Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(3): 169-175
Received: 12-03-2016
Accepted: 13-04-2016

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Evaluation of anti-diabetic activity of *Strobilanthes cuspidata* in alloxan induced diabetic rats and the effect of bioactive compounds on inhibition of *α*-amylase enzyme

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Abstract
The antidiabetic effect of hydro alcoholic extract of the leaves of *S. cuspidata* were investigated in alloxan induced diabetic rats and the bioactive compounds were tested for their antidiabetic potential *in vitro* by inhibition of *α*-amylase enzyme. The extract and the standard hypoglycemic drug metformin (250 mg/kg) were dissolved in isotonic saline solution (NaCl, 0.9% w/v) and were administered orally. Diabetes was induced in by a single intraperitoneal injection of 120 mg/kg alloxan monohydrate. Hyperglycemia was confirmed by the elevated glucose levels determined at 72 h after alloxan injection. The rats having blood glucose levels above 200mg/dl were used for the anti diabetic study. The extracts and standard drug were administered orally for 14 days. The fasting blood samples were collected by retro orbital sinus puncture under mild ether anesthesia and serum was separated by centrifugation at 4000 rpm for 15 min. Fasting blood glucose estimation was measured on day 0, 7 and 14 of the study. The results of OGTT reveal that the extract at both the dose levels (150 and 300 mg/kg) cause a significant decrease in the blood glucose level within 60 min after oral administration to normal rats. The extracts produce a significant attenuation (p<0.001) in the blood glucose level at 60 min to 120 min when compared with normal control. The results of the blood glucose level reveal that the extract at both the dose levels (150 and 300 mg/kg) show a significant (p<0.001) time dependent decrease in blood glucose level after oral administration at 7 and 14 days when compared to the diabetic control group. After 14 days of daily treatment with the extract (150 and 300 mg/kg), the results show significant dose-dependent fall in blood sugar levels by 38 and 41%, respectively. These results indicate that hydro alcoholic extract of *Strobilanthes cuspidata* have favorable effects in bringing down the severity of diabetes**.** The isolated compounds were tested for their antidiabetic potential *in vitro* by inhibition of *α*-amylase enzyme. Total saponins, Lupeol and stigmasterol showed higher alpha amylase inhibitory activity which confirms its antidiabetic potential.

Keywords: Anti-diabetic Activity, *Strobilanthes cuspidata,* Alloxan –induced Diabetic Rats, *α*-amylase enzyme

Introduction
Diabetes mellitus is a worldwide epidemic, is growing at an alarming rate and is the fifth leading cause of death in the world. India has been declared as the "Diabetic Capital of the World" and is one of the world's oldest known diseases. In 1997, diabetes prevalence was introduced as a "basic health indicator" for member states by the WHO, which estimated that a number of people with diabetes in the world would reach 300 million by 2025 (Hilary *et al.*, 1998) [46]. Chemical agents are available in the market to control and to treat diabetic patients but are unsafe. Present trend manages this deadly disorder through nutraceutical approaches as it is safe without any serious side effects.

Diabetes mellitus commonly known as diabetes is one of the oldest known diseases. In 1997, diabetes prevalence was introduced as a basic health indicator" for member states by the WHO, which estimated that a number of people with diabetes in the world would reach 300 million by 2025. Recently diabetic patients are also found to increase as predicted by WHO with increased microvascular complications such as diabetic neuropathy, nephropathy and retinopathy.

All form of diabetes has very serious effects on health. In addition to the consequences of abnormal metabolism of glucose (e.g., hyperlipidemia, glycosylation of protein, etc,) there are a number of long term diseases associated with the disease. These include depression cardiovascular, peripheral vascular, ocular, neurologic and renal abnormalities, which are responsible for morbidity, disability and premature death. Though diabetes is believed to be a lifestyle disease it also results due to genetic disturbances.

Diabetes mellitus is a group of metabolic disorder characterized by hyperglycemia and alternation in the carbohydrate, fat and protein metabolism associated with absolute or relative deficiencies in insulin secretion or insulin action. This dreadful disease is found in all parts of the world and is becoming a serious threat to mankind.

Currently available treatment of Diabetes, in addition to insulin supplement include many oral hypoglycemic agents like sulfonylureas, biguanides, thiazolidinedione, D-phenylalanine derivatives meglitinides & α-glucosidase inhibitors along with appropriate diet exercise (Rodney and Donald., 2001) [54]. Due to undesired side effects, continuous efforts are been made to develop new compounds for treatment of diabetes, especially of herbal origin.

In the light of the literature on *S. cuspidata* we made an attempt for the first time to study the effect of S. *cuspidata* ethanolic extract in antidiabetic activity by Alloxan induced diabetes rat model and the isolated compounds were tested for their antidiabetic potential *in vitro* by inhibition of α-amylase enzyme. *Strobilanthes cuspidata* (Benth.) T. Anderson is endemic to India (Nayar *et al*., 2006) [50]. The tribal people of Nilgiri hills use this plant for joint pain and inflammation. The *in-vitro* anti-inflammatory, anti-osteoarthritis activity and analgesic potential of *Strobilanthes cuspidata* was reported by Brahma Srinivasa Rao Desu (2011 and 2012) [28] by comparing the ethanolic extract with marketed herbal formulation Shallaki. With a view to develop a human friendly herbal drug to treat this order and related complications, the present study attempts were made to evaluate the efficacy of *S. cuspidata* in the management of diabetes.

Materials and Methods

Plant material and extraction

S. cuspidata (Acanthaceae) leaves were collected from Niligiris. The leaves (plant material) were dried, coarsely powdered and extracted (1kg) separately with ethanol (70%) in a soxhlet extractor for 24 h. The extracts were concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40-45 °C).The nature and yields of the extract were noted and stored in a refrigerator at 4 °C for further studies.

The column chromatographic technique is widely used for separation, isolation, purification of natural compounds. The fractions was chromatographed over silica gel (60-120) mesh of column length 100 cm and diameter 3 cm. Elution was carried out with solvent and mixtures of increasing polarities. The fractions were collected in four equal volumes and monitored on TLC (silica gel Gas adsorbent, with suitable solvent system) and similar fractions were pooled together based upon their TLC profile. Based on gradient elution the compounds were eluted with silica gel F to give a single spot with R_f value and it was designed as compound S1-Stigmasterol (2.5mg), S2- Lupeol(4 mg) and S3-E-3(3-Hydroxy-4-methoxy phenyl) acrylic acid(5mg).Total saponins were obtained by gravimetric analysis. These compounds were subjected to *in vitro* inhibition of α - amylase enzyme.

In-Vivo Studies

Experimental Animals

Healthy Wistar rats of either sex, weighing 180-220 g, were procured from the animal house, JSS College of Pharmacy, Ootacamund, India. The animal house was well ventilated and animals had 12 ± 1 h day and night schedule. The animals were housed in large spacious hygienic cages during the course of the experimental period and room temperature was maintained at 25 ± 1 °C. The animals were fed with standard rat feed and water *ad libitum*. The experiments were conducted as per the guidelines of CPCSEA, Chennai.

Oral glucose tolerance test (OGTT)

(*Bonner-Weir, 1988; Barham and Trinder, 1972*)

The oral glucose tolerance test was performed in overnight fasted (18 h) normal rats. Fasted rats were divided into four groups (n=6/group). These rats were orally treated in the following manner: Group I normal control (0.9% (w/v) saline), II (250mg/kg of Metformin), III (150 mg/kg of plant extract) and IV (300 mg/kg of plant extract. Glucose (3 g/kg) was fed 30 min after the administration of extract. Blood was withdrawn from the retro orbital sinus at 0, 30, 60 and 120 min of glucose administration and blood glucose levels were estimated by glucose oxidase–peroxidase method.

Alloxan induced diabetes (*Yadav et al., 2008*)

Preparation of test samples

The extract and the standard hypoglycemic drug metformin (250 mg/kg) were dissolved in isotonic saline solution (NaCl, 0.9% w/v) and were administered orally.

Induction of diabetes in rats by Alloxan monohydrate

Diabetes was induced in overnight fasted adult Wistar albino rats weighing 180–220 g by a single intraperitoneal injection of 120 mg/kg alloxan monohydrate (Loba Chemie, Bombay)freshly dissolved in normal saline (0.9%, w/v). Hyperglycemia was confirmed by the elevated glucose levels determined at 72 h after alloxan injection. The rats having blood glucose levels above 200mg/dl were used for the antidiabetic study.

After induction of diabetic the experimental animals were divided into five groups, each consisting of six rats.

Group I: Normal control (saline)

Group II: Diabetic control (Alloxan150 mg/kg.ip)

Group III: Alloxan (150 mg/kg.ip) + Metformin 250 mg/kg

Group IV: Alloxan (150 mg/kg.ip) + Plant extract (150 mg/kg, p.o)

Group V: Alloxan (150 mg/kg.ip) + Plant extract (300mg/kg, p.o)

All the extracts and standard drug were administered orally for 14 days. The fasting blood samples were collected by retro orbital sinus puncture under mild ether anesthesia and serum was separated by centrifugation at 4000 rpm for 15 min. Fasting blood glucose estimation was measured on day 0, 7 and 14 of the study.

Statistical analysis

The results are presented as means ± SEM with n=6.Statistical analysis was performed using Two-way ANOVA followed by Bonferroni multiple comparison test. $P<0.05$ are considered to be statistically significant.

In vitro inhibition of α - amylase enzyme

The study was carried out with porcine pancreatic α-amylase with starch as substrate. Acarbose was selected as the standard drug for comparison of results.

Principle

Alpha amylase will digest the starch in reaction mixture to yield maltose. This maltose will reduce the 3, 5-

dinitrosalicylic acid in the coloring agent to 3-amino-5-nitrosalicylic acid. This reaction will produce a colour change from orange to red. The intensity of red colour will be directly proportional to the amount of maltose produced. When an enzyme inhibitor is present in the reaction mixture, digestion of starch, production of maltose and intensity of red colour produced will be less.

Reagents used

- 20 mM phosphate buffer of pH 6.9 (prepared with sodium phosphate monobasic and sodium chloride)
- 1.0% starch solution (prepared in phosphate buffer by boiling)
- Colour reagent (prepared by slowly adding sodium potassium tartarate solution (prepared in the ratio 12g of solid dissolved in 8ml of 2M sodium hydroxide) to 20 ml of 96mM 3,5-dinitrosalicylic acid (prepared in distilled water) and then diluting the mixture to 40 ml with distilled water
- Enzyme solution (0.5mg/ ml) prepared in phosphate buffer.

From 1mg/ ml stock solution different concentrations (0.05-6.4 µg/ml) of Test samples were prepared in dimethyl sulfoxide. 500 µl of test/standard was added to 500 µl of α-amylase (0.5mg/ ml) and was incubated for 10 minutes at room temperature. 500 µl of 1.0% starch solution was added and incubated for another 10 minutes.q1ml of the colouring reagent was added to the reaction mixture and heated in a boiling water bath for 5 minutes. After cooling 10 ml of distilled water was added for dilution. To measure the absorbance of the coloured extracts, blank was prepared for each set of concentration of test sample by replacing the enzyme solution with buffer. Control incubations representing 100% enzyme activity was prepared by replacing the test drug with dimethyl sulfoxide. The absorbance was then measured at 540 nm. The α-amylase inhibition was expressed as percentage of inhibition and the IC_{50} values determined by linear regression plots with varying concentration of Test sample against percentage inhibition (Rao *et al.,* 2008) [52].

Formula to calculate the percentage inhibitory activity:

Percentage inhibition =

$$100 - \left\{ \frac{(AC - AC\ blank) - (AT - AT\ blank)}{(AC - AC\ blank)} X\ 100 \right\}$$

Where,
AC is absorbance of control
AC blank is absorbance of control without enzyme
AT is absorbance of Test
AT blank is absorbance of Test without enzyme.

Results

The leaves (plant material) were dried, coarsely powdered and extracted (1kg) separately with ethanol (70%) in a soxhlet extractor for 24 h. The extracts were concentrated to dryness in a rotavapor under reduced The air-dried and finely grounded leaves were The alcoholic extract was screened for various phytoconstituents like glycosides, alkaloids, flavonoids, saponins, tannins, triterpenes and resins.

Effect of extracts on OGTT

The results of OGTT are given in Table 1 and Figure 1. The data reveal that the extract at both the dose levels (150 and 300 mg/kg) cause a significant decrease in the blood glucose level within 60 min after oral administration to normal rats. The extracts produce a significant attenuation (p<0.001) in the blood glucose level at 60 min to 120 min when compared with normal control.

Effect of extracts on blood glucose level

The results of the blood glucose level are given in Table 2 and Figure 2. The data reveal that the extract at both the dose levels (150 and 300 mg/kg) show a significant (p<0.001) time dependent decrease in blood glucose level after oral administration at 7 and 14 days when compared to the diabetic control group. After 14 days of daily treatment with the extract (150 and 300 mg/kg), the results show significant dose-dependent fall in blood sugar levels by 38 and 41%, respectively.

Table 1: Effect of extract on the OGTT in normal rats

Groups	Treatment	0 min	30 min	60 min	120 min
I	Normal control	66.5±0.88	85.33±1.68	106±3.72	91.66±2.49
II	Metformin(250mg/kg)	69.33±0.66	83.5±1.54	74.5±0.61***	70.33±0.61***
III	Plant extract (150 mg/ kg)	71.33±0.66	86.83±1.47	78.66±0.66***	76.33±0.61***
IV	Plant extract (300 mg/ kg)	69.33±0.66	84.5±0.92	72.5±3.42***	70.16±0.60***

Values are expressed as mean± SEM (n = 6).
*** p<0.001, compared with normal control.
Two way ANOVA followed by Bonferroni's multiple comparison tests

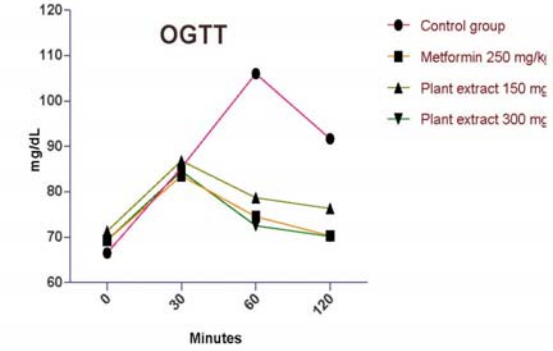

Fig 1: Effect of extract on the OGTT in normal rats

Table 2: Effect of extract on the blood glucose level in diabetic rats

Groups	Treatment	0 Day	7th Day	14th Day
I	Normal control	66.16±0.47	67.33±0.33	67.50±0.71
II	Diabetic control	213±0.85###	231.33±0.98###	249.16±1.42###
III	Metformin (250mg/kg)	211.5±0.61	174.83±3.31***	125.66±1.74***
IV	Plant extract (150 mg/kg)	218±1.91	187.66±4.08***	134.16±1.66***
V	Plant extract (300 mg/kg)	213.33±0.84	172.33±1.89***	125.33±1.43***

Values are expressed as mean± SEM (n = 6). ###p<0.001 compared with normal control, *** p<0.001, compared with diabetic control. Two way ANOVA followed by Bonferroni's multiple comparison tests.

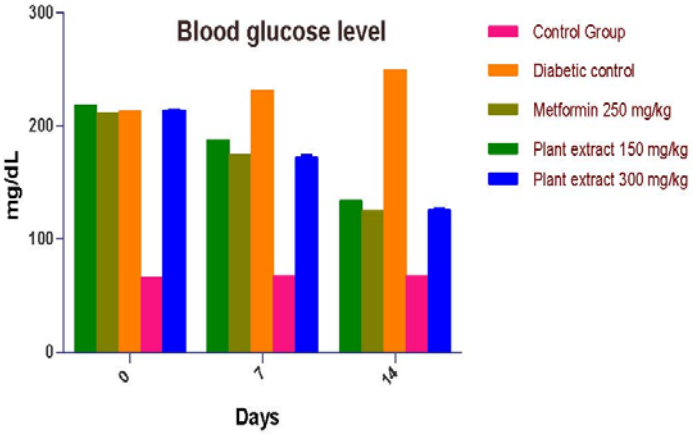

Fig 2: Effect of extract on the blood glucose level in diabetic rats

The isolated compounds were tested for their antidiabetic potential *in vitro* by inhibition of *α*-amylase enzyme. The study was carried out with porcine pancreatic *α*-amylase with starch as substrate. Acarbose was selected as the standard drug for comparison of results. Total saponins, Lupeol and stigmasterol showed higher alpha amylase inhibitory activity which confirms its antidiabetic potential.

Table 3: Alpha amylase inhibitory activity.

Concentration (µg/Ml)	% Inhibition				
	Standard Acarbose	Compound-1	Compound-2	Compound-3	Total Saponins
0.05	8.4±3.15	7.7±0.06	7.2±0.06	4.4±0.06	4.5±0.01
0.1	19.2±4.35	9.9±0.07	10.5±0.04	5.8±0.05	7.2±0.03
0.2	24.0±6.06	11.4±0.03	16.2±0.04	9.4±0.05	10.7±0.03
0.4	35.1±4.65	13.7±0.02	22.7±0.04	11.6±0.08	15.9±0.08
0.8	43.9±5.82	16.7±0.05	29.7±0.04	16.5±0.05	17.5±0.04
1.6	58.8±0.64	19.2±0.03	46.6±0.06	19.4±0.06	34.2±0.07
3.2	74.3±1.33	22.3±0.03	52.6±0.04	22.9±0.06	55.5±0.06
6.4	89.8±1.35	22.9±0.04	73.05±0.04	27.1±0.05	72.4±0.07
IC_{50} (µg/ml)	1.3 ±0.13		2.1±0.046		1.8±0.04

Values are expressed as mean ± SEM of triplicate measurement

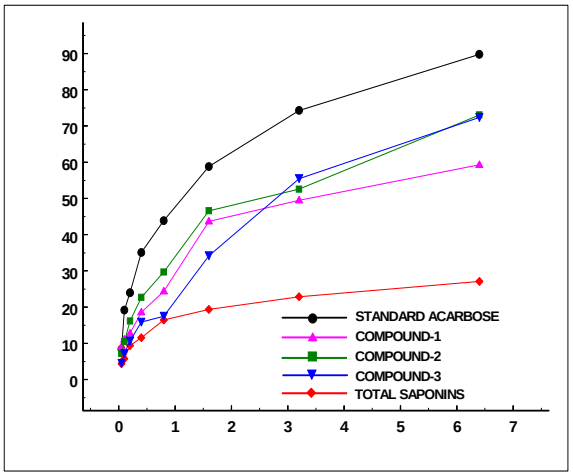

Fig 3: *In vitro* inhibition of α –Amylase enzyme

Compound 1- Stigmasterol
Compound 2- Lupeol
Compound 3- E-3-(3-Hydroxy-4-methoxy phenyl) acrylic acid / (3-Hydroxy-4-methoxy phenyl) cinnamic acid

Discussion

Diabetes mellitus is possibly the world's largest growing metabolic disease, and as the knowledge on heterogeneity of this disorder is advanced, the need for more appropriate therapy increases (Baily *et al*., 1986) [32]. Traditional plant medicines are used throughout the world for a range of diabetic populations. The study of such medicines might offer a natural key to unlock a diabetologist's pharmacy for the future.

In the light of the literature on S. cuspidata we made an attempt for the first time to study the effect of S. cuspidata ethanolic extract in antidiabetic activity by Alloxan induced diabetes rat model and the isolated compounds were tested for

their antidiabetic potential *in vitro* by inhibition of *α*-amylase enzyme. Alloxan induces "chemical diabetes" in a wide variety of animal species by damaging the insulin secreting pancreatic β-cell, resulting in a decrease in endogenous insulin release. Numerous studies demonstrated that a variety of plant extracts effectively lowered the glucose level in alloxan-induced diabetic animals, (Ji Su Kim *et al*., 2006) [47]. Oral glucose tolerance test (OGTT) measures the body's ability to use glucose, the body's main source of energy and can be used to diagnose prediabetes and diabetes, (Du Vigneaud *et al*., 1925) [42].

In the present study the effect of extracts on OGTT reveal that the extract at both the dose levels (150 and 300 mg/kg) cause a significant decrease in the blood glucose level within 60 min after oral administration to normal rats. The effect of extracts on blood glucose level reveal that the extract at both the dose levels (150 and 300 mg/kg) show a significant (p<0.001) time dependent decrease in blood glucose level after oral administration at 7 and 14 days when compared to the diabetic control group. After 14 days of daily treatment with the extract (150 and 300 mg/kg), the results show significant (p<0.001) dose-dependent fall in blood sugar levels by 38 and 41%, respectively. The isolated compounds were tested for their antidiabetic potential *in vitro* by inhibition of *α*-amylase enzyme. Total saponins, Lupeol and stigmasterol showed higher alpha amylase inhibitory activity which confirms its antidiabetic potential.

Results of antidiabetic activity of S. cuspidata leaf extracts established the scientific basis for the utility of this plant in the treatment of diabetes. The extract have shown significant reduction in blood glucose levels in both glucose loaded and alloxan induced diabetic rats. The isolated compounds such as stigmasterol, lupeol showed good antidiabetic activity. Total saponins isolated from the hydroalcoholic fraction revealed α-amylase inhibitory activity, therefore it is obvious that the fraction has enriched active principles.

The significant antidiabetic activity of S. cuspidata may be due to the presence of hypoglycemic saponins and triterpenes. It could be conceived that the plant extracts may also contain some biomolecules that may sensitize the insulin receptor to insulin or stimulates the β-cells of Langerhans to release insulin which may finally lead to the improvement of carbohydrate metabolizing enzymes towards the re-establishment of normal blood glucose level.

Diabetes is known to affect large number of metabolic pathways, including lipid metabolism, by altering the activities of various enzymes involved in these pathways. Insulin deficiency (type-1 diabetes) or decreased insulin action (type 2-diabetes) results in decrease in glucose utilization by insulin requiring tissue like liver and an increase in glucose production through an increased rate of gluconeogenesis; both resulting in hyperglycemia. As a consequence of increased glucose and decreased insulin level in blood plasma, hepatic regulation of lipid metabolism is greatly altered, (Umesh CS *et al.*, 2004) [58]. Diabetes is associated with profound alterations in the plasma lipid and lipoprotein profile with an increased risk of coronary heart disease, (Betteridge. J *et al.*, 1997) [35].

Insulin is an important regulator of many enzymes involved in lipogenesis, its deficiency causes major changes in the activity of these enzymes thereby affecting the lipid profile of various tissues. Kidney is insulin –independent tissue involved in the transport of glucose in the cells and thus gets severely affected due to increased blood glucose.

The isolation of insulin by Banting and Best was considered as the beginning of a new era for the treatment of diabetes by insulin (Bloom and Ireland 1992) [36] however, existence of NIDDM (type 2-diabetes),where long term insulin therapy becomes ineffective due to insulin resistance, and severe hypoglycemia particularly affecting the brain (Cryer 1992: Reichard *et al.* 1993) [40, 53] from insulin therapy in case of type- 1 diabetes, led the march for the search of an alternative to insulin thearpy, (Ramasarma,1996). It is well established that complications associated with diabetes can be markedly reduced through good glucose control.

Certain metal elements such as vanadium, selenium, molybdenum, tungstate, Zinc and manganese, (Heyligar *et al.* 1985; Baquer *et al.* 1998; Ezaki 1990; Goto *et al.* 1992; Shisheva *et al.* 1992; Baquer *et al.* 2003) [45, 33, 43, 44, 57, 34] with potential hypoglycemic activities have been studied earlier. However, vanadium and its various complexes have been particularly favoured for their inusulinometic effects (Ramasarma, 1996). Similarly, extracts from various plant materials have been tested in animal model system and their hypoglycemic effects have been elucidated (Murthy, 1995) [48]. The absence of toxic effects of plant extracts makes the use of such natural products for their antidiabetic properties favourable. Plant extracts which have been studied so far including Allium sativum Linn. (Sheela and Augusti 1992) [55], Momordica charantia Linn., fruit extract (Shibib *et al.* 1993; Ahmed *et al.* 1998) [56], Trigonella foenum-greacum Linn., seed powder (Moorthy *et al.*, 1989; Raju *et al.* 2001) [48, 51] among others, have been confirmed to possess antidiabetic properties.

Conclusion

The present work revealed that S. cuspidata is a good antidiabetic herbal remedy, antidiabetic effect in rats and their effect was comparable to that of Metformin in *in vivo* studies and Acarbose in *in vitro* studies. Therefore this plant is considered to be effective and alternative treatment for diabetes.

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