ORIGINAL ARTICLE

Inhibitory activity of leaf extract of *Tinospora cordifolia* and magnoflorine on aldose reductase for control of diabetes

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Abstract

Introduction: Diabetes mellitus is a metabolic disorder that affects a large population around the world, as declared by the World Health Organization. There is a need for discovery of natural, non-toxic agents with minimum side effects such as medicinal plants. *Tinospora cordifolia* (Guduchi) has been used as an antidiabetic medicine in Ayurveda and traditional folk medicine. The present study is an integrative approach of plant sciences, medicinal chemistry, and screening assays for the development of treatment for diabetes. It investigates the analysis and antidiabetic efficiency of the leaf extract of *T. cordifolia* and its alkaloidal component magnoflorine. **Materials and Methods:** Initially, the active fraction of *T. cordifolia* (AFTC) of methanolic leaf extract was analyzed through fractionation to isolate the active molecules through the column and thin-layer chromatography. The primary compounds were characterized through high-performance liquid chromatography and their chromatographic profiles established using appropriate standards. Subsequently, the plant extract of *T. cordifolia* and one of its alkaloidal components, magnoflorine was tested on streptozotocin (STZ)-induced diabetic rats for their antidiabetic property by assessing the inhibition of aldose reductase. **Results:** Four active molecules (alkaloids) were isolated from the plant extract of *T. cordifolia* through Column chromatography and thin-layer chromatography (TLC) and high-performance liquid chromatography studies confirmed their identity as magnoflorine, jatrorrhizine, palmatine, and berberine. Antidiabetic potential of plant extract and its component alkaloid, magnoflorine was tested on STZ-induced diabetic rats. Treatment with plant extract or magnoflorine decreased the serum glucose to normal level similar to that of the standard drug metformin and also remarkably prevented their weight loss to almost the same extent as those treated with metformin. Significant inhibition of aldose reductase activity was also observed by the plant extract or magnoflorine. **Discussion:** The study indicated that the extract of *T. cordifolia* and magnoflorine help in the maintenance of body weights and blood glucose levels of STZ-induced diabetic rats and also demonstrated a significant aldose reductase inhibition activity similar to that of Metformin (the standard antidiabetic drug). Hence, the plant extract and magnoflorine have immense antidiabetic potential. **Conclusion:** The plant extract and magnoflorine have an immense antidiabetic property, and magnoflorine can be developed into a potent antidiabetic drug after further trials. The pure form of magnoflorine is scarcely available, and the development of a purification process may be beneficial.

Key words: Aldose reductase, diabetes, leaf extract, magnoflorine, *Menispermaceae*, *Tinospora cordifolia*

INTRODUCTION

Diabetes mellitus is a metabolic disorder that affects huge chunks of population around the world, as declared by the World Health Organization.[1] It is characterized by elevation of both fasting and post-prandial blood sugar levels (hyperglycemia), and chronic hyperglycemia causes glycation of body proteins that, in turn, leads to secondary complications affecting eyes, kidneys, nerves, and arteries.[2] These complications may be prevented by maintaining normal blood glucose values. Insulin therapy is majorly used for the management of diabetes mellitus. Recent antidiabetic research has suggested

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that aldose reductase inhibitors (ARI) address complications caused by hyperglycemia. Drugs like metformin are used for control of hyperglycemia and limit intestinal glucose absorption but may cause hypoglycemia, liver problems, lactic acidosis, and diarrhea at higher doses. Hence, there is a need for discovery of natural, non-toxic agents with minimum side effects. Use of medicinal plants for prevention or cure of diseases has been increasing, but scientific evidence in terms of molecules/compounds is lacking.

Tinospora cordifolia (Willd.) Miers, which is known by the common names guduchi and giloy, is a herbaceous vine of the family *Menispermaceae* indigenous to the tropical areas of India, Myanmar and Sri Lanka and has a long history of use in Ayurvedic medicine (the traditional medicine of India) as an antidiabetic, anticancer, immune stimulating, cholesterol-lowering, and liver protectant.[3-5] *T. cordifolia* is sold by herbalists in open market for this purpose. The stem extract of *T. cordifolia* has an antidiabetic effect.[6] *T. cordifolia* contains various commercially important chemical compounds including alkaloids.[8,9] However, these have not yet been incorporated into pharmaceutical dosage forms. Identity of bioactive compounds and study of their mechanisms of action are very recent.[5,7-11] Therefore, the present study was carried out to identify primary compounds present in the leaves of *T. cordifolia* and to provide proof for the antidiabetic nature of plant extract and its component magnoflorine through investigation of their ARI activity in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Primary compounds present in the active fraction of *T. cordifolia* Alkaloidal fraction of *Tinospora cordifolia* leaves were isolated by fractionation through column and TLC and characterized through high-performance liquid chromatography (HPLC) using appropriate standards. The aqueous plant extract and its component magnoflorine were then screened for antidiabetic hypoglycemic effect and inhibition of the aldose reductase in STZ-induced diabetic rats.

Characterization of Alkaloids in Plant Extract by Column Chromatography, TLC, and HPLC

Preparation of plant extract for phytochemical analysis

The plant identified by the Head, Department of Botany, Osmania University, Hyderabad, India, as *T. cordifolia* (Willd.) Miers, belonging to family *Menispermaceae*, located in the Botanical garden of Osmania University, Hyderabad, was used for the experiments. Geolocation information for Hyderabad in terms of latitude and longitude is projected as 17°, 22' 31" N and 78° 28' 27" E. The voucher specimen with number 192 dated January 16, 2014, was deposited at the Osmania University herbarium. For the preparation of plant extract, healthy leaves were collected, dried in shade and ground to a powder. 5 g of leaf powder was extracted with methanol in Erlenmeyer flasks kept on an orbital shaker for 24 h. The extract was filtered through Whatman No. 1 filter paper and used for phytochemical analysis.

Analysis of plant extract and characterization of alkaloids (by column chromatography, TLC, and HPLC)

The methanolic plant extract was extracted with Mayer's reagent to isolate the alkaloidal fraction of *T. cordifolia* (AFTC and individual alkaloids were separately collected and identified by column chromatography, TLC, and later by HPLC.

Column chromatography

The chromatographic column was initially filled with lower polarity mobile phase (chloroform), and the polarity was increased (by adding methanol in ratios of 9:1, 8:2, and 7:3 chloroform: methanol) to detect the individual compounds and elute them.

TLC

The alkaloids were detected through TLC under ultraviolet (UV) light with different ratios of chloroform and methanol as mobile phase, and retardation factors (R_f values) recorded. The compounds were collected from the TLC plate and dissolved in solvent (methanol).

HPLC

HPLC was performed using the standards of magnoflorine and jatrorrhizine (NCBI PubChem CID 73337 and 72323, respectively) (Wuhan Chem Faces Biochemical Co., Ltd., China) and palmatine and berberine (NCBI PubChem CID 19009 and 2353, respectively) (Sigma-Aldrich, India). Acetonitrile, methanol (HPLC grade), and phosphoric acid (analytical-reagent grade) (HiMedia, India) were used. The compounds in the sample's chromatogram were identified by comparing the peaks with those of the standards. Quantity of each compound was determined from retention time and peak area of the chromatogram using the calibration curve plot of concentration versus the peak area of the standard with the following formula and expressed as a percentage.

$$\frac{\text{Peak area of sample}}{\text{Peak area of standard}} \times \frac{\text{concentration of standard}}{\text{concentration of sample}} \times \text{Purity of standard}$$

Study of Antidiabetic Effect of the Plant Extract and Magnoflorine

The antidiabetic effect of the plant extract of *T. cordifolia* and its component alkaloid was studied on STZ-induced diabetic rats.

Preparatzion of plant extract

Fresh leaves of *T. cordifolia* were collected and dried in shade. They were then ground to a fine powder and stored at 4°C in airtight containers until used for oral administration.

Experimental design

The experiments were performed on healthy, developing Wistar rats procured from Jeeva Life Sciences, Hyderabad. The animals received humane care in compliance with the guidelines of CPCSEA. The protocols were approved by the Institutional Animal Ethical Committee and conducted according to the CPCSEA guidelines on the use and care of experimental animals. The rats were acclimatized for 1 week to the laboratory conditions before starting the experiments. Their blood glucose levels were estimated before the start of the experiment to confirm their normalcy. Age of the rats was 2 months and weight ranged from 80 to 160 g. Rats were randomly divided into five groups; with 20 rats in each group (both male and female were grouped together). They were housed in polypropylene cages (5 rats/cage) under an ambient temperature of 25 ± 5°C at 60–65% relative humidity, with 12:12 h light and dark cycle. They were kept on standard pellet diet (chow) and water *ad libitum*, before the dietary manipulation. Blood glucose levels were estimated before the start of the experiment to establish the non-diabetic nature of all the rats. To induce diabetes, STZ was injected to the rats of all groups (except control group) by a single intraperitoneal injection at 60 mg/kg body weight (b.w.) (dissolved in 0.1 M sodium citrate buffer of pH 4.5) in overnight fasting animals.[12] STZ is a broad-spectrum antibiotic extracted from *Streptomyces acromogenes*. The STZ-induced diabetes causes destruction of β cells of the islets in the pancreas, which leads to a reduction in the release of insulin. An insufficient release of insulin causes hyperglycemia (high blood glucose). Diabetes was confirmed 48 h after injection of STZ by checking the blood of fasting animals with an Accu-Check glucometer.[13] Rats with blood glucose levels of more than 200 mg were considered diabetic and included in the study. Control animals were injected intraperitoneally with 0.1 M physiological saline. Except for the STZ group, other diabetic groups were then treated with the respective test compound: Metformin (an antidiabetic drug) was used as positive control and administered orally by mixing in physiological saline. Preliminary testing of the hypoglycemic activities of plant extract and magnoflorine in normal healthy rats helped plan their dosage. The plant extract (powder form) or magnoflorine was administered orally by mixing in 1–2 ml physiological saline. Metformin/plant and extract/magnoflorine were administered to fasted rats for 4 weeks at a dose of 100/200/100 mg/kg b.w., respectively.[14] The details of the groups are given below.

- Group 1: Control group (non-diabetic normal rats).
- Group 2: STZ group (STZ-induced diabetic rats by single STZ dose of 60 mg/kg b.w.).
- Group 3: Metformin group (diabetic rats treated with metformin at 100 mg/kg b.w.).
- Group 4: Plant extract group (diabetic rats treated with plant extract 200 mg/kg b.w.).
- Group 5: Magnoflorine group (diabetic rats treated with magnoflorine 100 mg/kg b.w.).

The experimental data pertaining to body weights, blood glucose levels, and NADP+(for aldose reductase inhibition) were recorded as explained below.

Body weights and blood glucose levels

The weights were recorded in grams initially before starting the first dose and also on the day of their last dose (28th day). Blood samples were drawn from the tail vein and plasma glucose estimation was done by the glucose-oxidase method. Pre- and post-prandial blood glucose levels were estimated after the 28th day of the experiment with Accu-Check glucometer.

Aldose reductase inhibition assay

Presently, ARI activity of test compounds was studied on all groups using *in vitro* method in which, the enzyme was prepared from the brain homogenates. Aldose reductase oxidizes NADPH and based on the concentration of NADPH oxidized, the ARI activity of test compounds was indirectly assessed.

Procedure

Hayman and Kinoshita protocol[15] was followed with slight modifications. The rat brain homogenate (10%) was taken in 50 mM potassium phosphate buffer (pH 6.2) and centrifuged at 25 kg for 30 min at 4°C. Potassium phosphate buffer (500 µl), lithium sulfate (10 µl), β-mercaptoethanol (50 µl), glyceraldehyde (100 µl), and water were added to the supernatant to make up to 1 ml. After incubating at 37°C for 5 min, NADPH (50 µl) was added and readings recorded at 340 nm. The activity of the enzyme was determined by the amount of NADP converted from NADPH per unit times at 37°C and 7.0 pH. The µM of NADPH oxidized/min/ml can be calculated as OD*dilution factor/sample taken as *6.22.

Statistical Analysis

Statistics analysis was carried out with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Results from three independent experiments were expressed as mean ± standard deviation. Comparisons between different groups were performed using Student's *t*-test. $P < 0.05$ was considered statistically significant. Results of test Groups-3, 4, and 5 were compared with that of diabetic control (STZ Group-2).

RESULTS AND DISCUSSION

The present study is an integrative approach of medicinal chemistry and *in vitro* screening assays for the development

of treatment for diabetes. Four alkaloids were isolated from the alkaloid fraction of leaves of *T. cordifolia* (AFTC) and magnoflorine (whose content was the highest) was chosen for testing its antidiabetic activity.

Characterization of Alkaloids in Plant Extract by Column Chromatography, TLC, and HPLC

Fractionation of methanolic AFTC was carried out by column chromatography, compounds isolated through TLC and characterized by HPLC using appropriate standards. Palmatine and berberine were isolated using a column with a mobile phase of chloroform and methanol in 9:1 ratio. Jatrorrhizine was isolated with a ratio of 8:2 of chloroform and methanol. Magnoflorine was isolated with a ratio of 7:3 chloroform and methanol. The purity of isolated components was checked by TLC on aluminum and preparative TLC plates [Figure 1]. Eight spots were observed on TLC plates under UV light. Two of these were fluorescent yellow, identified as palmatine (R_f value: 0.7) and berberine (R_f value: 0.62). Jatrorrhizine (R_f value: 0.5) was orange in color and magnoflorine (R_f value: 0.42) was pale and almost colorless. The results support previous reports.[16,17]

In the HPLC analysis, the retention time of four observed peaks of AFTC was recorded at 265 nm with several runs, compared with standards and identified as magnoflorine, jatrorrhizine, palmatine, and berberine [Figures 2 and 3] with the solvent system of chloroform and methanol (in 9:1 and 8:2 v/v).

However, good resolution was reported[18] on hexane and ethyl acetate (in 40:10 v/v) and also with n-Butanol, acetic acid, and water (50:10:40).[19] Presently, the compounds were identified at 265 nm and differed from the report[20] where, magnoflorine was identified at 267 nm. Very high retention time for magnoflorine (35 min.) was reported[21] and one of the reasons could be the different column used. The quantity of the four alkaloids, magnoflorine, jatrorrhizine, palmatine, and berberine (0.18, 0.01, 0.03, and 0.05 mg/ml [w/w], respectively) shows the remarkably high content of magnoflorine, which was, therefore, chosen as a test compound along with plant extract to test for antidiabetic efficacy. In contrast, others[4,22,23] reported a very low quantity of berberine in *T. cordifolia*.

Study of Antidiabetic Effect of the Plant Extract and Magnoflorine

The study of the antidiabetic effect of plant extract of *T. cordifolia* and magnoflorine was undertaken in STZ-induced diabetic Wistar rats through parameters such as weights of rats, blood glucose levels, and the extent of inhibition of aldose reductase. Weights of the rats in all the groups recorded before starting the experiment and again on the 28th day (the day of their last dose), show a visible increase

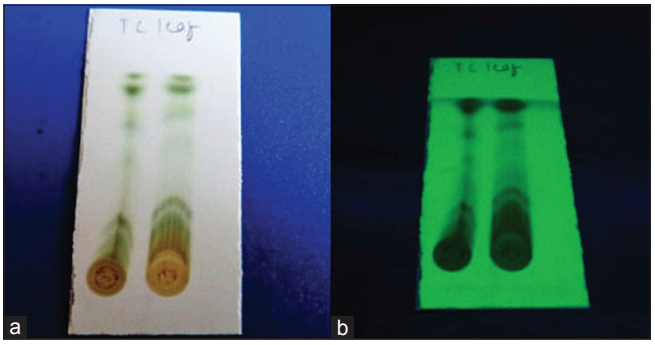

Figure 1: Thin-layer chromatography of leaf extract of *Tinospora cordifolia* (a=under visual light and b=under ultraviolet light)

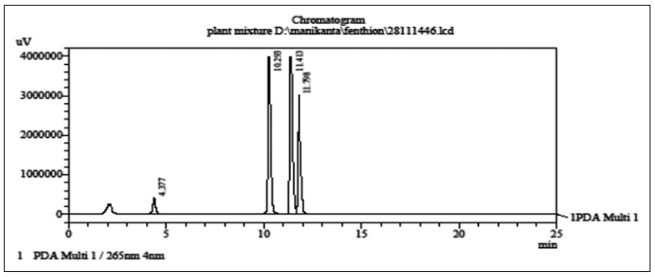

Figure 2: High-performance liquid chromatography of the standards of magnoflorine, jatrorrhizine, palmatine, and berberine

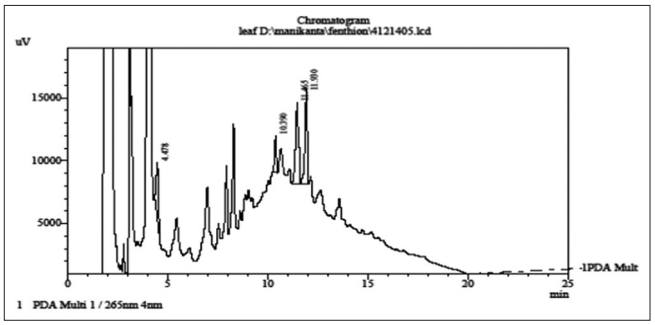

Figure 3: High-performance liquid chromatography analysis of AFTC (of leaf extract of *T. cordifolia*)

in body weights of the control group and a significant weight loss in the STZ group [Table 1]. Weights of the metformin group increased satisfactorily and the plant extract and magnoflorine groups also recorded remarkable weight gain indicating that the weight loss could be controlled by treatment with plant extract or magnoflorine.

Blood glucose levels (pre- and post-prandial) of all the rats in all groups were estimated after the 28th day of the experiment [Table 1]. Optimum levels of blood sugar were observed in the control group at both pre- and post-prandial levels in contrast to the significantly high values in diabetic STZ rats. Blood glucose levels of metformin group were maintained on par with the control. When compared with STZ group, the plant extract and magnoflorine groups recorded significantly lower blood glucose levels that were on par with those of the metformin group [Table 1].

Table 1: Data of body weights, hypoglycemic effect (on the 28th day), and aldose reductase inhibition of plant extract of *Tinospora cordifolia* and magnoflorine on STZ-induced diabetic rats

Treatment/dose (mg/kgb.w.)	Body weights		Hypoglycemic effect (blood glucose levels)		Aldose reductase inhibition (µmoles of NADPH oxidized/min/ml) mean±SD
	Initial weight (g) on day-1 mean±SD	Final weight (g) on day-28 mean±SD	Pre-prandial blood glucose levels (mg/dl) mean±SD	Post-prandial blood glucose levels (mg/dl) mean±SD	
Control	*96.25±12.50	*166.25±10.30	*133.50±4.65	*161.50±9.29	*0.015±0.001
STZ (diabetes induced by a single dose of 60 mg/kg b.w.)	*150.00±8.16	*122.50±9.57	*243.25±29.37	*341.25±32.75	*0.03±0.0006
Metformin (100 mg/kg b.w. per day)	**130.00±9.12	**166.25±8.53	**131.25±13.14	**169.75±11.15	**0.015±0.001
Plant extract (200 mg/kg b.w. per day)	**131.25±8.53	**172.50±15.54	**130.25±6.39	**168.00±5.72	**0.016±0.001
Magnoflorine (100 mg/kg b.w. per day)	**135.00±9.12	**175.00±16.83	**133.25±6.29	**169.25±5.62	**0.016±0.002

*$P<0.05$ when the STZ-induced diabetic group is compared to the control (untreated) group. **$P<0.05$ when the STZ group (as the negative control) is compared to metformin (the positive control), plant extract, or magnoflorine group. STZ: Streptozotocin

Although weight loss could be controlled by plant extract, magnoflorine treatment obviously contributed to weight gain since it reduced the amount of absorbable carbohydrates leading to decreased post-prandial glucose and demand for insulin.[9] A reduction in blood glucose level was reported[8,11] after treating Wistar rats with dried plant extract of *T. cordifolia*, which supports the present results demonstrating the antihyperglycemic activity. Whereas most of the representative isoquinoline alkaloids exhibited potent antidiabetic, anti-inflammatory, and antioxidant activities, magnoflorine could presently exert antioxidant and antiglycemic effects *in vivo*.[9] The significant reduction of blood glucose may either be due to insulin-like behavior of plant extract or magnoflorine or due to stimulatory effect to increase the secretion of insulin.[24]

Aldose reductase catalyzes the reduction of glucose to sorbitol in the presence of cofactor NADPH, which results in oxidation of NADPH to $NADP^+$ in the polyol pathway.[25,26] This pathway is an alternative route for the metabolism of glucose when glycolytic cycle becomes saturated. The high concentration of glucose in tissues is metabolized into sorbitol in the absence of insulin. The sorbitol produced is converted to fructose by sorbitol dehydrogenase. Since aldose reductase oxidizes NADPH, estimation of oxidized NADPH indirectly measures ARI activity of the compounds *in vitro*. Presently, the plant extract and magnoflorine were assessed and found to possess efficient and significant ARI activity [Table 1]. The STZ group recorded a low ARI activity compared to other treated groups in terms of oxidized NADPH levels. ARI by plant extract and magnoflorine groups was almost equal to the metformin group. Several reports suggest that accumulation of sorbitol can lead to diabetic complications and the ARI activity of some natural products reduces the accumulation in tissues, thus preventing osmotic swelling, alteration in membrane permeability and oxidative stress-mediated tissue injury in diabetes, which leads to cataract, retinopathy, neuropathy, and nephropathy.[6,27-29] Presently, the extract of *T. cordifolia* and its component alkaloid, magnoflorine significantly maintained body weights, decreased fasting serum glucose, suppressed the increase of blood glucose levels, and recorded high ARI similar to metformin suggesting that they could be developed into an efficient antidiabetic drug(s) after further trials. Pure form of magnoflorine is scarcely available and is also expensive; therefore, the development of the purification process may be beneficial.

CONCLUSION

The present study is an integrative approach of medicinal chemistry and *in vitro* screening assays for the development of treatment for diabetes. Four alkaloids, magnoflorine, jatrorrhizine, palmatine, and berberine were isolated from the leaf extract of *T. cordifolia*. Results of antidiabetic activity indicate that and its component alkaloid, magnoflorine

help maintain body weights and blood glucose levels of STZ-induced diabetic rats and also demonstrate a significant inhibition of aldose reductase similar to metformin suggesting that they have immense antidiabetic properties and can be developed into potent antidiabetic drugs after further trials. The pure form of magnoflorine is scarcely available, and the development of a purification process may be beneficial.

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