Experimental model proposed to deduce pharmacological evidence of the beta-cell neogenesis activity of *Argyrolobium roseum* vis-a-vis an evaluation of its hypoglycemic activity

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Abstract

Background: *Argyrolobium roseum* is described in texts for its botanical aspects with no mention of its therapeutic uses. Recently, a solvent fraction of this plant has been reported to possess insulin secretagogue activity. **Objective:** Primary objective of the study was to evaluate the hypoglycemic activity of different fractions of *Argyrolobium roseum* plant. Secondary objective was to devise a model for detecting its beta-cell neogenesis activity. **Materials and Methods:** Alcoholic fraction (AR) was studied in fasting, glucose-loaded and streptozotocin (STZ)-treated hyperglycemic rats. For deducing beta-cell neogenesis activity, a sub-lethal dose of STZ, causing delayed death in the animals, was administered. **Results:** AR in a dose of 200 mg/kg showed hypoglycemic activity in fasting and STZ-treated rats. With sub-lethal dose of STZ, recovery from hyperglycemia was seen. **Conclusion:** AR of *Argyrolobium roseum* possesses hypoglycemic and a possible beta-cell neogenesis activity. Further studies are required to confirm its beta-cell neogenesis potential by cytological methods and to establish its role in the treatment of type 2 diabetes.

Key words: *Argyrolobium roseum*, D-pinitol, hypoglycemic, regeneration, streptozotocin

Introduction

Despite the advances made in medical science, diabetes mellitus continues to be a life-threatening disease. Insulin and hypoglycemic agents have increased the life span of diabetic patients, but the associated complications continue to be the cause of morbidity and mortality. Beta cell function is gradually lost, requiring increasing doses of oral hypoglycemic drugs and/or requiring insulin.

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In search of new remedies for diabetes, hundreds of plants have been investigated for the hypoglycemic activity. The plant *Argyrolobium roseum* came under authors' investigation on the basis of information that a person residing in a distant hilly area had treated some cases of diabetes by 2-week treatment with a plant growing in that area. On visit to the said place, the concerned person was contacted, the plant locally known as FLY JARI was collected, botanically identified, and a sample was preserved in the herbarium of the institute labeled as ASC-29.

On review of literature, it was found that though the plant had been mentioned in some texts,[1-3] no clinical use was attributed to this plant. However, a fraction of *Argyrolobium roseum* has now been reported for its insulin secretagogue activity.[4] When tested on normal fasting rats, the plant powder and its alcohol and acetone extracted fractions exhibited hypoglycemic activity. Since merely the hypoglycemic effect of the plant could not be accounted for recovery of the patient from diabetes by 2-week treatment, this led us to think that the plant

possibly possessed beta-cell neogenesis activity. To explore this activity, an experimental model with parameters indicative of the status of the beta cells was devised and the plant was put to test for the said activity. The results so obtained have proved rewarding.

Materials and Methods

Material

Argyrolobium roseum (Family–Leguminosae; sub-family–Fabaceae) is a small ground level growing plant. The whole plant was plucked from ground level in the flowering month of March, shade dried, powdered, and kept in the refrigerator before it was processed. The powdered plant was percolated four times with methyl alcohol (90%), vacuums dried, coded as AR, and was employed for detailed study. From this extract, a number of other fractions were obtained. These were evaluated for hypoglycemic activity and compared with tolbutamide and glipizide as the reference standards. The test material was given orally in the form of a fine homogenized aqueous suspension prepared with 1% (w/v) gum acacia.

Isolation and identification of active constituents

Review of literature of the hypoglycemic activity possessing medicinal plants and their active constituents revealed that some cyclitols including D-pinitol having insulin-like activity are present in some natural sources like pine needles, chickpeas, alfalfa, soya beans, and other legumes and in Bougainvillea spectabilis.[5-7] As the plant under study belongs to leguminosae family, it was deemed possible that active constituent present could be a cyclitol; study was planned accordingly to isolate it. AR was washed with petroleum ether followed by wash with chloroform; five washes with both were given. The residue was dissolved in deionized water and subsequently filtered. The filtrate was washed five times with n-butanol in a separating funnel. The aqueous part was collected and passed through a column (60 cm height x 2.5 cm diameter) filled with ion exchange resin—first basic, i.e., Amberlite (IR 400) followed by acidic (IR 120). The resins retain reducing sugars, pigments, and ions, while cyclitols including pinitol elute out. The eluted solution was vacuum-dried, dissolved in methanol, and crystallization was allowed to occur at 4^0C followed by filtration of mother liquor and subsequently, the crystalline material collected was air dried.

Identification/Finger printing of active constituent(s)

The crystallized substance was subjected to determination of its melting point and High-performance liquid chromatography (HPLC) (Shimadzu, Chennai, India). The separation was carried out on Rezex RSO-oligosaccharide Ag+4% column of size 200 x 10 mm. HPLC-grade water was used as mobile phase and pumped at a flow rate of 0.3 ml/min. Pinitol standard was first injected and the peak was detected at 34.033 minutes. Confirmation of presence of pinitol was done by matching the retention time of the peak in the sample.

Animals

Wistar rats, male and female, weighing 200 ± 10 g fed on standard pellet diet, water *ad libitum,* and maintained at 24 to 28^0C room temperature with 12-hour day and night cycle were used. Animals deprived of food for 18 hours were used as fasting animals. Permission was obtained from the Institutional Animal Ethical Committee for the said study.

Evaluation of hypoglycemic activity

Hypoglycemic activity was evaluated in both normal and hyperglycemic rats. The hyperglycemic rats employed were streptozotocin (STZ) treated, 40 mg/kg i.p. dissolved in 0.01M citrate buffer, 10 days after STZ treatment.[8] Blood was withdrawn by retro-orbital puncture using a fine sterile capillary tube for glucose estimation, before and 3 hours after test drug treatment. Dose of alcoholic fraction of AR used was 200 mg/kg po. Vehicle (1% w/v gum acacia aqueous suspension) in equivalent amount as per body weight served as the control. Glipizide in a dose of 0.5 mg/kg p.o. served as the reference standard. Serum was separated within 30 minutes and samples were assayed for glucose estimation by the Trinder's method.[9]

Onset and duration of hypoglycemic effect

Five groups of fasting rats, with six rats in each group, were employed. Each group of rat was for the specific interval of time, i.e., ½, 1, 2, 3, and 5 hours. The blood sugar level of rats in each group before and after treatment with AR for each interval of time was determined. This method is different from the conventional methods of using the same group of animals repeatedly for different intervals of time, as the acute stress associated with repeated loss of blood and infliction of injury due to repeated withdrawal of blood from the same animal is likely to distort the blood sugar level of the animals and thereby interfering with the effect of the test drug.

Evaluation of beta cell neogenesis activity

Literature was reviewed to find out a working model for evaluating the said activity. STZ is reported to induce diabetes with features similar to that associated with uncontrolled diabetes mellitus in human subjects.[8] STZ has been employed at different doses in different species of animals,[10-12] and reported to damage beta cell by generation of free radicals[13-16] and IL-IB.[16] It has also been reported

that the diabetogenic action of STZ is dose dependent.[17] One of the recognized models for beta cell neogenesis is the neonatal rat injected with STZ at the time of birth.[18] The evidence for beta cell neogenesis in this model was based partly on cytological and partly on pharmacological investigations. The former involved immunochemical and stereological morphometric methods.

We had neither the facilities nor the expertise to carry out the cytological investigation; so, it was compelling on our part to devise a reliable and relevant model for generating pharmacological evidence on the neogenesis of beta cell. It was deemed more relevant to include adult rats as in them STZ caused pathological pattern that resembled type 2 diabetes.[19,20] The dose of STZ reported for induction of diabetes is 50 to 60 mg/kg by intraperitoneal (ip) or intravenous (iv) routes, with death of animals within a week.[10] It was decided to use such a dose of STZ that caused mortality over a period of 3 to 4 weeks (sublethal dose), permitting at least two intervening weeks for the treatment of animals with test drug, before the animal dies. For this, different doses of STZ were tried and a dose of 40 mg/kg/ip was found to be appropriate. The use of this dose and route had been reported earlier also.[21]

On day 0, the animals showing blood sugar level in normal range were treated with the said dose of STZ. On day seven, their blood sugar was determined and accordingly, the animals were distributed into respective groups so that the mean blood sugar value of each group was close to each other. Treatment with test drug or controls was started from day seven and continued till day 21. The drug treatment was discontinued after day 21, but the animals were monitored for their beta cell status for the next three weeks. At the end of every week, blood sugar level, mortality, and body weight were recorded as indicator of functional status of the beta cell. On day 42, the response of the survived animals to the hypoglycemic effect of AR or Glipizide was tested as this reflected the status of the beta cells. The data were analyzed using students *t* test. Results were expressed as mean ± SEM and *P* value <0.05 was considered statistically significant.

RESULTS

Hypoglycemic activity
Fall in blood sugar levels were 24, 29, 21, and 18 mg/dl with plant powder, its alcoholic extract, acetone extracted fraction, and glipizide, respectively, in normal fasting rats [Table 1]. The aqueous and alkaloid fractions did not show this activity (data not shown). When hypoglycemic activity of AR was tested on diabetic rats, AR showed significant fall of blood sugar in STZ-treated hyperglycemic rats. This hypoglycemic activity continued from half-hour of AR treatment till 5th hour (last recording) [Table 2].

Toxicity
No adverse effect or mortality was observed on any animal treated with AR or plant powder in the dose of 200 and 2 500 mg/kg po, respectively.

Beta cell neogenesis activity
In control group(s), blood sugar continued to rise and remain at high level, the body weight continued to fall, and there was 100% mortality by day 28 in male group and 40% in female group by day 42. In the AR-treated group, blood

Table 1: Hypoglycemic activity of *Argyrolobium roseum* and its fractions on fasting rats

Treatment (mg/kg/p.o)	Blood glucose in mg/dl (Mean ± SE)			
	0h	**3rd**	**Fall**	**P***
Control (Vehicle)	86.1 ± 15.2	87.1 ± 14	1 (↑)	-
Plant powder (2500)	90.6 ± 8.7	66.6 ± 8.9	24 ± 12.3	<0.001
Alcohol extract (200)	95.2 ± 14.5	70.3 ± 15.8	29.9 ± 3.8	<0.001
Acetone fraction (200)	95.6 ± 12.8	74.5 ± 17.3	21 ± 18.5	<0.02
Glipizide (0.5)	87.7 ± 7.3	69 ± 8.9	18 ± 4.2	<0.01

Drug treatment: Just after drawing blood at 0h; *n* = 6 in each group, *After 3rd hour compared to value at 0 hour

Table 2: Onset and duration of hypoglycemic effect of alcoholic extract of AR on fasting rats

Treatment (AR 200 mg/kg p.o)	Blood glucose in mg/dl (Mean ± SE)					
	0h	**½ h**	**1 h**	**2 h**	**3 h**	**5 h**
I	83 ± 5	49 ± 4	-	-	-	-
II	81 ± 6	-	49 ± 5	-	-	-
III	80 ± 30	-	-	50 ± 5	-	-
IV	85 ± 4	-	-	-	59 ± 6	-
V	86 ± 4	-	-	-	-	67 ± 3

Each group was for one specific interval of time after treatment with AR, *n* = 6 in each group

sugar after initial rise and body weight after initial reduction returned back to normal and there was no mortality. On day 42 of study, AR-treated group animals responded to hypoglycemic effect of AR equivalent to that recorded in normal rats, indicating thereby that the functional status of beta cells was as good as that of normal animals [Tables 3 and 4].

To check if hypoglycemic effect of AR was contributing in any way to its beta cell neogenesis activity, glipizide as a hypoglycemic agent was used as a positive control. In glipizide-treated group, decrease in blood glucose levels was less marked during the course of treatment as compared with AR group and it increased more after the treatment was stopped. The mortality was 75% as compared with nil mortality in AR-treated group. Body weight continued to decrease as compared with its rise in AR-treated group. On day 42, glipizide tested on lone surviving rat showed hypoglycemic effect [Table 5].

Table 3: Beta cells neogenesis by AR in streptozotocin-treated male rats

Group (*n* = 5)	Parameters	Day 0	Days after treatment with streptozotocin						3h after treatment with AR on day 42
			7	14	21	28	35	42	
Control	Blood sugar (mg/dl)	83 ± 9	338 ± 11	495 ± 53	570	-	-	-	-
	Mortality	-	-	2	2 ± 2	4 ± 1	-	-	-
	Body weight (g)	174 ± 80	153 ± 6	140 ± 0	130	-	-	-	-
AR 200 mg/kg p.o	Blood sugar (mg/dl)	84 ± 5	336 ± 95	248 ± 104	199 ± 80	162 ± 43	156 ± 44	116 ± 9	69 ± 7
	Mortality	-	0	0	0	0	0	0	
	Body weight (g)	180 ± 9	157 ± 11	160 ± 7	169 ± 11	177 ± 13	186 ± 6	193 ± 2	-

All values in mean ± S.E, treatment with AR: Daily from day 7 to day 21, Day 42: AR tested in above dose for hypoglycemic effect as determinant of beta cells neogenesis

Table 4: Beta cells neogenesis by AR in streptozotocin-treated female rats

Group (*n* = 5)	Parameters	Day 0	Days after treatment with streptozotocin						3 h after treatment with AR on day 42
			7	14	21	28	35	42	
Control	Blood sugar (mg/dl)	82 ± 2.7	316 ± 83	460 ± 46	411 ± 57	496 ± 26	535 ± 141	412 ± 81	357 ± 44
	Mortality	-	0	0	0	0	1	1 ± 1	-
	Body weight (g)	166 ± 17	150 ± 18	147 ± 16	143 ± 12	140 ± 12	138 ± 5	128 ± 3	-
AR 200 mg/kg p.o	Blood sugar (mg/dl)	85 ± 2.1	330 ± 12	229 ± 136	188 ± 85	150 ± 61	145 ± 21	121 ± 12	88 ± 16
	Mortality	-	0	0	0	0	0	0	-
	Body weight (g)	165 ± 5	148 ± 7	172 ± 5	186 ± 12	188 ± 17	196 ± 12	199 ± 8	-

All values in mean ± S.E, treatment with AR: Daily from day 7 to day 21, Day 42: AR tested in above dose for hypoglycemic effect as determinant of beta cells neogenesis

Table 5: Beta cells neogenesis by AR vs glipizide in streptozotocin treated male rats

Group (*n* = 4)	Parameters	Day 0	Days after treatment with streptozotocin						3 h after treatment with AR/Glipizide on day 42
			7	14	21	28	35	42	
Glipizide 0.5 mg/kg p.o	Blood sugar	80 ± 8.1	427 ± 99	370 ± 80	384 ± 56	470	410	390	350
	Mortality	-	0	0	1	1 ± 2	0	0	-
	Body weight	197 ± 13	181 ± 16	168 ± 14	155 ± 13	165	150	145	-
AR 200 mg/kg p.o	Blood sugar	82 ± 3.3	394 ± 60	213 ± 179	147 ± 58	135 ± 23	145 ± 29	144 ± 41	105 ± 37
	Mortality	-	0	0	0	0	0	0	-
	Body weight	-	190 ± 18	166 ± 24	165 ± 24	170 ± 17	179 ± 18	199 ± 15	-

Blood sugar in mg/dl, Body weight in grams, all values in mean ± S.E, treatment with AR/Glipizide: Daily from day 7 to day 21, Day 42: AR/Glipizide tested in above doses for hypoglycemic effect as determinant of beta cells neogenesis

Table 6: Beta cells neogenesis with low dose of AR in streptozotocin-treated female rats

Group (*n* = 4)	Parameters	Day 0	Days after treatment with streptozotocin					
			7	14	21	28	35	42
Control	Blood sugar	82 ± 3.3	300 ± 35	469 ± 39	-	-	-	-
	Mortality	-	0	2	2 ± 2	-	-	-
	Body weight	161 ± 11	141 ± 6	135 ± 7	-	-	-	-
AR 100 mg/kg p.o	Blood sugar	80 ± 8	286 ± 35	487 ± 12	362 ± 105	345 ± 277	340 ± 296	-
	Mortality	-	0	0	0	2	2 ± 0	2 ± 2
	Body weight	188 ± 19	155 ± 21	140 ± 26	130 ± 16	127 ± 10	125 ± 5	-

Blood sugar in mg/dl, Body weight in grams, all values in mean ± S.E, treatment with AR: Daily from day 7 to day 21

Tests were repeated with half the dose of AR used as compared with earlier tests, i.e., 100 mg/kg compared with 200 mg/kg employed in earlier test. Although the dose of 200 mg restored the blood sugar and body weight back to near normal without any mortality and the animals responded positively to the hypoglycemic effect of the AR on day 42 of study [Tables 3 to 5], the dose of 100 mg of AR did not produce the effects to the same extent, though the effect was better than the control group [Table 6].

Finger printing of active constituent

HPLC of the AR showed a peak retention time of 34.033 minutes, which is the same as that of D-pinitol (34.017). Thus, the active constituent was identified as D-pinitol.

Discussion

The deduce hypoglycemic activity can be attributed to D-pinitol isolated from AR. D-pinitol (3-O-methyl chiro-inositol) is an inositol, a key component of the insulin-related phosphoglycans released from cell membrane on the binding of insulin with its receptor and likely to be participating in the release of phosphoglycans and thereby enhance the activity of insulin or overcome the insulin resistance. This explains for insulin-like effects observed with D-pinitol to improve glycemic control in a number of experimental and clinical studies reported.[7,22-31] D-pinitol is reported to be present in pine needles, chickpeas, alfalfa, soya beans, and other legumes and in Bougainvillea spectabilis used in traditional medicine for conditions associated with diabetes.[5]

The model employed to explore beta cell neogenesis activity is based on causing apoptosis of beta cell with STZ. It was decided to use such a dose of STZ that led to hyperglycemia, weight loss, and mortality but not before 2 to 3 weeks, the time period which was required for a test drug to act and show its beta cell neogenesis activity, if possessed. Accordingly, dose of 40 mg/kg i.p. was worked out. The time to start the treatment was also very crucial. As such, one week time was allowed for diabetes to be established, i.e., allow time for the beta cell to undergo apoptosis before the treatment was started, then continued for 2 weeks for the drug to act, observe in next 3 weeks without drug treatment for the recovery from diabetes, i.e., neogenesis of beta cells, and then determine the status of beta cells at the end, i.e., on day 42 by testing the response of a hypoglycemic agent, as has been done in this study and AR-treated animals which were found as recovered, i.e., beta cells regenerated as compared with controls. Thus, the pharmacological evidence recorded by way of restoration of blood sugar and body weight to near normal

and absence of mortality as compared with controls and response of AR-treated animals to a hypoglycemic agent at the end of 6 weeks are indicative of beta cell neogenesis. The experimental model developed is all the more relevant since diabetes is first allowed to develop in adult rats as is the case with type 2 diabetes appearing in adult age. The recognized model of beta cell neogenesis in STZ-treated neonatal rats[18] has a drawback that damage to beta cells by STZ in new born is followed by a rapid remission from neonatal diabetes starting from day 3 to 5 after birth.[19,32] Adult rats with STZ exhibit decreased beta cell mass and a chronic pathological pattern that presents functional similarities to type 2 diabetes.[19,20] Secondly, the model developed can be put to use by those who do not have the facilities for carrying out cytological studies involving immunochemical and morphometric methods.

The pancreatic beta cell responsible for maintenance of body's glucose level within a narrow range, their number, and functioning can be said to be dynamic, i.e., undergoing both replication and apoptosis in a balanced way.[33] One determinant in the development of diabetes is inadequate mass of beta cells, either absolute in Type 1 diabetes or relative in Type 2 diabetes.[18] A better understanding of regeneration and factors responsible for stimulating regeneration and replication can lead to new therapeutic strategies for the treatment of diabetes. If beta cell neogenesis by AR is further confirmed by cytological evidence, it will provide a new dimension to the therapeutic intervention of diabetes mellitus.

Beta cell neogenesis means the regeneration of new beta cells from precursor cells. Neogenesis from duct epithelium is the most currently described and best documented process of differentiation of precursor cells into beta cells. This process contributes not only to beta cell mass expansion during fetal and neonatal life, but it is also involved in the maintenance of beta cell mass in adults and further a number of factors controlling the differentiation of precursor cells have been identified.[34-36] Recently, the incretin hormone glucagon-like peptide (GLP-1) and its long-acting analogue exendin-4, known to enhance glucose-stimulated insulin release and glucose disposal in peripheral tissues, have been reported to stimulate the proliferation of INS-1 cells *in vitro*,[37] and increase beta cell mass in adult rodents *in vivo*.[38-40] It is likely that D-pinitol present in AR is exerting both hypoglycemic and beta cell neogenesis by augmenting the release of GLP-1, as GLP-1 has been reported to mediate its action by promoting DNA synthesis, activation of phosphotidylinostol 3-kinase and by increasing transcription factor.[37]

Follow-up study for recording cytological evidence on beta cell neogenesis by AR or its active constituents is warranted. It is also hoped that the experimental model developed for evaluating the beta cell neogenic activity of a test material is going to prove useful as a screening model for the said activity.

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