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RESEARCH ARTICLE

Evaluation of *in vitro* antioxidant and antidiabetic activities from *Amomum nilgiricum* leaf extract

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ABSTRACT
In the present study, hexane, ethyl acetate and methanol fraction of *Amomum nilgiricum* leaf was evaluated for antidiabetic efficacy, scavenging activities, followed by estimation of total phenol, total flavonoids. In the present study, a significant amounts of total phenolics (79.92±1.58 mg/g) and flavonoids (21.74± 0.89 mg/g) were showed from Ethyl acetae fraction. H_2O_2 scavenging activity (97.62±2.89%) with IC_{50} value of 78.57 µg/ml concentrations. Ethyl acetate fraction exhibited maximum inhibitory activity of glucose movement into outer solution across dialysis membrane at 250 µg/ml as compared to the control. The ethyl acetate fraction revealed maximum insulin secretory activity (130.5±3.66%) in RIN-m5F cells. Methanol fraction recorded maximum glucose uptake percent in yeast cells (67.08±1.68%) when compared to standard metronidazole (68.06±0.73%). This study scientifically validates the antidiabetic activity of *A. nilgiricum*. Hence, in view of its comparative hypoglycemic strength, it can work as a valuable healing agent in treating diabetes.

Introduction

Diabetes is an utmost widespread and prolonged endocrine disease disturbing the metabolism of carbohydrate, lipid and protein (1). The abnormal metabolic disarray characterized by hyperglycemia occurs while either the pancreatic cells do not secrete adequate insulin, or while body cells are unable to successfully use the insulin formed (2). The long-term problems of diabetes contain heart disease, stroke, atherosclerosis leading to dysfunction and organs failure. Based on 2015 World Health Organization (WHO) reports, globally, about 422 million people (aged 20–79) were living with diabetes, and by 2040 it may rise to 650 million (3, 4). In India, diabetes population is estimated to be 61.3 million, and it may increase up to 101.2 million until 2030 (5). This large number which accounts for nearly 20% of the global patients, has deemed India as the world's capital of diabetes (6, 7). During the hyperglycemic condition, there is a constant formation of reactive oxygen species (ROS). It is evident that diabetes influences modifications in the actions of the antioxidant enzymes in numerous tissues. This oxidative stress further involved in the improvement and advancement of diabetes related problems. Antioxidants show significant functions in free radicals scavenge with defending cells by

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oxidative pressure (8–10). The treatment of diabetes remains a global health challenge. Early-stage diabetes treatment includes the postprandial reduction hyperglycemia by delaying the breakdown of hydrocarbons into absorbable monosaccharides over the prevention of hydrocarbons degrading enzymes *viz*., α-amylase and α-glucosidase present in the gastrointestinal strip. Consequently, declining the pace of glucose absorption also, in turn, dampening the postprandial glucose increase (11).

At present, there are a variety of therapeutic approaches comprising hypoglycemic agents *viz*., α-glucosidase inhibitors (miglitol, voglibose and acarbose), insulin secretagogues (sulfonylureas and glinides), insulin sensitizers (thiazolidinediones – metformin and pioglitazone). These drugs have limitation in their usage such as generating hypoglycemia at more doses, liver cell injury, lactic acidosis, headache and diarrhoea (12–16). Control of diabetes mellitus (DM) without any side effects is still a challenge to the remedial system. Medicinal plant/plant extracts are effectively used since ancient time for management of diabetes as they are reported to be more effective, less side effects and relatively low cost. According to WHO (2002), medicinal plant/plant extracts are being used to treat diabetes in about 90% of the population in developing countries. Therefore, identifying and evaluating such plants have become more important (17–20). The WHO has registered 21,000 medicinal plants used around the world and 150 plant species used commercially. Among these 2500 species are reported from the Indian subcontinent. There are about 800 species reported to have antidiabetic activity (21). Most of the plants are known to possess antioxidant with antidiabetic activities. It has been observed that the reduction of lipid peroxidation plus improved antioxidant could decrease diabetes complaints (22).

Amomum nilgiricum V. P. Thomas & M. Sabu is a recently described species of the family *Zingiberaceae* (23), from the Western Ghats, Kerala, India. The family comprises with 53 genera with more than 1200 species, numerous of them are generally known for therapeutic uses (24). *A. nilgiricum* is robust, clump forming habit, stout and non-stoloniferous rhizomes, lanceolate to elliptic-lanceolate, 32–41 × 6.5–8 cm. Lamina, 2–8 mm long, petiole, tomentose beneath leaves, 4.5–9 cm long and persistent ligule, corolla tube is longer than labellum, labellum is not trilobed, uniformly yellow and emarginate anther crest and reduced staminodes (23).

During the current study, the antidiabetic and antioxidant activity of different extracts from *A. nilgiricum* leaves was performed. To assess the efficacy of *A. nilgiricum* extract as antidiabetic agents, we examined the influence on the ability of glucose transport across yeast cell membrane, glucose diffusion and insulin secretion.

Materials and Methods

Reagents

Folin Ciocalteu reagent, $NaNO_2$, hydrogen peroxide, gallic acid, NaCl, Na_2CO3, glucose, KCl, NaCl, $MgCl_2$, NaH_2PO_4, $CaCl_2$.

Plant material collection and extraction

Amomum nilgiricum was obtained from the region of Western Ghats, Kerala, India at an altitude of 1150 m mean sea level (MSL). The leaves of the plants were rinsed in water and washed in sterilized distilled water and shade dried. The dehydrated leaf samples were powdered and used for the extraction process. Powdered leaves (100 g) were extracted using a water-ethanol mixture (ratio of 80:10) on a magnetic stirrer for 24 hr. The leaf extract was filtered and dried at 40 ºC using vacuum evaporator. The dried crude leaf extract was dissolved in 250 ml of sterilized distilled water for further fractionation. The extracts were separated with methanol, hexane and ethyl acetate and these fractions were dried using vacuum evaporator and used for further studies.

Estimation of total phenols

The whole content of phenol was estimated by Folin Ciocalteu (FC) as per the standard method (25). In brief, 100 µl of fractions was made up to 2 ml with sterile distilled water and 500 µl of FC reagent was added and incubated for 3 min. After incubation, 2 ml of Na_2CO_3 was added and the sample was incubated in warm water for 1 min. The content was allowed to cool and the absorbance was recorded at 765 nm using a spectrophotometer (Elico-Japan). The total phenol content was stated as mg gallic acid equivalents (GAE) $g^{-1.}$

Determination of total flavonoids

The total flavonoids content was assessed as per the standard procedure (26). The reaction mixture comprises 100 µl of different fractions and the solution was made up to 2 ml with the distilled water and incubated at room temperature for 3 min. Further, 300 µl of $AlCl_3$ and 300 µl of $NaNO_2$ was added to the above mixture and the mixture was allowed to stand for 6 min. Then, 1 M of NaOH solution (2 ml) was added and the final final volume of the reaction mixture was prepared to 10 ml with sterile water. The mixture was allowed to stand for 15 min and the absorbance was measured at 510 nm. and flavonoid concentration expressed as mg quercetin equivalent g^{-1} extract. The total flavonoid content was calculated from a calibration curve and the result was expressed as mg rutin equivalent per g dry weight.

Estimation of antioxidant activity by in vitro methods

Hydrogen peroxide scavenging capacity

The hydrogen peroxide (H_2O_2) scavenging activity of the fractions were performed as per the standard procedure (27). Hydrogen peroxide (40 mM) in phosphate buffer was prepared and

fractions at different concentrations (0.78–100 µg/ml) were added with 0.6 ml of distilled water prepared to 3 ml (pH 7.4). After 30 min, absorbance was recorded at 230 nm against a blank solution. Gallic acid was used as as a positive control. The % H_2O_2 scavenging activity was evaluated by the following formula:

$$\% \text{ Radical scavenging activity} = \frac{\text{Absorbance of the control - absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

Estimation of antidiabetic activity

Glucose diffusion measurement:

The Glucose diffusion method was performed according to the standard method (28). The experiment was performed in a dialysis bag (7.6 cm x 15 mm, dialysis tubing cellulose membrane, Sigma Aldrich), 6 ml of fractions was added into dialysis bag, along with 2 ml 0.15 M Sodium chloride containing 0.22 mM glucose. The closed dialysis tubing positioned in a centrifuge tube comprising 100 ml of 0.15 M NaCl in an orbital shaker and incubated for 3 hr at 37 °C. Amount of glucose inside the dialysis tube was recorded and control contains 2 ml of 0.15 M NaCl having 0.22 mM glucose with 1 ml distilled water. The glucose movement into the outer solution was examined each half an hour using glucose oxidase peroxidase diagnostic kit (Sigma-Aldrich). The glucose concentration was verified every 30 min for 3 hr. The glucose diffusion retardation index (GDRI) was calculated using the following formula: GDRI = (100 − glucose content (µg/ml) in external solution in the presence of sample /glucose content (µg/ml) in external solution in the absence of sample) ×100.

Glucose uptake in yeast cells

Glucose uptake in yeast cell was evaluated as per the standard procedure (29). The baker's yeast (*Saccharomyces cerevisiae*) was subjected to repetitive centrifugation (Thermo Fischer) (3000 rpm, for 5 min) in distilled water until the supernatant fluids were was clear and a 10% (v/v) uspension was prepared in distilled water. Leaf extract fractions of 50–250 µg/ml + 1 ml of 5, 15 and 25 mM glucose solution added and incubated at 37 °C for 10 min. The reaction was initiated by addition of 100 µl yeast suspension to the combination of glucose and fractions was mixed and incubated at 37 °C for 1 hr. It is centrifuged at (2500 rpm for 5 min), and the quantity of glucose in the sample is assessed by using a spectrophotometer at 520 nm. The % of glucose uptake was determined by:

$$\text{Glucose uptake (\%)} = \text{Abs control} - \text{Abs fractions} / \text{Abs control} \times 100$$

Insulin secretion assay

The amount of insulin released by RIN-m5F cells was estimated (30). Cells RIN-5F cell line was obtained from National Centre for Cell Science, Pune, India. The RIN-m5F cells (10^5 cells/ml) was isolated in fresh RPMI-1640 growth medium (supplementary with 1 mM sodium pyruvate, 10% FBS, 10 mM HEPES) 90%; fetal bovine serum 10%, antibiotics (100 µg/ml streptomycin, 100 IU/ml penicillin G, and 2.5 µg/ml amphotericin B) added 0.2 ml into 96-well plates. Plate kept in a 5% CO_2 incubator at 37 °C for 48 hr, after incubation, the medium was removed and cells incubated in glucose-free Krebs-Ringer (KRB) buffer (3.6 mM KCl, 1.5 mM $CaCl_2$, 135 mM NaCl, 5 mM $NaHCO_3$, 0.5 mM $MgCl_2$ 0.5 mM NaH_2PO_4) added with 1 mg/ml BSA and 10 mM HEPES incubated for 2 hr. The growth medium removed and substituted by 100µl of glucose-free KRB comprising different concentrations (50–250 µg/ml) fractions. Glibenclamide (1, 10, 100 µM) acts as a positive control. After one hr of incubation, aliquots of 25 µl of media was collected from every well and assessed for the amount of insulin. The insulin content was estimated by DRG diagnostic insulin ELISA kit by: % of insulin secreted = [(Abs treated cells/Abs control cells) × 100] - 100. The quantity of insulin secreted was expressed as µU/ml.

Result

Total phenolic and flavonoid contents

The total phenolic and flavonoid contents from ethyl acetate, hexane, methanol, and aqueous fractions of *A. nilgiricum* leaves were shown in Fig. 1. The ethyl acetate fraction showed maximum phenolic content (79.92±1.58 mg/g) while methanol, hexane and aqueous fractions showed 47.67±0.66 mg/g, 37.46±1.12 mg/g and 65.51±1.83 mg/g respectively. The ethyl acetate fraction showed maximum flavonoid content (21.74±0.89 mg/g) while methanol, hexane and aqueous fractions showed 19.76 mg/g, 18.64 mg/g and 9.51 mg/g respectively.

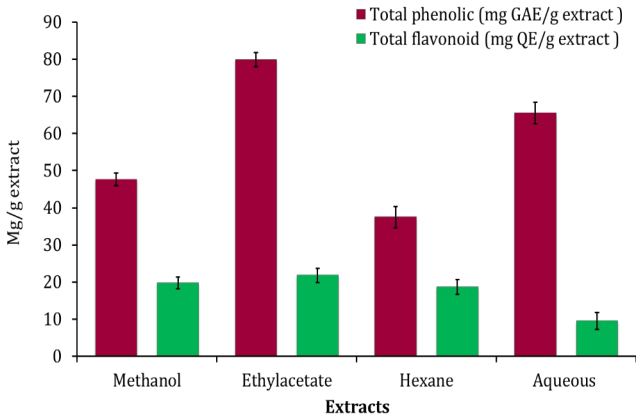

Fig. 1. Determination of total phenolic and flavonoid from different fractions of *A. nilgiricum* leaves.

H_2O_2 scavenging activity

The scavenging capacity of fractions on hydrogen peroxide was shown in Fig. 2. The *A. nilgiricum* leaves fractions were capable of scavenging H_2O_2 in a dose dependent manner. The ethyl acetate fraction showed maximum H_2O_2 scavenging activity of 97.62±2.89% followed by methanol (90±1.33 %), hexane (86.43±1.56%) and water fraction was revealed lowest H_2O_2 scavenging activity of 85±1.89%. The IC_{50} values as follows: ethyl acetate – 78.57 µg/ml, methanol – 83.62 µg/ml, hexane – 92.38 µg/ml and

aqueous fraction – 115.57 µg/ml. The standard, gallic acid, showed 62.33 µg/ml.

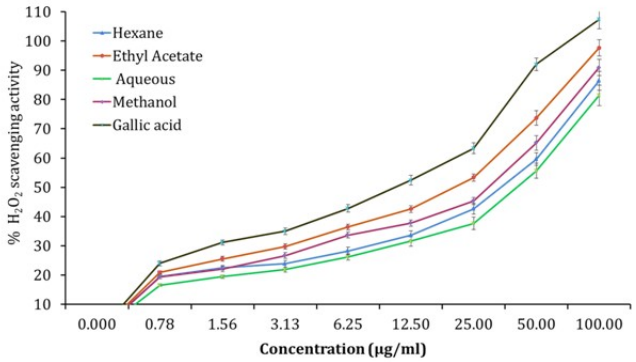

Fig. 2. H_2O_2 scavenging activity of different fractions of *A. nilgiricum* leaves.

In vitro glucose diffusion

The effects of different fractions tested upon glucose diffusion *in-vitro* are shown in Fig. 3a. Glucose diffusion assay was conducted to examine the influence of different fractions of leaf extracts with respect to its glucose retardation activity across the dialysis tube. The glucose diffusion at the different times assayed was significantly different between the fractions. The amount of glucose diffusion was recorded to increase from 30 to 180 min. Ethyl acetate fraction showed inhibitory effects on transmission of glucose into the outside solution across the dialysis membrane and aqueous fraction was showed minimum inhibition to the diffusion of glucose as related to the control. For all fractions, the rate of glucose transfer into external solution was lesser than control. The fractions were exhibited GDRI between 19% and 26%. GDRI (%) reduced with decreasing concentration of the fractions (Fig. 3b). The aqueous fraction was revealed to show better GDRI at 250 µg/ml concentration.

Glucose uptake in yeast cells

The effect of fractions on glucose uptake across yeast cell membrane was determined *in-vitro* system containing yeast cells suspended in a 5, 15, 25 mM glucose solution at different concentrations (Fig. 4).The result of metronidazole on glucose uptake by the yeast cells at 25 mM glucose was a little higher as compared to the fractions. All the fractions increased yeast cells glucose uptake. Among the fractions, methanolic fraction was showed maximum glucose uptake of 67.08±1.68% which was nearly close to the standard metronidazole, i.e. 68.06±0.49%, 71.06±2.62%, 71.06±2.62% concentrations at 5 mM, 15 mM, 25 mM respectively (Table 1).

Insulin secretion assay

The effect of fractions on insulin secretion was evaluated on RIN-m5F cell lines. The fractions significantly stimulated the insulin secretion in RIN-m5F pancreatic cells in comparison to Glibenclamide (Fig. 5). The ethyl acetate fraction was revealed maximum insulin secretory effect on RIN-m5F cells when when compared to other fractions. A dose-dependent increase in insulin secretion was significantly improved from different concentrations of 62.5 µg/ml to 500 µg/ml as compared to Glibenclamide (1, 10, 100 µM).

Table 1. Percentage of glucose uptake in yeast cells treated with *A. nilgiricum* leaf extracts fractions with 5, 15 and 25 mM glucose concentration.

Extracts	Concentration (µg/ml)	Inhibition (%) at 5 mM glucose	Inhibition (%) at 15 mM glucose	Inhibition (%) at 25 mM glucose
Metronidazole	50	44.87±0.98	38.92±0.66	25.78±0.57
	100	49.78±1.28	44.97±0.57	28.46±0.68
	150	54.95±1.16	50.65±089	32.66±0.89
	200	65.98±1.33	62.891.34	35.12±0.76
	250	77.66±1.125	71.54±1.66	36.34±0.98
Ethyl acetate	50	35.74±0.98	31.32±0.57	18.67±0.57
	100	37.89±1.37	32.87±0.66	21.43±0.56
	150	38.65±1.56	39.66±0.78	25.54±0.43
	200	51.42±1.89	48.32±1.12	29.76±0.89
	250	69.06±2.13	61.54±1.56	34.67±0.66
Methanol	50	36.76±1.46	33.23±0.66	21.66±0.6
	100	39.99±1.63	35.76±0.57	25.87±0.89
	150	47.75±1.83	42.18±1.32	28.45±0.57
	200	59.42±2.26	52.65±1.45	33.23±0.98
	250	71.06±2.62	65.87±2.12	36.56±0.57
Hexane	50	28.64±0.66	21.78±0.89	19.34±0.66
	100	32.97±1.32	28.54±0.66	21.34±0.56
	150	36.85±1.11	32.76±0.89	24.91±0.89
	200	45.44±1.34	38.33±0.87	29.45±0.54
	250	61.43±1.89	59.43±1.46	31.66±0.48
Aqueous	50	29.51±0.98	21.43±0.78	17.67±0.33
	100	35.23±1.21	27.45±0.66	19.32±0.78
	150	37.62±1.16	31.89±0.57	21.43±0.66
	200	49.77±1.57	40.65±1.33	26.87±0.98
	250	63.55±1.98	48.76±1.63	29.78±0.73

Results are expressed as mean ± SE (n=3) of three independent experiments.

Discussion

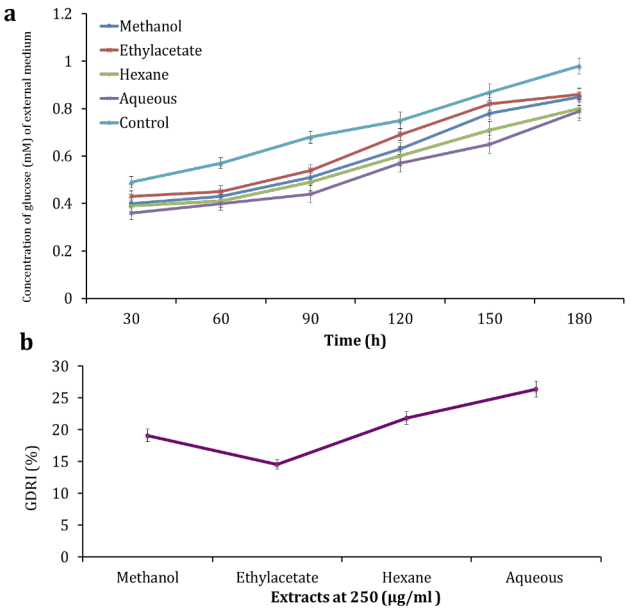

Fig. 3. Effect of different fractions of *A. nilgiricum* leaves on the diffusion of glucose across the dialysis membrane. **a)** Diffusion of glucose across the dialysis tube at 250 µg/ml at different time intervals. **b)** Glucose diffusion retardation index after 3 hr at 250 µg/ml concentration.

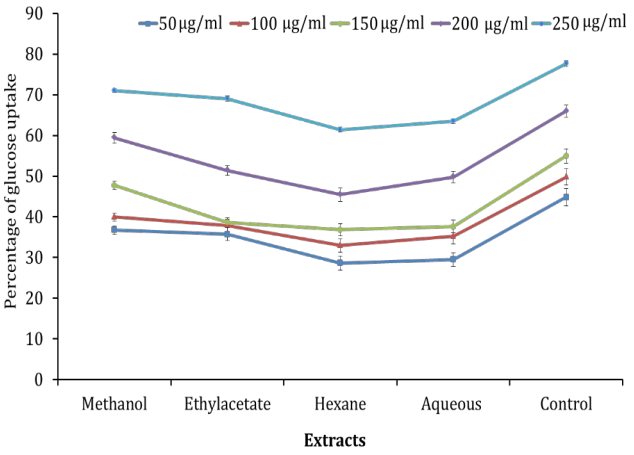

Fig. 4. Percentage of glucose uptake in yeast cells treated with different fractions of *A. nilgiricum* leaves.

Diabetes mellitus is one of the growing metabolic disorder worldwide. The defectiveness in the energy regulation and metabolism of the body because of insulin resistance otherwise scarcity to elevated blood sugar points leading to numerous problems in diabetes (31). Medicinal plants have been used in traditional/ayurvedic usage to treat Diabetes mellitus, especially in developing countries (32). The phytocomponents of the plant extracts which show hypoglycemic effect may work separately or synergistically (33, 34). Further, the secondary plant metabolites associated with antioxidants prevent the damage of pancreatic beta cell due to oxidative stress.

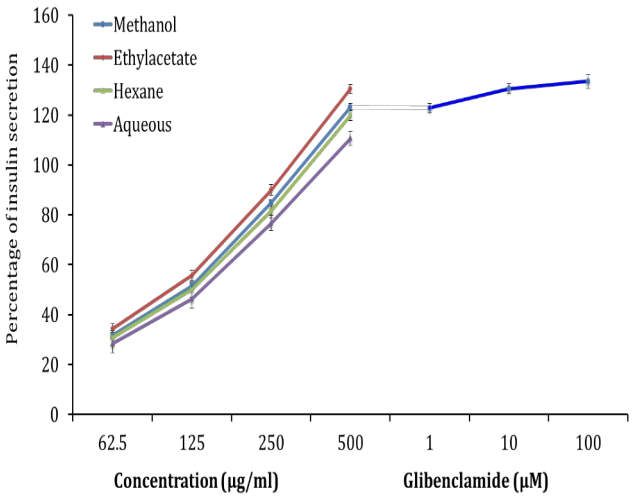

Fig. 5. Effect of different fractions of *A. nilgiricum* leaves on insulin secretion in RIN-m5F pancreatic cells. Glibenclamide was used as a positive control in glucose free medium.

The α-amylase and α-glucosidase enzymes hydrolyze α-1,4-glucosidic linkages to liberate α-glucose. Inhibition of these enzymes expressively reduces the postprandial acceleration in blood glucose (11). Earlier studies suggested that plant-derived phenolics have significantly inhibited *α*-amylase and *α*-glucosidase activities (35–37). The side effect of diabetes drugs is mainly due to extreme inhibition of pancreatic *α*-amylase resultant in the irregular bacterial fermentation of unused sugars in the colon (38).

The plant fractions dose-dependently increased the glucose uptake in yeast cells. The transport of glucose across the yeast cell membrane is an effective model to evaluate the hypoglycemic effect (39). Glucose uptake is mediated by stereospecific membrane carriers (40). The methanol fraction of *A. nilgiricum* leaves exhibited the maximum glucose transport across yeast cells followed by ethyl acetate, hexane and aqueous fractions.

A. nilgiricum leaves fractions inhibited the amount of glucose diffusion across the membrane; hence, they can act as a promising inhibitor in dropping the blood glucose level by inhibiting the movement of glucose molecule across the plasma membrane into the blood vessel. Ethyl acetate fraction exhibited potent inhibitory effects compared to control. This indicates that *A. nilgiricum* leaves extract can significantly inhibit glucose diffusion (41). The glucose GDRI is beneficial *in-vitro* index to calculate the efficiency of fractions on the delay in glucose absorption in the intestinal tract (42, 43). The aqueous fraction was recorded to have the maximum GDRI value at 250 µg/ml concentration. Similar studies have been reported in soluble fibres, for example, oats, wheat bran and psyllium husk were showed to prevent between 10 and 23% glucose transmission (44).

Insulin secretory activity could be due to natural substances present in the plant extract, which stimulates insulin secretion or protects the intact functional β-cells from further deterioration, so that they remain active and continue producing insulin. Terpenoids and polyphenols from medicinal plants are known to stimulate the pancreatic beta-cell resulting in secretion of insulin (45, 46).

Conclusion

The result of the current research indicates *A. nilgiricum* leaf extracts exhibited antioxidant property, exhibited *in-vitro* glucose diffusion, glucose uptake by yeast cells and stimulated insulin-secreting effect on RIN m5F pancreatic cell lines. Further, isolation and evaluation of bioactive molecules could be helpful for the discovery of new medicines to control diabetes mellitus.

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Authors' contributions

NK, UAC, SK did the experiments, NK, UAC, KCG, VG, NS, SC, NSR wrote the manuscript, NK, UAC, KCG, VG, NS, SC, NSR and SK read the manuscript and made suitable changes.

Conflict of interests

The authors declare that they have no conflict of interest.

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