Journal of Research in Pharmacy
Research Article
www.jrespharm.com

Evaluation of anti-diabetic potential of *Ficus arnottiana* Miq. leaves against *in-vivo* and *in-vitro* experimental models of diabetes

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Received: 31 July 2023 / Revised: 09 October 2023 / Accepted: 12 October 2023

ABSTRACT: The present study was aimed to investigate the potential anti-diabetic effects of Ficus arnottiana Miq. leaves against streptozotocin-induced diabetic model in experimental animals. The leaves of Ficus arnottiana were collected and processed into a powdered form, which was subsequently extracted using methanol as the solvent. The anti-diabetic activity of the resulting extract was evaluated at two oral doses (200 and 400 mg/kg) at two different doses (200 and 400 mg/kg). The parameters assessed in this study included blood glucose levels, C-peptide levels, and serum insulin levels. Furthermore, pancreatic tissue samples were subjected to histopathological analysis. Additionally, in vitro determination of α-amylase and α-glucosidase inhibition by the extract was carried out. The phytochemical screening of the extract revealed the presence of alkaloids, tannins, flavonoids, phenolic compounds, reducing sugars, and saponins. For anti-diabetic activity, the test extract significantly reduced blood glucose levels while increasing both serum C-peptide and serum insulin levels. Histopathological analysis of extract treated group depicted improved islets of Langerhans and an increase in centrally located beta cells. Furthermore, the extract significantly inhibited α-amylase and α-glucosidase enzyme in-vitro. Based on these findings, it can be concluded that Ficus arnottiana (Miq) leaves extract exhibits antidiabetic activity. And the anti-diabetic effect of Ficus arnottiana (Miq) at 400 mg/kg was comparable to that of the standard drugs Glibenclamide (in-vivo) and acarbose (in-vitro).

Key words: Anti-diabetic activity; STZ induced diabetes; histopathological evaluation; α-amylase; α-glucosidase; Ficus arnottiana (Miq).

1. INTRODUCTION

Both preventative and curative pharmaceutical preparations for humans rely heavily on medicinal plants. India, known as the "Botanical Garden of the World," is the country that produces the most medicinal plants. Since ancient times, it has been popular in India to treat illnesses with various portions of plants. For millennia, medicinal plants have found application in various medical traditions like Ayurveda, Siddha, Unani, and more [1].

Diabetes, also known as diabetes mellitus, is the most common and increasing illness worldwide and comprises a group of metabolic disorders marked by persistent high levels of blood sugar (hyperglycemia). Diabetes arises due to either an overproduction of insulin by the pancreas or the

How to cite this article: Inamdar SK, Anwar PS, Fatima K, Ahmad M, Tazneem B, YadavP, Sarafroz M, Khan A, Taleuzzaman M. Evaluation of anti-diabetic potential of *Ficus arnottiana* Miq. Leaves against *in-vivo* and *in-vitro* experimental models of diabetes. J Res Pharm. 2024; 28(5): 1844-1854.

http://dx.doi.org/10.29228/jrp.857
ISSN: 2630-6344

body's cells being inefficient in utilizing insulin. Insulin, a hormone responsible for facilitating the entry of glucose from food into cells to serve as fuel [2,3]. For individuals with type 2 diabetes or those at the borderline, managing blood glucose levels can be improved by inhibiting alpha-glucosidase and alpha-amylase, enzymes involved in carbohydrate digestion. This inhibition can significantly reduce the post-meal surge in blood glucose levels [5]. Type I diabetes mellitus (DM) is commonly managed utilizing insulin replacement therapy, whereas type II DM is typically addressed through the administration of oral hypoglycemic agents. The primary pharmacological intervention for type II diabetes mellitus involves the utilization of various classes of medications, including insulin secretagogues, biguanides, insulin sensitizers, alpha glucosidase inhibitors, incretin mimetics, amylin antagonists, and sodium-glucose co-transporter-2 (SGLT2) inhibitor [5]. Currently, there is a renewed interest in functional foods and plant-based remedies that can effectively manage physiological responses in treating and preventing diabetes and obesity. There are numerous plants in the plant kingdom, making it easy to find natural, effective oral hypoglycaemic medications with minimal or no negative impacts. Over 1200 plant species have been identified as being empirically utilised for their suspected hypoglycaemic properties globally [4].

Ficus arnottiana (Miq) belongs to genus Ficus and the family Moraceae, is a large group of trees or shrubs, distributed across the tropical regions of both hemispheres. In India alone, approximately 65 species of Ficus are present, showcasing the remarkable diversity of the genus across various habitats. Among them are impressive botanical giants like the Banyan tree, peepal tree, and Indian rubbers, as well as delicate, climbers such as *Ficus pumila* and *Ficus scandens* Roxbs. This genus is a rich source of beneficial compounds, including flavonoids, xanthones, tannins, flavonoid glycosides, and numerous other phenolic compounds. Pharmacologically it has been reported to have anti-ulcer, hypoglycaemic Anti-oxidant, aphrodisiac demulcent, emollient, effect [6].

The study's objective was to assess the anti-diabetic effects of Ficus arnottiana (Miq) leaves by considering both the ethnopharmacological knowledge shared by the tribal community and the reported phytochemical composition.

2. RESULTS

As presented in Table 1, the percentage yield of the crude extract was found to be 11.1% (40 gm of extract was obtained from 360 gm of powdered plant material).

2.1. Preliminary phytochemical Screening

The result of the preliminary phytochemical analysis of the extract revealed the presence of alkaloids, tannins, flavonoids, phenolic compound, reducing sugar, and saponins as shown in Table 1.

Table 1: Phytochemical screening of crude extract of Ficus Arnottiana (Miq)

S. N	Phytoconstituents	Test	Results
1	**Carbohydrates**	Banford's test	-
		Molisch's test	-
2	**Alkaloids**	Mayer's test	+
		Dragendorff's test	+
		Hager's test	-
		Wagner's test	+
3	**Flavonoids**	Alkaline test	+
		Ferric chloride test	+
4	**Phenolic compound**	Ferric chloride test	+
		Lead acetate test	+
		Bromine water test	+
5	**Reducing sugar**	Benedict's test	+

		Fehling's test	+
6	**Tannins**	Gelatin test	+
		Bromine water test	+
7	**Saponins**	Foam test	+

2.2. Antidiabetic activity of test extract against STZ induced diabetic rats

Marked rise in blood glucose level was observed after STZ administration. When measure after 30 min of treatment, *Ficus arnottiana* 400 mg/kg BW and Glibenclamide 5 mg/kg significantly reduced blood glucose level in diabetic rat compared to disease control rat ($p<0.01$); *Ficus arnottiana* 200 mg/kg also reduced blood glucose level compared to disease control rat ($p<0.05$).

When measured at 60 and 90 min, the reduction of blood glucose level by *Ficus arnottiana* 400 mg/kg and glibenclamide 5 mg/kg was highly significant compared to disease control rat ($p<0.001$); and the reduction of blood glucose level by *Ficus arnottiana* 200 mg/kg BW was significant at $p<0.01$, compared disease control rats.

Furthermore, when measured at 180 min, *Ficus arnottiana* 400 mg/kg BW and glibenclamide 5 mg/kg significantly reduced blood glucose level compared to disease control ($p<0.05$), whereas *Ficus arnottiana* 200 mg/kg BW does not reduced the blood glucose level compared to diseased control rat. From the result it is demonstrated that the effect of *Ficus arnottiana* 400 mg/kg was nearly equivalent to that of glibenclamide 5 mg/kg. Data are presented in Table 2.

Table 2: Effect of *F. arnottiana Miq.* leaves extract on blood glucose level in Streptozotocin (STZ) induced diabetic rats.

Group	0 min (mg/dl)	30 min (mg/dl)	60 min (mg/dl)	90 min (mg/dl)	120 min (mg/dl)	180 min (mg/dl)
Control	116.3±2.2	109.6±3.4	114.1±4.4	116.6±3.5	110.3±4.8	113.8±2.6
Disease control	234.6±3.1	239.2±2.7	239±2.0	238.8±2.2	239.8±1.7	238.3±2.2
Glibenclamide (5 mg/kg, BW)	237.7±2.8	227.2±2.1**	225.1±1.9***	223.2±3.2***	225.7±3.2**	228.5±3.1*
***Ficus arnottiana* 400 mg/kg**	236.6±2.4	225.9±1.6**	225.4±2.5***	221.8±2.0***	226.7±2.1**	229.4±2.2*
***Ficus arnottiana* 200 mg/kg**	237.5±2.5	230±2.5*	226.8±2.3**	226.8±2.3**	228.7±2.5*	231.5±1.6

All the values are mean ± SEM, n=6, one-way Analysis of Variance (ANOVA) followed by multiple comparison Tukey's test. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ v/s diseased control group.

2.3. Serum insulin level

As presented in Table 3, administration of STZ, significantly decreased serum insulin level. *Ficus arnottiana* extract 400 mg/kg BW and Glibenclamide 5 mg/kg400 treated group displayed a significant increase in serum insulin level ($p<0.001$) when compared to disease control group. *Ficus arnottiana* extract 200 mg/kg BW treated rat showed increased serum insulin level ($p<0.05$) compared to disease control group.

Table 3: Effect of *Ficus arnottiana Miq*. leaves extract on serum Insulin level in Streptozotocin (STZ) induced diabetic rat

Group	Serum Insulin level (µ IU/mL)
Control	22.4 ± 0.21
Disease control	15.83 ± 0.27
Glibenclamide 5 mg/kg	20.67 ± 0.45***
***Ficus arnottiana* 400 mg/kg BW**	21.40 ± 0.36***
***Ficus arnottiana* 200 mg/kg BW**	17.32 ± 0.20*

All the values are mean ± SEM, n=6, one-way Analysis of Variance (ANOVA) followed by multiple comparison Tukey's test. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ v/s disease control.

2.4. Serum C-peptide level

Marked decrease in serum C-peptide level was found in disease control rats. *Ficus arnottiana* 400 mg/kg and 200 mg/kg treated rats showed increased serum C-peptide level compared to disease control ($p<0.01$ and $p<0.05$ respectively). And Glibenclamide treated rats showed increased serum C-peptide level to most significant level ($p<0.001$) compared to disease control group.

Table 4: Effect of *Ficus arnottiana Miq*. Leaves extract on serum C-peptide level in Streptozotocin (STZ) induced diabetic model

Group	C- peptide level (ng/ml)
Control	0.59 ± 0.02
Disease control	0.40 ± 0.02
Glibenclamide 5 mg/kg	0.53 ± 0.01***
***Ficus arnottiana* 400 mg/kg BW**	0.50 ± 0.02**
***Ficus arnottiana* 200 mg/kg BW**	0.49 ± 0.01*

All the values are mean ± SEM, n=6, ns=Not significant, one-way Analysis of Variance (ANOVA) followed by multiple comparison Tukey's test. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ v/s disease control

2.5. Histopathology of pancreas

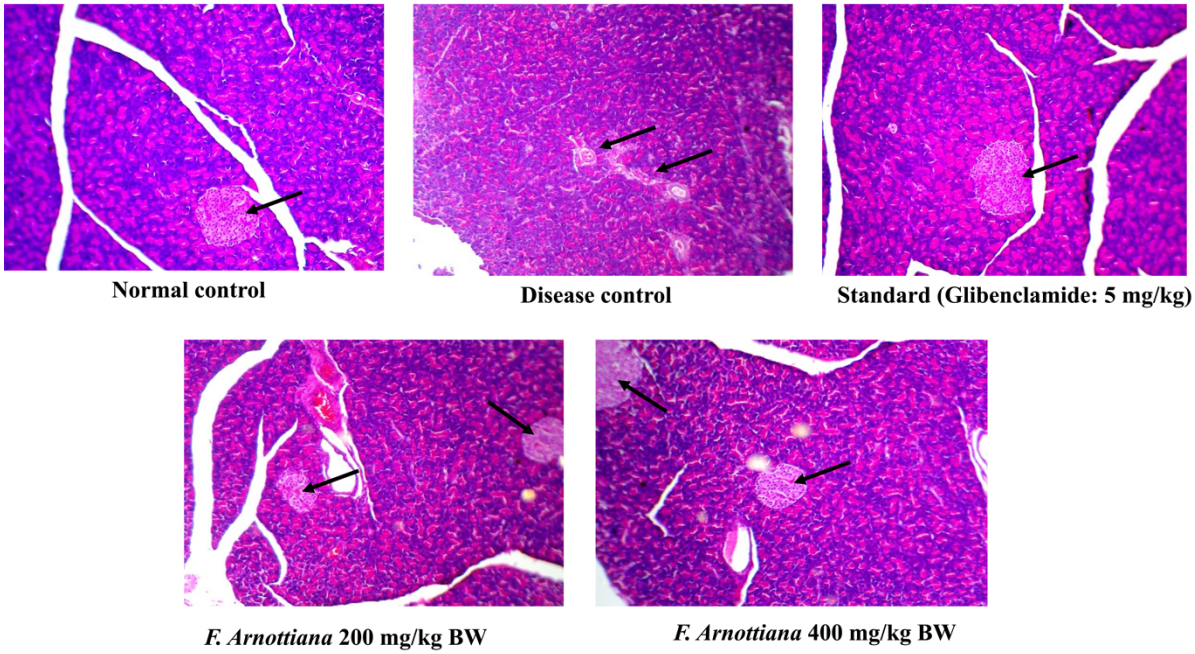

Figure 1: Histopathological analysis of pancreas. Normal control group, standard glibenclamide, and Ficus arnottiana 400 mg/kg shows normal-sized islets of Langerhans and normal density of islet cells and centrally located β-cells. F. arnottiana 200 mg/kg treated group showing partial recovery of the tissue with well-defined islets of Langerhans. Diabetic STZ induced group showing distortion of the islets of Langerhans.

The disease control group displayed distorted islets of Langerhans, characterized by prominent necrotic and degenerative changes in the β-cells. Additionally, there was an infiltration of inflammatory lymphocytes, and the blood vessels appeared severely dilated and congested.

For the group treated with glibenclamide, a well-defined islet of Langerhans was observed, with a proliferation of β-cells located between normal pyramidal acidophilic pancreatic acini.

In the case of the Ficus arnottiana (200 mg/kg) treated group, there was partial recovery of the tissue, with well-defined islets of Langerhans and a moderate increase in the number of proliferated β-cells (indicated by a red arrow). Furthermore, the Ficus arnottiana (400 mg/kg) treated group exhibited a remarkable recovery of the pancreatic tissue, with a significant increase in the number of centrally located β-cells.

2.6. *In-vitro* inhibition of α-amylase and α-glucosidase

As presented in Table 5, the percent inhibition of α-amylase by *Ficus Arnottiana Miq* extract at the concentrations 20, 40, 80 µg/ml was found to be 2.4%, 30.4%, 58%. Meanwhile, the standard compound acarbose showed 6%, 27%, and 80% of α-amylase inhibition at the same concentration. The percent inhibition of α-glucosidase by *Ficus arnottiana Miq* extract at the concentrations 20, 40, 80 µg/ml was found to be 12%, 38.4%, 56% and that of with standard compound acarbose it was found to be 18.8%, 42.4%, and 74%.

Table 5: *In-vitro* inhibition of α-amylase and α-glucosidase by *F.Arnottiana* extract and acarbose.

Conc (µg/ml)	% Inhibition of α-amylase		% Inhibition of α-glucosidase	
	F. Arnottiana Miq	Acarbose	*F. Arnottiana Miq*	Acarbose
20	2.46	6	12	18.8
40	30.4	27	38	42.4
80	58	80	56	74

3. DISCUSSION

Diabetes mellitus is a chronic medical illness that is associated with several consequences, including neuropathy, nephropathy, and retinopathy. Additionally, it is important to note that there is an elevated susceptibility to cardiovascular ailments associated with the presence of either a relative or absolute deficiency of insulin, which subsequently leads to hyperglycemia, glycosuria, hyperlipidemia, ketonemia, and ultimately contributes to the development of heart failure, stroke, and peripheral vascular disorders. [7] Pharmaceutical treatments for diabetes do have some drawbacks, including drug resistance leading to reduced effectiveness, potential side effects, and even toxicity. As a result, there has been a growing recommendation for the incorporation of medicinal plants as a potential treatment option. Numerous plants contain valuable compounds such as carotenoids, flavonoids, terpenoids, alkaloids, and glycosides, which often demonstrate beneficial effects in managing diabetes. The use of these plants is attributed to their ability to improve the functioning of pancreatic tissue, either by promoting insulin secretion or by reducing glucose absorption in the intestines, thereby leading to anti-hyperglycaemic effects. [8]

In present study, *Ficus arnottiana* (Miq.) leaves Moraceae were assessed for its anti-diabetic potential. This plant was chosen as it is reported to have active phytoconstituents such as sitosterol, glycol acetate, friedelin, bioactive isoflavone glycoside and its antidiabetic potential has not been studied earlier. [9] Initially , the leaves of the plant was extracted using methanol and the resulting extract had a yield of 11.1%. The extract further underwent phytochemical screening which revealed the presence of alkaloids, tannins, flavonoids, phenolic compounds , reducing sugars, and saponins. Afterwards, anti-diabetic activity the extract was assessed using streptozotocin induced diabetes model, *in-vitro* analysis of α-amylase and α-glucosidase inhibition and histopathological analysis of pancreas.

Streptozotocin enters the β-cells by utilizing the glucose transporter GLUT2 and causes DNA alkylation. The development of diabetes due to streptozotocin primarily occurs through the activation of poly ADP-ribosylation rather than direct DNA damage. Poly ADP-ribosylation leads to decrease in cellular NAD + and ATP levels. When streptozotocin is administered, increased ATP dephosphorylation provides a substrate for xanthine oxidase, which produces superoxide radicals. Consequently, hydroxyl radicals and hydrogen peroxide are generated. Additionally, streptozotocin releases high levels of nitric oxide, which inhibits aconitase activity and contributes to DNA damage. As a result, β-cells undergo necrosis. This models has been considered suitable for inducing diabetes. [10]

After inducing diabetes, various parameters such as blood glucose levels, serum insulin levels, serum C-peptide levels, and pancreatic histopathology were examines. *Ficus arnottiana* demonstrated a significant reduction in blood glucose levels and an increase in insulin and serum C-peptide levels compared to the disease control group. Moreover, the effects of *Ficus arnottiana* at dosage of 400 mg/kg were similar to those of the standard drug Glibenclamide 5 mg/kg, BW. Histopathological analysis revealed that treatment with test extract, especially at 400 mg/kg, resulted in well-defined islets of Langerhans with an increased number of centrally located β-cells, indicating its potential as an anti-diabetic agent.

The *in-vitro* anti-diabetic activity was evaluated using inhibition of α-amylase and α-glucosidase. α-Amylase is an enzyme responsible for breaking down starch into oligosaccharides, while α-glucosidase converts oligosaccharides into monosaccharides, which are then absorbed, leading to an increase in blood glucose levels. Therapeutically focusing on these enzymes has proven to be successful in the management of type 2 diabetes mellitus and in regulating spikes in postprandial blood glucose. Various enzymatic inhibitors like acarbose, miglitol, and voglibose have shown efficacy in selectively inhibiting these enzymes. Consequently, there is significant interest among researchers in developing potent inhibitors specifically targeting α-amylase [11, 12].

In line with this, the extract under investigation was evaluated for its potential to inhibit above mentioned enzymes. The leaves extract of *Ficus arnottiana Miq*., particularly at 400 mg/kg BW, significantly inhibited both α-amylase and α-glucosidase enzymes. The degree of inhibition observed with the extract was comparable to that of the standard inhibitor acarbose, as quantified by percent inhibition of both enzymes.

The study findings indicate that the extract of *Ficus arnottiana* Miq has noteworthy potential in combating diabetes, as demonstrated in both *in-vivo* and *in-vitro* experiments. This anti-diabetic effect is likely due to the presence of alkaloids, flavonoids, and phenolic compound in the extract.

Scientific investigations have revealed that herbal medicines contain bioactive compounds like flavonoids, terpenoids, glycosides, and alkaloids, which exhibit diverse anti-diabetic effects. These effects involve enhancing insulin secretion, reducing insulin resistance, promoting liver glycogen synthesis, and displaying antioxidant and anti-inflammatory properties [13]. Numerous research studies have demonstrated that herbal medicines contain these biologically active macromolecules, which play a crucial role in their effectiveness against diabetes. These compounds influence insulin secretion, enhance insulin sensitivity, promote liver glycogen synthesis, and exhibit antioxidant and anti-inflammatory effects [13].

4. CONCLUSION

The findings of this study suggest that *Ficus arnottiana Miq* extract exhibits strong anti-diabetic potential. Its ability to lower blood glucose levels, increase serum insulin and serum C-peptide levels, and improve pancreatic histopathology indicate its efficacy in managing diabetes. The observed inhibitory effects on α-amylase and α-glucosidase enzymes further support its anti-diabetic activity. These beneficial effects may be attributed to the presence of alkaloids, flavonoids, and phenolic compounds in the extract. Further research is warranted to elucidate the underlying mechanisms and isolate the bioactive components responsible for the observed effects. *Ficus arnottiana Miq* extract holds promise as a potential therapeutic agent for the treatment of diabetes.

5. MATERIALS AND METHODS

5.1. Chemicals

All the chemicals used in phytochemical screening, streptozotocin, starch were purchased from Hi-media pvt ltd. Mumbai, α-Amylase and α-glucosidase were purchased from Sigma Aldrich, USA.

5.2. Plant material

Leaves of *Ficus arnottiana (Miq).* were gathered from local area of Kalaburagi, Karnataka, India. The plant material was identified and authenticated by botanist Dr. N.G Patil, Associates Professor, Veeramma Gangasiri Women's College Kalaburagi, Karnataka. The leaves were coarsely powdered and stored in air locked container for further investigation.

5.3. Extraction of plant material

250 g of the powdered leaves were extracted with methanol at 55 °C for 18 hours using Soxhlet. The resulting extract was then dried using a rotary evaporator. The final crude extract obtained was weighed and kept in refrigerator until further analysis.

5.4. Phytochemical screening of the crude extract

The *Ficus arnottiana (Miq)* extract was subjected to biochemical testing to identify its active principal compounds, including saponins, alkaloids, flavonoids, tannins, phenols, and lipids. Standard procedures were employed for these biochemical tests on the methanolic extract of *Ficus arnottiana (Miq).* To carry out the tests, the extract was dissolved in phosphate buffer saline with the addition of 1% tween 80.

5.4.1. Detection of carbohydrates

Barford's test: 1 ml of the extract was combined with an equal volume of Barford's reagent. The resulting mixture was then subjected to heating for a duration of 2 minutes. Subsequently, the formed mixture was examined for the existence of a red precipitate.

Molisch' test: 2 ml of the extract was mixed with two drops of alcoholic α-naphthol, and then 1 ml of concentrated H_2SO_4 was carefully added down the side of the test tube. The resulting mixture was examined for the formation of a violet ring.

5.4.2. Detection of alkaloids

Mayer's test: A small amount of extract was gently combined with 1-2 ml of Mayer's reagent, poured down the side of the test tube. The resulting mixture was examined for the occurrence of a creamy white-yellow precipitate.

Dragendorff's test: A small quantity of the extract was mixed with 1-2 ml of Dragendorff's reagent. The resulting mixture was then examined for the existence of a reddish-brown precipitate.

Hager's test: A small volume of the extract was combined with 1-2 ml of Hager's reagent. The resulting mixture was observed for the presence of a creamy white precipitate.

Wagner's test: A small amount of the extract was gently mixed with 1-2 drops of Wagner's reagent, ensuring it was added along the sides of the test tube. The resulting mixture was observed for the formation of a brown reddish precipitate.

5.4.3 Detection of flavonoids

Alkaline test: To 1 ml of extract, a few drops of sodium hydroxide and dilute hydrochloric acid were introduced. The resulting mixture was examined for the presence of a yellow-colored precipitate.

Ferric chloride test: To 1 ml of extract, a few drops of a 10% ferric chloride solution were added. The resulting mixture was observed for the formation of a green-colored precipitate.

5.4.4. Detection of phenolic compounds

Lead acetate test: To a small quantity of extract, a few drops of freshly prepared lead acetate solution were added. The resulting mixture was observed for the presence of a white precipitate.
Bromine water test: A drop of extract was mixed with a small amount of bromine water. The resulting mixture was observed for any changes in colour.

5.4.5. Detection of reducing sugar

Benedict's test: 0.5 ml of extract was combined with a few ml of Benedict's reagent, and the mixture was heated for 2 minutes. The resulting reaction mixture was examined for the presence of a green-colored precipitate.
Fehling's test: 1 ml of extract was mixed with Fehling's solution A and B, and the mixture was heated on a water bath. The resulting reaction mixture was examined for the presence of a red precipitate.

5.4.6. Detection of tannins

Gelatin test: The extract was solubilized in 5 ml of sulfuric acid, along with 10% gelatin solution and 10% NaCl. The resulting reaction mixture was examined for the formation of a white precipitate.
Bromine water test: 0.5 ml of extract was mixed with 10 ml of bromine water. The resulting reaction mixture was observed for decolouration.

5.4.7. Detection of saponins

Foam test: 1 ml of extract was combined with 4 ml of distilled water. The resulting mixture was vigorously shaken, leading to the formation of foam. The foam should persist for a duration of 10 minutes [14].

5.5. Experimental Animals

Wistar albino rats weighing between 180 to 200 grams were obtained from animal house at HKES's Matoshree Taradevi Rampure Institute of Pharmaceutical Sciences, located on Sedam Road, Gulbarga, Karnataka. These rats were housed in polypropylene cages under controlled conditions with an ambient temperature of 25±2 °C and relative humidity of 55–65%. They were kept under a 12 hour light/dark cycle and provided with free access to food and water. The animal study was conducted following the approved protocol (HKE'S/MTRIPS/IAEC/125/2021-22) in accordance

with the guidelines of the Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

5.6. Anti-diabetic activity:

5.6.1. Antidiabetic activity of test extract against Streptozotocin (STZ) induced diabetic rats

Induction of diabetes:

The rats were subjected to an overnight fasting period, after which they received a single dose of freshly prepared streptozotocin (STZ), (55 mg/kg BW, i.p). Following the STZ administration, the rats were allowed to access food and water after 30 minutes. To prevent hypoglycemic shock, the rats were orally administered a 5% glucose solution after 6 hours. Four days after the STZ administration, the fasting blood glucose levels of the rats were measured and those with the blood glucose levels more than 200 gm/dl were selected for further studies. The rats were categorized into following groups:

Group I (normal control) : Distilled water (10 ml/kg, p.o)
Group II (disease control): STZ (55 mg/kg, i.p)
Group III (Standard): Glibenclamide (5 mg/kg, p.o) + STZ (55 mg/kg, i.p)
Group IV and V (test): *Ficus arnottiana* (200 mg/kg, p.o) + STZ (55 mg/kg, i.p) and *Ficus arnottiana* (400 mg/kg, p.o) + STZ (55 mg/kg, p.o), respectively.
Afterwards, the blood glucose level was measured at 30, 60, 90, 120, and 180 minutes using glucometer [15].

5.6.2. Estimation of rat C-Peptide by using Radioimmunoassay (RIA)

The experimental setup included total radioactive tubes, nonspecific binding (NSB) tube, Rat C-Peptide (RCP) standard (25-1600 pM), quality controls, and unknown samples. The buffer used consisted of phosphate/saline buffer (pH 7.4) containing 0.05 M EDTA, 1000 KIU/ml Trasylol, and 1% BSA. The assay was conducted in following manner: 100 µl of each concentration of RCP (0, 25, 50, 100, 200, 400, 800, and 1600 pM) were pipetted into borosilicate glass tubes. Quality control and unknown samples were taken in same concentration. The 300 µL of buffer was added to the NSB tubes and remaining assay tubes , except for total radioactive tubes. Appropriately diluted (35-45% B/B0 binding) RCP anti-serum was added to all tubes except the NSB tubes. The tubes were then covered and incubated at 40 °C for 18-24 hours followed by addition of RCP tracer (15000 cpm/100µL). The tubes were again incubated for 24 hours at 40°C. A second antibody precipitation was done during a 2 hour incubation to separate radiolabeled RCP bound to the antibody from free ligand. 1 mL of 0.05 M phosphate buffer (without BSA) was added to all tubes, except for the total radioactive tubes. The tubes were shaken vigorously and then centrifuged (3000 g) for 20 min at 40 °C. The supernatant was decanted, and the pellets were counted using an automated gamma counter (Ape Automatic Gamma Counter, Micromedic Systems, Inc., Costa Mesa, CA). Calculation for each sample were performed by the automated gamma counter using a data reduction system with log/logit transformation [14].

5.6.3. Estimation of serum insulin level using ELISA

The assay was based on Sandwich-ELISA principle. The micro-ELISA plate was pre-coated with an antibody specific to Rat serum. Standard and sample with their specific antibodies were filled in the micro-ELISA plate. Subsequently, rat serum specific biotinylated detection antibody and Avidin-horseradish peroxidase (HRP) conjugate were added sequentially to each well and incubated. The mixture was washed to remove any unbound component, and a substrate solution was added to each well. The well containing rat serum, biotinylated detection antibody, and Avidin-HRP conjugate displayed a blue colour. The enzyme-substrate reaction was halted by adding a stop solution, resulting in appearance of yellow color. The optical density (OD) was then measured spectrophotometrically at a wavelength of 450 nm to determine the results [16].

5.6.4. Histopathology of pancreas

The tissue sample were prepared into 5µm thick paraffin slices, fixed with 20% paraformaldehyde buffered with 10% neutral buffered saline. The tissue sample was then stained with haematoxylin and eosin (H & E) for observation under a light microscope [17].

5.6.5. In-vitro antidiabetic assay

Estimation of alpha amylase Inhibition

A solution was prepared containing porcine pancreatic α-amylase at a concentration of 1 U/mL in phosphate buffer (pH 6.8). Test extracts were also prepared at concentration of 20, 40, and 80 mg/ml. To initiate the experiment, 250 µL of the α-amylase sample solution was mixed with 100 µL of the test extract and acarbose. This reaction mixture was then incubated at 37°C for 20 minutes. Following the incubation period, 250 µL of a solution containing 0.5% starch was added to the mixture and was further incubated at 37°C for additional 20 minutes. To halt the chemical reaction, 1 mL of dinitrosalicyclic acid was added to the reaction tubes, which were then placed in a boiling water bath for 5 minutes and cooled down. Afterwards, the absorbance of the samples were taken at 540 nm. The absorbance of the control α-amylase, with a concentration of 1 U/mL and without any inhibitor, represented 100% enzyme activity. Acarbose, known as an α-amylase inhibitor, was used for comparison. The percentage inhibition of α-amylase was calculated as [18]:

$$\%\ inhibition\ of\ \alpha - amylase = \frac{(Absorbance\ of\ the\ control - absorbance\ of\ the\ test\ sample)}{Absorbance\ of\ the\ control} \times 100$$

Estimation of Alpha-Glucosidase Inhibition

0.5 U/mL of α-glucosidase was dissolved in a phosphate buffer solution (pH 6.8). Test extracts were also prepared at concentration of 20, 40, and 80 mg/mL. For the experiment, a mixture was made consisting of 125 µL of α-glucosidase solution, 50 µL of the test sample with varying concentration, and 700 µL of phosphate buffer, followed by incubated at 37 °C for 15 minutes. Subsequently, 125 µL of a 5mM solution of p-nitrophenyl glucopyranoside was added to the reaction mixture and the mixture was again incubated at 37°C for 15 minutes. To stop the reaction, 1000 µL of Na_2CO_3 was added, and the absorbance was read at 405 nm. Acarbose was used for comparative purposes. The percentage inhibition of α-glucosidase was calculated as [18]:

$$\%\ inhibition\ of\ \alpha - glucosidase = \frac{(Absorbance\ of\ the\ control - absorbance\ of\ the\ test\ sample)}{Absorbance\ of\ the\ control} \times 100$$

5.7. Statistical analysis

Data was analysed using graph pad prism version 9 software. All the values were expressed as mean ± SEM, n=6, and analysed using one-way Analysis of Variance (ANOVA) followed by multiple comparison Tukey's test. Criteria of statistical significance was $p<0.05$.

Acknowledgement: The authors are thankful to Department of Pharmacy, RGUHS University for their continuous support. We are thankful to Dr Shivakumar Inamdar sir for carrying out anti- diabetic studies.

Author's contribution: Supervision – S.K.I.; Concept – S.K.I., M.T., ; Experimentation: P.S.A.., T.B., P.Y., M.S., A.K.; Analysis and interpretation – K.F., M.A., M.S.; Writing – A.K., M.S.; Proof reading – M.T.

Conflict of Interest: The authors declare that they have no conflict of interest.

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