

Antidiabetic Activity of Methanolic Extract of *Artabotrys suaveolens* Leaves in 3T3-L1 Cell Line

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Abstract

Artabotrys suaveolens is an evergreen shrub that belongs to the Annonaceae family, traditionally known for its use in regulating menstruation and in treating cholera. Other medicinal properties of this shrub remain to be studied and validated. The current study was aimed to evaluate the anti-diabetic property of methanolic extract of *Artabotrys suaveolens* leaves *in vitro* using 3T3L1 cell line. Anti-diabetic property of methanolic extract of *A. suaveolens* was evaluated *in vitro* by assaying for glucose uptake and the translocation of glucose transporter (GLUT4). The effect of *A. suaveolens* extract in inhibiting the activity of α -Glucosidase and α -Amylase enzymes was also evaluated using colorimetric assays. The cytotoxic effect of the extract was determined using the MTT assay. *A. suaveolens* (AS) extract treatment enhanced glucose uptake in 77.39 % of the cells, and GLUT4 expression was found in 74.87 % of the cells treated with AS extract. The extract inhibited α -amylase activity by 70.29 % and α -glucosidase activity by 74.06%. MTT assay showed that, AS extract had no cytotoxic effect on 3T3-L1. Our results attribute a promising anti-diabetic property for the methanolic extract of *A. suaveolens* leaves. Further studies that elucidate the molecular mechanism behind the anti-diabetic property of the extract must be carried out to recommend this as a possible alternative treatment for diabetes.

Keywords: *Artabotrys suaveolens*, Anti-diabetic, 3T3-L1, MTT assay, GLUT4, α -glucosidase, Diabetes mellitus

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Abbreviations: AS - *Artabotrys suaveolens*; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's Modified Eagle's medium; FBS: Fetal Bovine Serum; FITC: Fluorescein isothiocyanate. D-PBS: Delbuco's Phosphate Buffered Saline; FACS: Fluorescent Activated Cell Sorter, GLUT4: Glucose transporter 4; PNPG - p-nitrophenyl- α -D-glucopyranoside; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 2-NBDG: 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose; WHO-World Health Organization; μ g: Micrograms; mL: millilitre; SD: Standard Deviation.

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INTRODUCTION

Artabotrys suaveolens is an evergreen shrub that belongs to the *Annonaceae* family, traditionally known for its use in regulating menstruation and in treating cholera. The genus *Artabotrys* consists of 2,200 species^{1,2} and is geographically distributed in tropical and subtropical regions of East Asia and Africa^{2,3}. The roots and bark of *Artabotrys* have long been used as emmenagogue, and to relieve weakness after childbirth^{4,5}. The leaves of *Artabotrys* have also been used in traditional medicine. However, the anti-diabetic properties of *Artabotrys suaveolens* remain unexplored.

Diabetes mellitus (DM), an endocrine disorder characterized by impaired secretion and/or function of insulin that results in blood glucose levels high and perturbations in protein carbohydrate, and lipid metabolism⁶, will affect an estimated at 300 million by the year 2025^{7,8}. DM results in several complications, including cardiovascular diseases, renal failure, and bone related diseases⁹. Several strategies have been employed to treat Diabetes to maintain normal blood sugar levels. Inhibiting sugar metabolizing enzymes have been a vital approach¹⁰. There are synthetic drugs that work with this approach, but their adverse warrant the use of natural anti-diabetic compounds¹¹. Numerus enzyme changes that are directly related to diabetes mellitus focuses on the preliminary problem during the course of the disease. DM can be described as the disease with protean manifestations, includes coronary sclerosis, coronary artery disease, idiopathies, and renal, retinal and neurologic disorders. Patients diagnosed with premature arterial disease exhibits signs of preclinical or

subclinical diabetes treated for fasted blood sugar level or the tolerance of glucose.

In this study, we evaluated the antidiabetic activity of the methanolic extract of *Artabotrys suaveolens* leaves using biochemical and cellular assays. We assayed for the inhibitory effect of the extract on the activity α -glucosidase and α -amylase enzymes. We also analysed the cellular glucose uptake and GLUT4 expression levels and analysed the cytotoxic effects of the plant extract on 3T3-L1 cells.

MATERIALS AND METHODS

Chemicals and reagents

DMEM High Glucose (#AL219A, Himedia), DMEM without glucose (#AL186, Himedia), Fetal Bovine Serum (#RM10432, Himedia), D-PBS (#TL1006, Himedia), 2-NBDG (Invitrogen: Cat no. 13195), Metformin (#PHR 1084, Sigma), Acarbose (#A8980, Sigma), Anti-Mouse Glut4 –Alexa Fluor 488 Antibody (#NBPI-49533F, Novus Biologicals), MTT Reagent (# 4060, Himedia), DMSO (#PHR1309, Sigma), FACS Calibur (BD-Biosciences, USA), Microplate reader (#EC800, Biotek).

Collection and preparation of *Artabotrys suaveolens* plant extract

Artabotrys suaveolens was collected from the evergreen forests of Nilgiri in the Western Ghats, India and identified. The plant is also geographically distributed in Myanmar, Thailand, Malaysia, Indonesia and Philippines (Fig. 1).

A. suaveolens leaves were washed, shade-dried and grounded into fine powder using mortar and pestle. The powder was mixed with methanol such that 20 g of the powder was mixed with 100 mL of methanol and was extracted using Soxhlet extraction and was filtered using a Filtermann



Fig. 1. *Artabotrys suaveolens* is a scrambling or climbing, evergreen shrub

(125 mm) filter paper. The extract was then concentrated and dried using a rotary evaporator.

Cell culture

3T3-L1 (Mouse embryonic fibroblast) cell line was procured from NCCS, Pune. The cells were cultured in DMEM High Glucose supplemented with 10% FBS, 10,000 units of Penicillin G, 10000 µg/mL Streptomycin, and 10 mM HEPES. The cells were maintained at 37°C and 5% CO₂ incubator. The cells were passaged when they were 80 – 90% confluent.

Cytotoxicity Assay

The cytotoxic effect of AS extract on 3T3-L1 cells was analysed using the MTT assay. Briefly, 3T3-L1 cells were seeded at an initial density of 20*10⁴ cells per well/200µL in a 96 well plate and

incubated overnight at 37°C with 5% CO₂. The cells were then treated with different concentrations (25 to 400 µg/mL) of AS extract and was incubated for 24h at 37°C with 5% CO₂. Post-treatment, 200µL of MTT reagent (0.5 mg/mL) was added to the cells. The 3T3-L1 cells were incubated for 2h at 37°C and the crystals were dissolved by 20µL of DMSO. Absorbance at 570 nm was obtained using the microplate reader. Percentage viability of cells were calculated using the formula: % cell viability = (Mean OD of test at 570 nm/ Mean OD of Untreated cells at 570 nm) × 100

Glucose uptake assay

Glucose uptake assay was performed according to a previously described protocol¹². 3T3-L1 cells were seeded at an initial density of

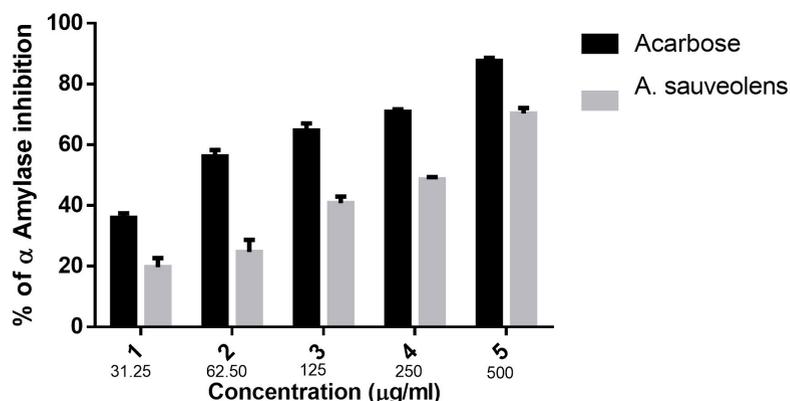


Fig. 2. Inhibition percentage of α-amylase against at different concentrations of Acarbose and *A. suaveolens*; the IC₅₀ value of the Acarbose and the extract were 49.19 ± 8.1µg/mL and 282.32±8.7µg/mL, respectively. Values are presented as mean ± SD.

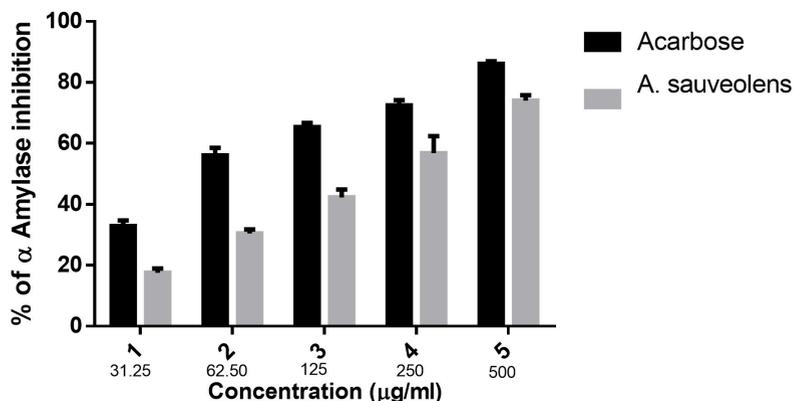


Fig. 3. Inhibition percentage of α-Glucosidase at different concentrations of *A. suaveolens* and Acarbose; Values are presented as mean ± SD.

2×10^5 cells/well/2mL medium. The spent medium was aspirated, and the cells were washed with D-PBS. The cells were then treated with the extract (100 μ g/mL) or Metformin (100 μ M), prepared in glucose-free DMEM, for 2h. To this, 100 μ M of 2-NBDG was added. The cells were then trypsinized and the pellet was resuspended in 0.5 mL of D-PBS. Cellular uptake of the glucose analog, 2-NBDG was evaluated using Flow cytometry (BD FACS Calibur) and the data was analysed using Cell Quest Pro software.

GLUT4 Expression studies

The translocation of GLUT4 to the cytoplasmic membrane was analysed using flow cytometry¹³. A 3T3-L1 cell were seeded at a density of 2×10^5 cells / well in a 6-well plate and was cultured overnight. The cells were then washed with D-PBS and were treated with the extract (100 μ g/mL) or Metformin (100 μ M) for 24h. Post treatment, cells were trypsinized and the pellet

was resuspended in 0.5mL D-PBS. The cells were incubated with Anti-Mouse Glut4-Alexa Fluor 488 antibody (#NBPI-49533F, Novus Biologicals) for 30 mins in the dark and unbound antibodies were washed with DPBS. FL1 channel of FACS Calibur was used to determine GLUT4 expression. Cell Quest Pro software was used for data analysis.

Inhibition of α -Amylase Activity

The α -Amylase inhibition assay was performed for different concentrations of the extract (31.25 to 500 μ g/mL) according to a previously described method with slight modifications¹⁴. Equal volumes of α -amylase and the mentioned concentration of the extract (100 μ L) were mixed and was incubated at 37°C for 10 min in microtubes. The reaction mixture was mixed with 100 μ L of 1% soluble starch for 30 min and was incubated at 37°C. The reaction was arrested using 200 μ L of dinitrosalicylic acid at 100°C for 5 min. From this mixture, 50 μ L of the mixture was transferred to 96-well microplate was further diluted with 200 μ L of distilled water in each well. Acarbose was used as positive control. Absorbance was measured at 540 nm using microplate reader (#EC800, Biotek). The inhibitory activity of the extract was calculated by the following formula: % inhibition = (Mean OD of Untreated Control - mean OD of test samples / Mean OD of Untreated Control) \times 100

Inhibition of α -Glucosidase activity

The α -glucosidase inhibition assay was performed with various concentrations of the extract (31.25 to 500 μ g/mL) according to a previously described method with slight modifications (15). 600 μ L of potassium phosphate buffer, and 25 μ L of enzyme (α -glucosidase, 1.2 EU/ml) were mixed with 100 μ L of test samples.

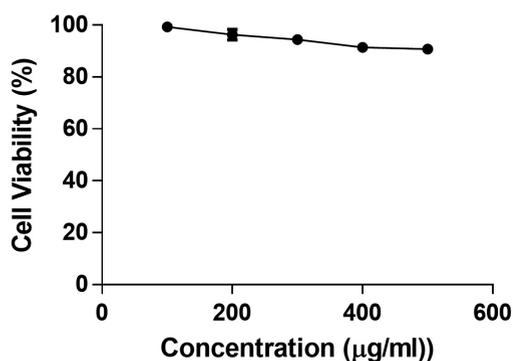


Fig. 4. The effect of *A. suaveolens* on 3T3-L1 cell line viability was analysed by MTT assay and the test compound didn't show high toxicity and IC50 couldn't be identified at given concentrations.

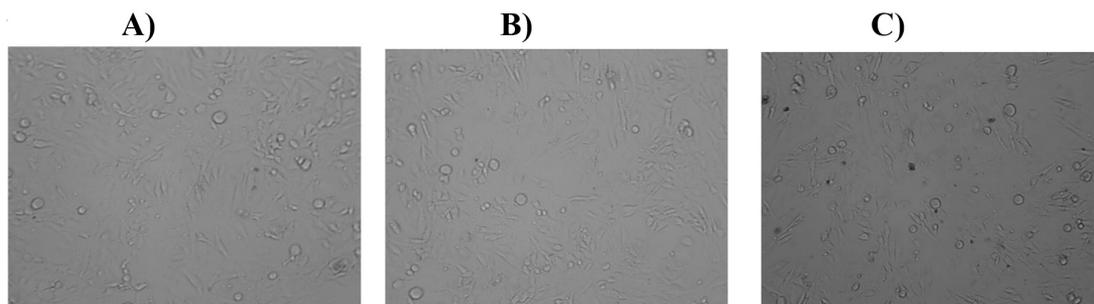


Fig. 5. Inverted light microscopy images of 3T3 L1 cell line after exposure to *A. suaveolens*, and Metformin. From 'A' to 'C' where A) control B) Standard Metformin drug and C) 400 μ g concentration of *A. suaveolens* extract.

The reaction mixture was incubated for 15 min at 37°C. After this, 25µL of PNPG was added to the reaction mixture and was incubated for 15 min at 37°C. The reaction was then arrested with 750µL of Na₂CO₃. The absorbance was measured at 405nm by using a micro-plate reader (#EC800, Biotek). The activity of the test sample was calculated by the following formula. Acarbose was used as positive control: % Inhibition = (Mean OD of Untreated Control - mean OD of test samples / Mean OD of Untreated Control) × 100

Table 1. Inhibition of α-amylase enzyme activity by *A. suaveolens* extract

Sample	Concen. (µg/mL)	% inhibition of enzyme activity	IC50 (µg/mL)
Acarbose	31.25	35.99±1.44	49.19 ±8.1
	62.50	56.22±2.05	
	125.00	64.70±2.3	
	250.00	70.84±0.89	
	500.00	87.65±0.96	
A. suaveolens extract	31.25	19.73±3.00	282.32±8.7
	62.50	24.71±3.97	
	125.00	40.75±2.18	
	250.00	48.63±0.73	
	500.00	70.29±1.81	

Statistical Analysis

The experiments were conducted in triplicate (n =3) and the results were expressed as mean ± SD. Graph-Pad prism 5 was used for statistical and linear regression analysis.

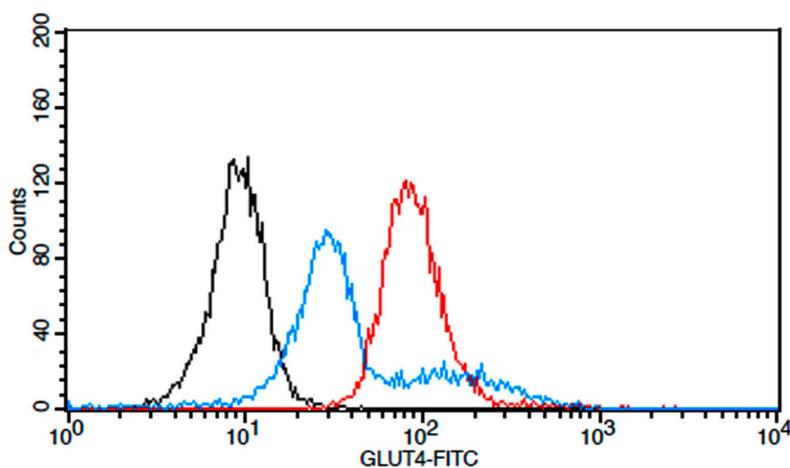
RESULTS

Inhibition of α-Amylase Activity

The percentage inhibition of α-amylase activity by the plant extract was estimated with

Table 2. Inhibition of α -Glucosidase enzyme activity by *A. suaveolens* extract

Sample	Concen (µg/mL)	% inhibition of enzyme activity	IC50 (µg/mL)
Acarbose	31.25	32.83±1.91	55.84±6.42
	62.50	56.07±2.42	
	125.00	65.39±1.27	
	250.00	72.49±1.72	
	500.00	86.15±0.78	
A. suaveolens extract	31.25	17.54±1.41	235.46±18.55
	62.50	30.45±1.37	
	125.00	42.32±2.56	
	250.00	56.79±5.54	
	500.00	74.06±1.68	



Key	Name	Parameter	Gate
—	3T3L1-CELL CONTROL	FL1-H	G1
—	3T3L1-STD CONTROL	FL1-H	G1
—	3T3L1-SAMPLE AS	FL1-H	G1

Fig. 6. Overlaid expression of GLUT4 in untreated 3T3L1 cells (black colour line) & standard drug treated cells (metformin 100µm) (red colour line) and test compound treated cells (cyan colour line).

Acarbose as the positive control. *A. suaveolens* extract exhibited 19.73% inhibition in α -amylase activity at 31.25 μ g/mL concentration and 70.29% inhibition at 500 μ g/mL concentration. The IC₅₀ value was found to be 282.32 \pm 8.7 μ g/mL. Acarbose, the positive control, exhibited 35.99% inhibition of α -amylase activity at 31.25 μ g/mL and 87.65% inhibition at 500 μ g/mL concentration. IC₅₀ value for Acarbose was found to be 49.19 \pm 8.1 μ g/mL shown in Table 1.

Inhibition of α -Glucosidase Activity

The percentage inhibition of α -glucosidase activity by AS extract was estimated with Acarbose as the positive control. AS extract exhibited, were 17.54% inhibition in α -glucosidase activity at 31.25 μ g/mL concentration and 74.06% inhibition at 500 μ g/mL concentration. The IC₅₀ value was found to be 235.46 \pm 18.55 μ g/mL. Acarbose, the positive control, exhibited 32.83% inhibition of α -glucosidase activity at 31.25 μ g/mL and 86.15% at 500 μ g/mL concentration. IC₅₀ value for Acarbose was found to be 55.84 \pm 6.42 μ g/mL [Table 2; Fig. 3].

Cytotoxic effect of *A. suaveolens* on 3T3-L1 cell line

The cytotoxicity of *A. suaveolens* extract on 3T3-L1 cells was tested using MTT assay. Briefly, the cells were treated with different concentrations of the extract for 24h. The extract treated cells showed 99.30% viability at 25 μ g/mL and 91.07% at 400 μ g/mL, suggesting that the extract had no cytotoxic effect on 3T3L1 cells. The concentrations of the test compound and its corresponding percent cell viability has been tabulated in Table 3 and Fig. 4,5.

GLUT 4 translocation study in 3T3-L1 Cell line

Translocation of GLUT4 receptors from

Table 3. Cell viability effects of *A. suaveolens* extract in 3T3-L1 cell line

Culture conditions	% of viability
Control	100.00 \pm 00
25	99.30 \pm 0.93
50	96.25 \pm 1.98
100	94.43 \pm 1.73
200	91.37 \pm 0.48
400	90.76 \pm 0.82
Metformin (100 μ M)	91.07 \pm 1.95

cytoplasm to the cell membrane is crucial to glucose absorption. Cells were treated with 100 μ g/mL of AS extract for 24h and GLUT4 expression was analyzed by Flow cytometry by probing with Anti-Mouse Glut4-Alexa Fluor 488 antibody (#NBPI-49533F, Novus Biologicals). Metformin was used as positive control at a concentration of 100 μ M. The fluorescence intensity of cells treated with plant extract was significantly higher than the negative control [Fig. 6]. On treatment with the plant extract, 77.39% of cells showed GLUT4 expression and Metformin induced GLUT4 translocation in 99.96% of cells [Fig. 7].

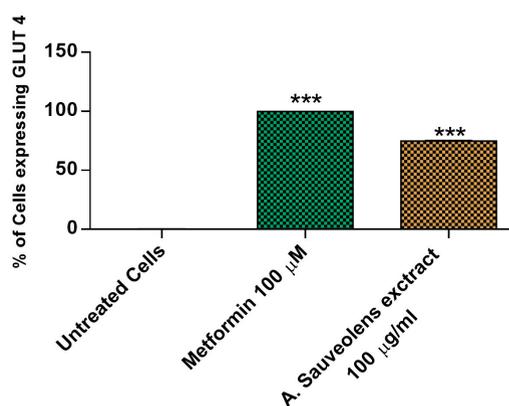


Fig. 7. GLUT Vs 3T3L1. The percentage of cells expressing GLUT4 comparison with Untreated, Metformin and *A. suaveolens* extract.

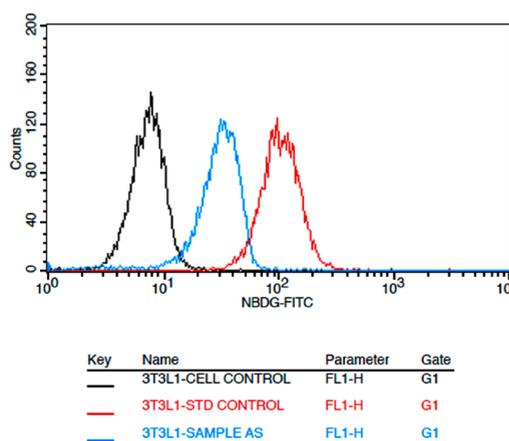


Fig. 8. Overlaid expression of given untreated 3T3L1 cells (black colour line) & standard drug treated cells (metformin 100 μ m) (red colour line) and test compound treated cells (cyan colour line) against the 2-nbdg stain.

Glucose uptake Assay

The effect of AS extract on glucose uptake was analyzed in 3T3-L1 cells using 2-NBDG, a fluorescent deoxyglucose analog. The 3T3-L1 cells were treated with 100 μ g/mL of plant extract or with 100 μ M of Metformin. The cells treated with 2-NBDG alone was considered as negative control. The fluorescence intensity of cells treated with the extract was significantly high compared to the untreated cells (Fig. 8). Moreover, the percentage of cells that took up the 2-NBDG in the presence of plant extract was similar to the positive control, Metformin [Fig. 9].

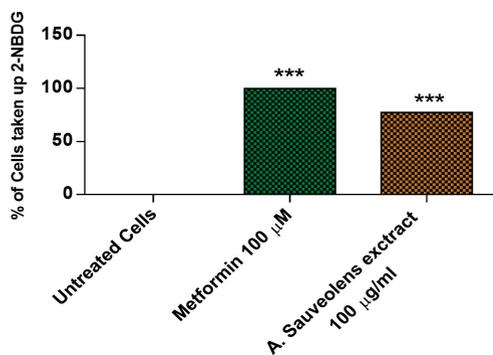


Fig. 9. The percentage of cells taken up 2-NBDG when treated with Metformin and test compound. Untreated cells are considered as negative control.

DISCUSSION

Diabetes mellitus has been a major cause of morbidity and mortality worldwide, with an estimated 382 million adult patients and 5.1 million deaths in 2013. Several studies have used multiple approaches to tackle DM. Amongst these inhibiting the enzymes that facilitate the absorption and metabolism of carbohydrates has been one approach. The important enzymes that are involved in glucose absorption from the gut are the pancreatic α -amylase enzyme and α -glucosidase enzyme. Inhibiting these enzymes reduces the absorption of glucose from the gut and thereby control the postprandial blood sugar levels^{16,17}. The antidiabetic drugs, that are available in the market specifically inhibit the activity of these enzymes include Miglitol, Voglibose and Acarbose. However, long-term use of these drugs has several effects including flatulence and abdominal bloating¹⁸. Using natural compounds,

which are extracted from medicinal plants, may not exert such effects¹⁹ and therefore play a vital role in treating ailments like Diabetes^{16,17,20,21}.

The species of *Artabotrys* is a traditionally used plant species for different clinical conditions such as, cholera, scrofula, enlarged spleen and malaria. In the presented study, we evaluated the antidiabetic properties of methanolic extract of *A. suaveolens* leaves. Our results suggest that methanolic extract of *A. suaveolens* effectively inhibits the activities of α -amylase and α -glucosidase enzymes. These effects were determined using Acarbose as the standard drug. Additionally the cytotoxic effects of *A. suaveolens* have been tested on 3T3-L1 cells, the findings of the study reveals that *A. suaveolens* did not show any cytotoxicity on these cells. These findings are consistent with the earlier reports on the effect of natural compounds in inhibiting the enzyme activities of α -amylase and α -glucosidase enzymes²²⁻²⁴.

CONCLUSION

In conclusion, AS extract significantly inhibited α -amylase and α -glucosidase enzyme activities with similar efficiency as the control, Acarbose. Glucose uptake and GLUT4 expression profile of 3T3-L1 cells treated with AS extract were also enhanced. Further, AS extract had no cytotoxic effects. These data suggest that methanolic extract of *A. suaveolens* has strong anti-diabetic properties and could possibly serve as a nutraceutical in treating Diabetes Mellitus. However, further studies must be carried out to establish these findings by identifying the specific modes or action and the specific constituents that drive these mechanisms.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AUTHORS' CONTRIBUTION

All authors listed have made substantial, direct and intellectual contribution to the work, and approved for publication

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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