinoma cell line (RINm5F) was obtained from National Centre for Cell Science (NCCS), Pune, India, which was used in the *in vitro* experiments. All reagents and chemicals were purchased from Sigma Chemical Co., Germany, unless otherwise specified. Rat Insulin ELISA kits were purchased from Mercodia, Sweden.

Insulin secretion in vitro

RINm5F cell line was used to evaluate insulin secretion (33,34). This cell line responds to a wide variety of insulinotropic stimuli including glucose, amino acids, hormones, neurotransmitters and drugs (33,35,36). Cells were seeded at a concentration of 1×10^6 cells/well in 24-well plates (NUNC A/S, Roskilde, Denmark) cultured in RPMI-1640 containing 11.1 mM glucose, 10% fetal calf serum and antibiotics (50 000 IU/L penicillin-streptomycin; 1000 IU/L nystatin) to allow attachment overnight prior to acute tests. Cells were washed thrice with Krebs-Ringer bicarbonate buffer (KRB; 115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl_2 , 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 24 mM NaHCO_3 , 10 mM Hepes-free acid, 1 g/L bovine serum albumin, 1.1 mM glucose; pH 7.4) and preincubated for 40 min at 37°C. Unless otherwise specified, cells were then incubated for 1 mL KRB with 1.1 mM glucose in the absence and presence of 5 mM streptozotocin + KA-133 (0.1, 0.5 and 1 mg/mL) and glibenclamide 0.02 mg/mL. In a separate set of the same experiments, vitexin was also tested in 0.1, 0.30, 0.010 mg/mL concentration for insulin secretion

effect. Following incubation, aliquots were removed from each well 3 h and 6 h after treatment and insulin estimation was done (37).

Cytotoxicity assay

The viability of RINm5F cells after treatment with KA-133 was assayed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as described previously (38). Briefly, cells were seeded in 96-well microtiter plates (1×10^4 cells per well in 200 μ L of medium), and left to adhere to the plastic plates overnight before being exposed to the active fraction KA-133. In each experiment, KA-133 was added in each well at a concentration of 0.1, 0.5, 1, 2, 4 mg/mL and incubated at 37°C for 24 h. After 24 h exposure to KA-133, 50 μ L of (5 mg/mL) MTT solution was added to each well and the cells were incubated in the dark at 37°C for an additional 4 h. Thereafter, the medium was removed, the formazan crystals were dissolved in 200 μ L of DMSO and the absorbance was measured at 570 nm in a micro plate reader (Spectramax plus 384, Molecular Devices 1311, Sunnyvale, C A, USA).

Statistical analysis

Data were evaluated by Student's unpaired t-test, one-way analysis of variance (ANOVA) or two-way analysis of variance where appropriate. Groups were considered to be statistically highly significant if $p < 0.001$.

RESULTS

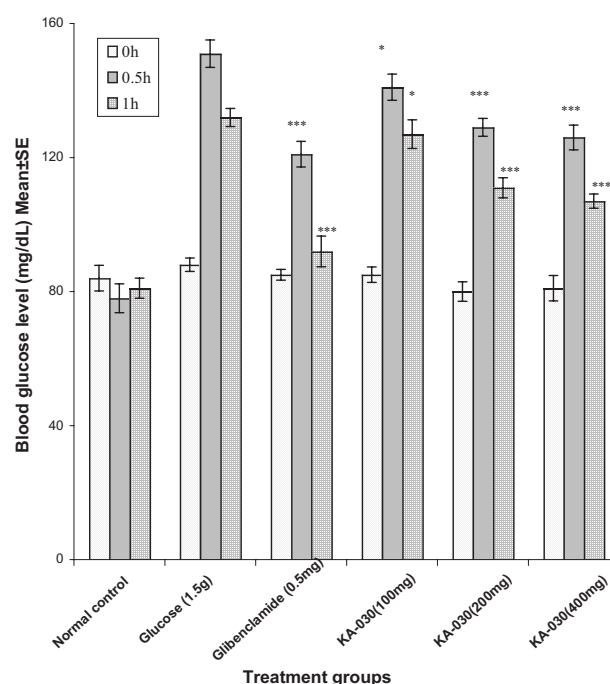
Studies in vivo

Effect of alcoholic extract (KA-030) on blood glucose level in oral glucose tolerance test

Ethanollic extract of *A. roseum* caused a dose related significant antihyperglycemic effect on OGTT in Wistar rats when compared to glucose (1.5 g/kg p.o.) primed group. At the 100 mg/kg p.o dose level, there was insignificant decrease in the blood glucose level ($p < 0.05$) after 0.5 and 1 h post glucose administration. However, at 200 and 400 mg doses, the blood glucose

level in glucose loaded rats was highly significantly ($p < 0.001$) reduced at both 0.5 and 1 h estimation (Fig. 3).

Figure 3. Effect of ethanolic extract of *Argyrobium roseum* (KA-030) on blood glucose level in glucose primed Wistar rats. Values are given as (mg/dL) mean \pm SE for 6 animals/group. Experimental group was compared with the glucose primed control: ** $p > 0.05$; *** $p < 0.001$ compared with 1.5 g/kg p.o. glucose primed control.



Effect of ethanolic extract (KA-030) on blood glucose level in streptozotocin treated Wistar rats

Prior to streptozotocin treatment, the blood glucose level in all the five test groups was within a close range. However, after 7 days of streptozotocin injection, the blood glucose level significantly increased. The normoglycemic group of animals served as non-diabetic control throughout the study period. The blood glucose level monitored weekly during the three-week treatment with KA-030 (100, 200 and 400 mg/kg p.o.) showed that KA-030 at 400 mg dose exhibited significant antihyperglycemia ($p < 0.01$ on days 14 and 21, $p < 0.001$ on day 28)

Figure 4. Effect of ethanolic extract of *Argyrobium roseum* (KA-030) on blood glucose level in streptozotocin Wistar rats. Values are given as (mg/dL) mean \pm SE for 6 animals/group. Experimental group was compared with the streptozotocin control; * $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with diabetic control.

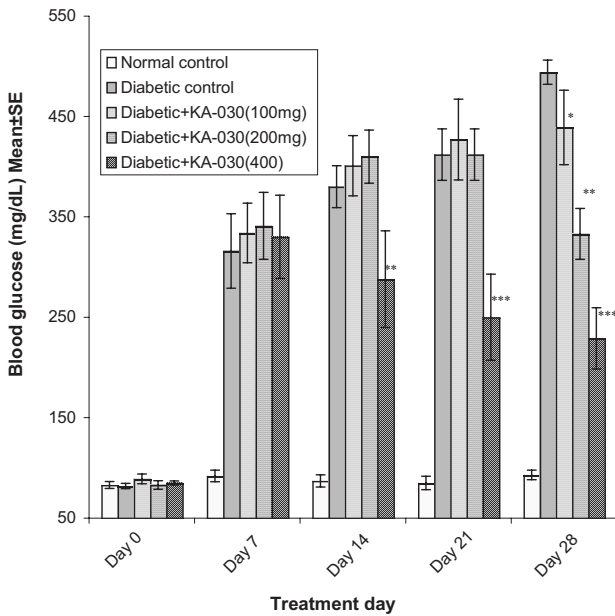


Figure 5. Effect of butanolic fraction of *Argyrobium roseum* (KA-133) on blood glucose level in glucose primed Wistar rats. Values are given as (mg/dL) mean \pm SE for 6 animals/group. Experimental group was compared with the glucose primed control; ** $p > 0.05$; *** $p < 0.001$ compared with 1.5 g/kg p.o. glucose primed control.

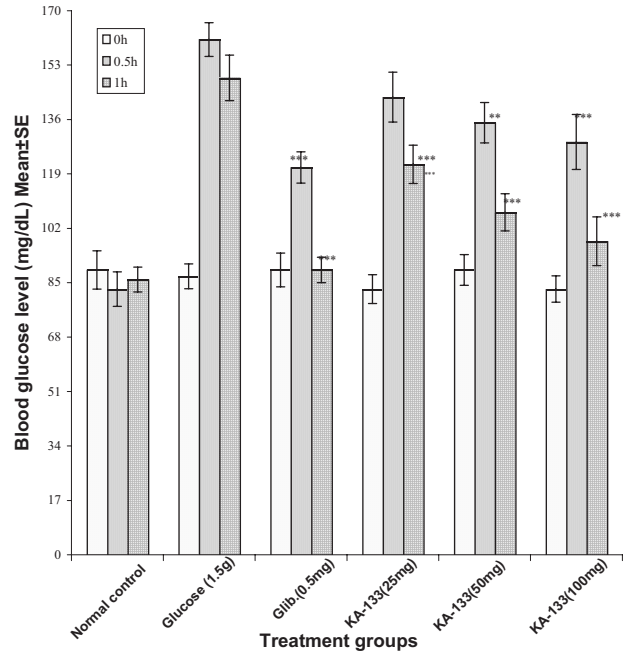


Figure 6. Effect of butanolic fraction of *Argyrobium roseum* (KA-133) on insulin level in glucose primed Wistar rats. Values are given as (U/L) mean \pm SE for 6 animals/group. Experimental group was compared with the glucose primed control; * $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with 1.5 g/kg p.o. glucose primed control.

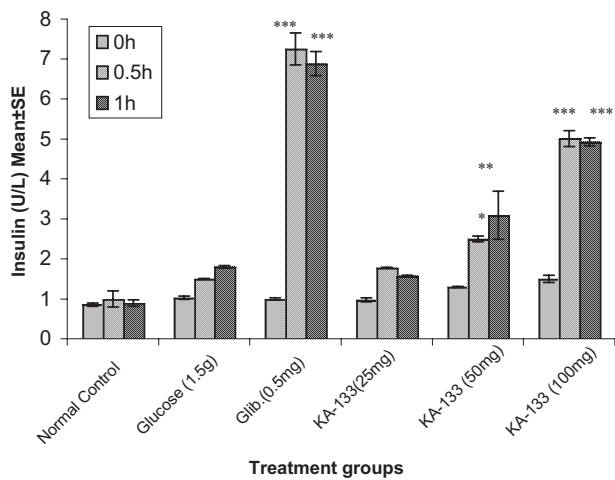
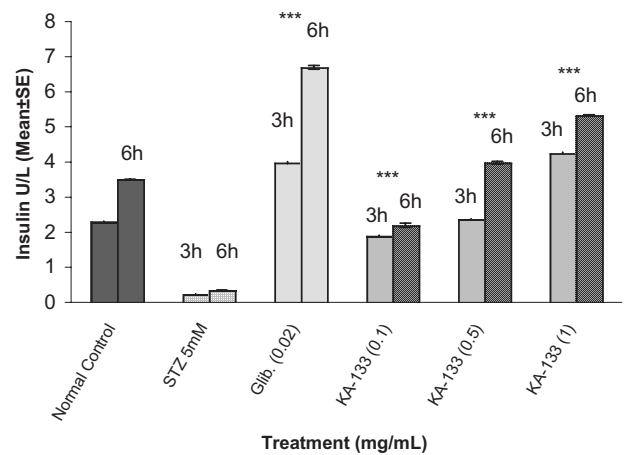


Figure 7. Effect of butanolic fraction of *Argyrobium roseum* (KA-133) on insulin level in RINm5F cells. Values are given as (U/L) mean \pm SE of 3 experiments. Test group was compared with 1.1 mM glucose treated cells; *** $p < 0.001$.



whereas 100 and 200 mg dose had a mild effect ($p < 0.01$ on days 14 and 21, $p < 0.001$ on day 28) (Fig. 4).

Effect of fractions of ethanolic extract of A. roseum (KA-030) on blood glucose level in oral glucose tolerance test

To confirm the insulin secreting effect of various fractions obtained from ethanolic extract of *A. roseum*, the KA-131, KA-132, KA-133 and KA-134 fractions were studied in doses depending upon their proportionate concentration in the parent extract KA-030 for their effect on blood glucose and insulin levels in OGTT. KA-133 was found to possess a dose related antihyperglycemic effect in glucose primed Wistar rats (Fig. 5). The KA-131, KA-132 and KA-134 fractions were found to be devoid of insulin secreting property (data not shown). The antihyperglycemic property of KA-133 was confirmed by its insulin secreting effect on *in vivo* insulin level in the same model (Fig. 6).

Insulin secretion *in vitro*

Effect of butanolic fraction (KA-133) on in vitro insulin secretion in RINm5F cells

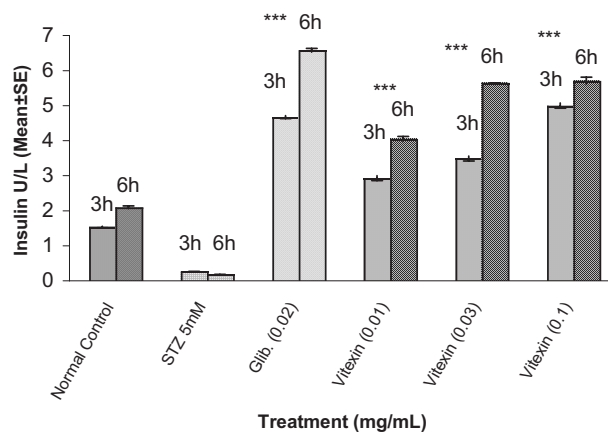
RINm5F cells were used to evaluate the mechanism underlying the effect of butanolic fraction KA-133 on antihyperglycemic property. The fraction when tested *in vitro* for insulin secretion effect alone exhibited a stimulatory effect on insulin secretion (0.1 to 1 mg/mL) in RINm5F cells (Fig. 7). The other fractions KA-131, KA-132, KA-134 tested in this test model did not affect insulin secretion (data not shown).

Effect of vitexin on in vitro insulin secretion in RINm5F cells

Vitexin, pure principle, isolated from the butanolic fraction of ethanolic extract of *A. roseum* was also evaluated *in vitro* for insulin secretion effect (0.01, 0.03 and 0.1 mg/mL) in RINm5F cells. Compared to STZ 5 mM control, vitexin significantly increased the insulin secretion. This effect of vitexin *versus* glibenclamide (0.02 mg/mL) was found to be less at

0.01, 0.03 mg/mL, whereas at a concentration of 0.1 mg/kg it was comparable to glibenclamide at 3 and 6 h post treatment (Fig. 8).

Figure 8. Effect of vitexin (KA-3) on insulin level in STZ treated RIN5f cells. Values are given as (U/L) mean \pm SE of 3 experiments. Test group was compared with 5 mM STZ treated RINm5F cells; *** $p < 0.001$.



Effect of butanolic fraction (KA-133) on RINm5F cell viability

KA-133 in the concentration of 0.1-4 mg/mL tested for cell toxicity by MTT assay showed that this fraction at various concentrations did not exert any cytotoxic effect in RINm5F cells.

DISCUSSION

Argyrobium roseum (Camber) *Leguminosae* (39,40), although mentioned in various texts on flora of the north-western Himalayan belt of the Indian subcontinent (29,30,41,42), was found to be not reported for its use in any form in the treatment of any ailment of mankind including diabetes mellitus. We were the first to evaluate this herb for antidiabetic effect with special emphasis on blood glucose level and insulin secretion. We found that the ethanolic extract of *A. roseum* showed a significant antihyperglycemic effect in the OGTT and streptozotocin induced diabetes model. The ethanolic extract, when demonstrating a promising antihyperglycemic effect as compared to glucose primed Wistar rats in OGTT

model and STZ diabetic Wistar rats in STZ induced diabetic model, was further fractionated to yield four fractions. These fractions were also tested using an *in vitro* model (RINm5F) for insulin secretion and *in vivo* model (OGTT) for antihyperglycemia and insulin secretion. Out of four fractions obtained from ethanolic extract of *A. roseum*, butanolic fraction (KA-133) was found to possess antihyperglycemic effect, which was also confirmed by its stimulatory effect on insulin secretion under both *in vitro* and *in vivo* conditions. The active fraction, KA-133, was further standardized after isolation and characterization of the pure principle, vitexin, which also exhibited a dose related stimulatory effect on insulin secretion *in vitro*.

This study has proved that the antihyperglycemic effect of ethanolic extract of *A. roseum* is mainly attributable to the subfractions and compounds extracted in butanol. The butanolic fraction which exhibited antihyperglycemic and insulin secretagogue activity was compared to glibenclamide, a second generation sulfonylurea, which increases the β -cell insulin release. The butanolic fraction has been

standardized on the basis of vitexin, a pure principle isolated from butanolic fraction also possessing the same degree of stimulatory effect, although in a higher dose than its proportionate concentration in parent fraction, on insulin secretion in STZ treated RINm5F cells.

In conclusion, our results showed that ethanolic extract after three-week treatment completely reversed diabetic conditions in STZ treated rats. The butanolic fraction from this extract markedly reduced hyperglycemia by stimulating the insulin release in OGTT. Chemoprofiling of butanolic fraction showed vitexin as one of the compounds which was also found to be an active insulin secretagogue in STZ treated RINm5F cells. These findings suggested that butanolic fraction of *A. roseum* and the pure compound(s) therein could be further explored to reveal a natural drug candidate to be useful in diabetes mellitus treatment.

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