

Annona stenophylla aqueous extract stimulate glucose uptake in established C2C12 muscle cell lines

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Abstract

Background: *Annona stenophylla* is a folk medicine popularly used in Zimbabwe for the treatment of many ailments. This study was carried out to determine some of the possible anti diabetic mechanisms of its action using in vitro cell culturing methods.

Methods: *A. stenophylla*'s effects on glucose uptake were tested using muscle cells (C2C12). Expression of glucose 4 transporters was determined by treating cell lines with plant extract. Total RNA was isolated and using RT-PCR, GLUT 4 expression levels were quantified. Translocation of GLUT 4 was assessed using FITC fluorescence measured by flow cytometry.

Results: Treatment of cells with plant extract significantly increased glucose uptake in a concentration dependent manner, with the highest concentration (250 µg/ml) giving 28% increased uptake compared to the negative control. The increase in glucose uptake (2.5 times more than control) was coupled to increase in GLUT 4 mRNA and subsequently GLUT 4 translocation. Wortmannin expunged the *A. stenophylla* induced increase in GLUT 4 mRNA and glucose uptake.

Conclusion: The results suggest that *A. stenophylla* aqueous extract increases glucose uptake partly through increasing the GLUT 4 mRNA and translocation potentially acting via the PI-3-K pathway. This study confirms the ethnopharmacological uses of *A. stenophylla* indicating potential for anti-diabetic products formulation.

Keywords: *Annona stenophylla*, glucose uptake, GLUT 4, diabetes, wortmannin.

DOI: <https://dx.doi.org/10.4314/ahs.v19i2.47>

Cite as: Taderera T, Chagonda LS, Gomo E, Katerere D, Shai LJ. *Annona stenophylla* aqueous extract stimulate glucose uptake in established C2C12 muscle cell lines. *Afri Health Sci.* 2019;19(2): 2219-2229. <https://dx.doi.org/10.4314/ahs.v19i2.47>

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Introduction

The rapidly increasing incidence of diabetes mellitus is becoming a serious threat to human health in all parts of the world. The control and treatment of diabetes and its complications mainly depend on chemical or biochemical agents¹. There is need for tight regulation of glucose which is the main source of energy for the body. After a meal insulin is released by the pancreatic islets inducing glucose uptake by tissues that are sensitive to it. Glucose transporter type 4 (GLUT4) is the major transporter in the GLUT family, that mediates glucose uptake by the muscle and adipose tissues². Under insulin resistance, translocation of insulin-sensitive GLUT4 is impaired, resulting in the consequent defect in the insulin-stimulated

glucose uptake, a rate-limiting step for glucose disposal^{3,4}. In skeletal muscle, insulin stimulates glucose uptake primarily by increasing translocation and redistribution of the GLUT4 from internal membrane to the plasma membrane^{5,6}. The increased expression of GLUT4 has previously been shown to lower blood glucose levels and enhance glucose transport and utilisation in skeletal muscles⁷.

While conventional treatments such as sulfonylureas, metformin and thiazolidinediones are effective, they have several limitations, including adverse side effects, secondary failure or the inability to halt further loss of insulin secretory capacity⁸ and in many cases the precise mechanism of action remains to be completely clarified⁹.

The insulin stimulation followed by cascade signalling enhances glucose uptake, utilisation and storage in various tissues¹⁰. Therefore, muscle glucose-uptake and suppression of gluconeogenesis could be considered as an excellent target for treatment of T2DM. Therapeutic approaches with natural products provide a fruitful source for searching safe, effective and relatively inexpensive new remedies for diabetes mellitus and associated metabolic disorders^{3,11}.

Annona stenophylla (family: Annonaceae), commonly known as the dwarf custard apple, is a small rhizomatous shrub found in some regions of Zimbabwe¹². This plant has been used in traditional medicine and scientific studies have validated some of the folkloric claims of different activities such as antiparasitic, anti-infective, antiviral and antidiabetic activities^{12,13,14}. Earlier in our laboratory we have demonstrated that *A. stenophylla* plant extract possess antioxidant activity, antimicrobial activity and has potential hypoglycaemic effects. The hypoglycaemic activity has partly been attributed to its ability to inhibit carbohydrate metabolising enzymes and a polyherbal formulation incorporating *A. stenophylla* has been formulated^{15,16,17,18,19}. The extract has been found to be relatively safe to use through brineshrimp lethality tests and subacute toxicity tests using a rat model^{20,21}.

New insights into the mechanisms of action in glucose lowering and insulin resistance may provide new treatment strategies for diabetes. In a bid to explore the observed effects of *A. stenophylla* plant extract, mechanisms of antidiabetic activity were investigated using established muscle cell lines.

Materials and methods

This was an experimental study performed at the University of Zimbabwe, Physiology laboratory.

Reagents

Dulbecco's Minimum Essential Medium DMEM, fetal bovine serum (FBS), trypsin-EDTA, antibiotic (strep/penicillin) and Phosphate buffered saline (PBS) reagents were from Gibco, USA. Whatman No. 1 filter paper Merck (South Africa) Antibodies to GLUT4 were from Indaba Biotechnology (South Africa)

Plant material and extraction

The plant roots of *Annona stenophylla* were collected from Mazowe District in Harare, Zimbabwe GPS coordinates were latitude 17 ° 24 14.25 Southings and longitude 30 ° 41 36.13 Eastings, using guidelines for sustainable harvesting of traditional medicinal plants in Zimbabwe^{23,24}. Identification and authentication was done with the aid of a botanist from the National Herbarium and Botanic Gardens of Zimbabwe, and a voucher specimen tagged (2540) was kept for reference. The roots were air dried for 4 weeks in the laboratory at ambient temperature, ground, and extracted overnight with distilled water at 37 °C. The filtered extract was lyophilized in a freeze dryer and stored at -20 °C until use.

Cell culture

C₂Cl₂ myocytes were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) and 1% antibiotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin) in a humidified atmosphere of air and 5% CO₂ at 37°C¹. The myocytes were left without change of medium for 6-7 days after seeding into 48 well plates for differentiation to occur and experiments were performed in the differentiated myotubes which express GLUT 4 insulin responsive transporters.

Glucose uptake

The determination of glucose uptake in C₂Cl₂ myotubes was performed using a modified method described previously²⁵. Briefly, media was removed from cell wells and replaced with 1ml of fresh medium containing increasing concentrations of *A. stenophylla* aqueous extract, PBS as negative control and positive control insulin, followed by an overnight incubation period. Glucose concentration

in the medium was determined after the incubation period by the glucose oxidase method using a commercial kit (KAT medicals) following the manufacturer's instructions. Test samples were tested in quadruplicates and repeated at a later occasion. Absorbances were read at 540nm in a microplate reader named (Anthos 2010). The amount of glucose taken up by the cells was regarded as being proportional to the absorbance readings. A standard curve using known glucose concentrations was constructed and used to extrapolate the glucose levels.

Extraction of RNA and analysis of gene expression

After exposure to plant or control treatments, total RNA was extracted from C₂Cl₂ myotubes (GeneJET RNA kit, Thermo Scientific). RNA was measured using a Qubit machine (Invitrogen) and normalised for cDNA synthesis (Revert Aid 1st strand cDNA KIT, Thermo Scientific). RT-PCR was done using Thermo Scientific Dream Taq Green following manufacturer's instructions. The PCR products were run on 1% agarose gels stained with ethidium bromide and quantified using ChemDoc Imager software. GAPDH was used as an internal control.

The primers used were:

GAPDH: 5'-AACTTTGGCATTGTGGAAGG-3' (forward) and 5'-ACACATTGGGGGTAGGAACA-3' (reverse)

Glut4: 5'-ACATACCTGACAGGGCAAGG-3' (forward) and 5'-CGCCCTTAGTTGGTCAGAAG-3' (reverse)

GLUT4 translocation

The levels of GLUT 4 transporters in C₂Cl₂ myotubes (non permeabilised) were measured by flow cytometry. After cells were treated with plant extract or the controls, they were harvested and washed twice with 2% FBS in PBS. Cells were blocked using CD16/32 for 15 min and then incubated with a conjugated anti-GLUT4 antibody

solution (1.0 µg/ml in PBS) for 1 h at 4 °C. Excess antibodies after labelling were removed by washing twice in ice-cold PBS as described by Wang et al²⁶. The cells on the surface membrane were measured in duplicate and assay repeated at a later occasion. The fraction of GLUT 4 was expressed as increase in FITC fluorescence with respect to untreated stained cells.

Statistical analysis

Values are given as mean ± SE. Analysis of statistical significance of differences in measurements between samples was done by one-way ANOVA followed by Dunnett's post hoc test (GraphPad Prism version 5). P < 0.05 was considered statistically significant.

Results

Effect of *A. stenophylla* plant extract on glucose uptake in C2Cl2 cells

There was a general dose dependent increase in glucose uptake after 2 and 24 h of both plant extract and insulin positive control administration (Figure 1). Significant differences in the 2h compared to 24h glucose uptakes were recorded for concentrations 125 µg/ml and 250 µg/ml (p = 0.011 and 0.014 respectively). *A. stenophylla* plant extract showed percentage increase in glucose uptake that was comparable to insulin (positive control).

Changes in glucose uptake were measured after 2h and 24hours for the plant extract compared to the positive control insulin. % increase in glucose uptake was calculated as a fraction of the untreated control. Each assay was done in triplicate (N=6) and repeated on a separate occasion and results are the average (± SD). * (P< 0.05) shows significant differences upon comparing 2 h percentage glucose uptakes to 24 h ones at a particular concentration.

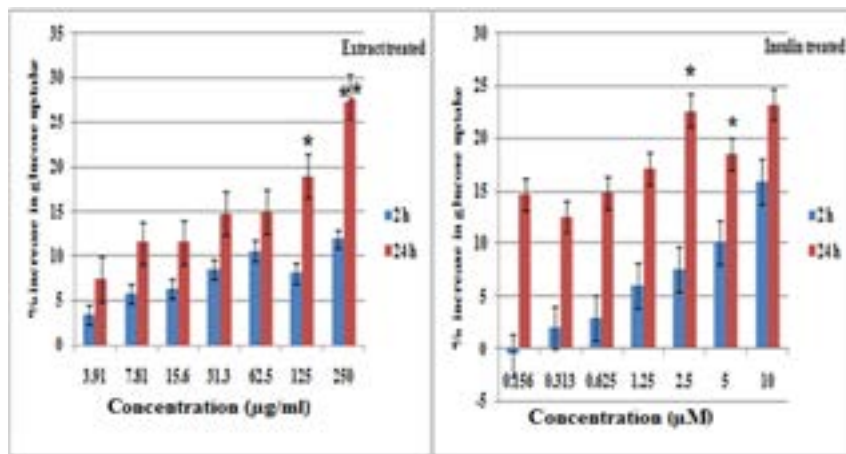


Figure 1: Effect of different treatments on glucose uptake in C2C12 muscle cells.

Effect of *A. stenophylla* extract on GLUT4 mRNA levels in C2C12 cells

Incubation of C₂Cl₂ cells with *A. stenophylla* and insulin resulted in increased mRNA levels of GLUT 4 when compared to the untreated control (Figure 2). The RT-PCR products were approximately 150 bp.

cDNA prepared from total RNA was amplified using GLUT 4 primer after treatment with plant extract (250 µg/mL) and insulin positive control (10 µM). Amplicon band sizes were equated to amounts of mRNA levels of the GLUT 4. cDNA loading was normalised by use of the housekeeping gene (GAPDH). Results are the average (± SD) of two assays (Each assay done in duplicate), N=4.

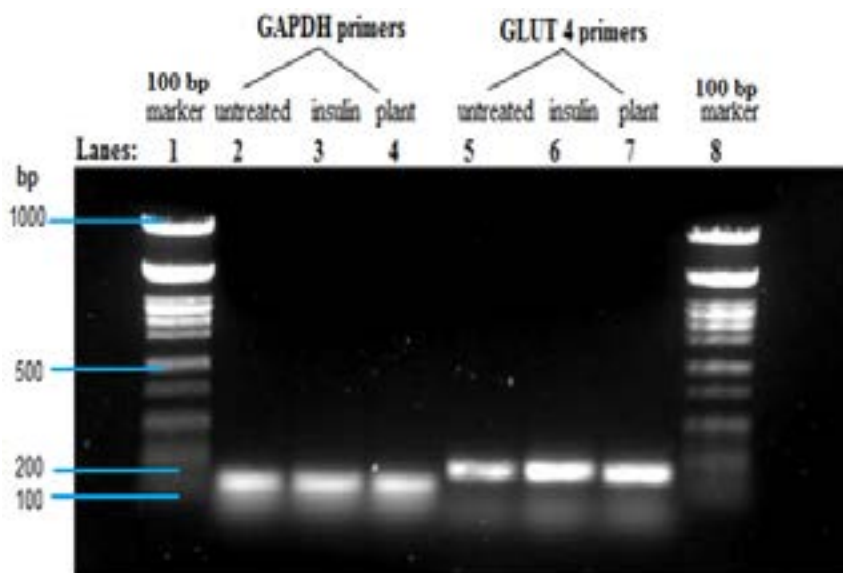


Figure 2: Effect of *A. stenophylla* extract on GLUT 4 mRNA levels in C2C12 cells using RT-PCR.

Equal amounts of cDNA material loaded were shown by analysis of the band sizes using Chemidoc Imager and this was confirmed by the almost equal amounts of band intensities in GAPDH (Figure 3). The untreated control

showed the least amount of amplicon band intensities when compared to those from plant extract treated and insulin treated cells. Differences in band intensities were not significant when treated samples were compared to the untreated control ($p > 0.05$)

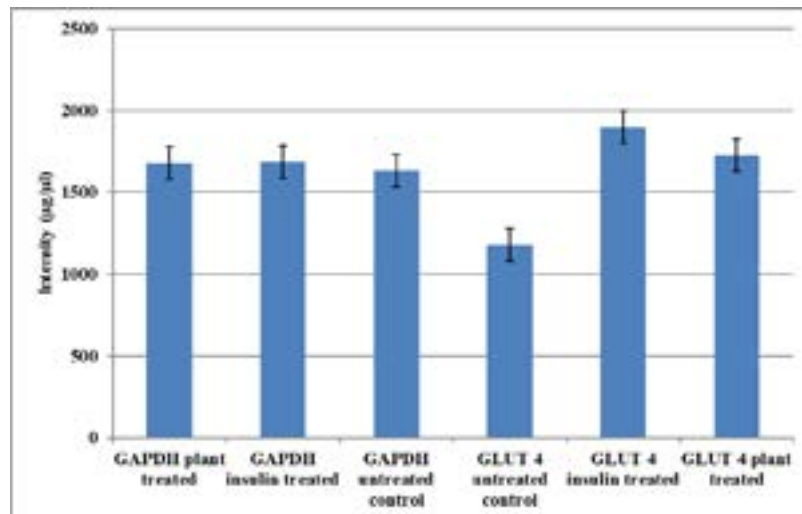


Figure 3: GLUT 4 mRNA amplicon intensities in C2C12 myotubes.

GLUT 4 mRNA amplicons resulting from cells treated with plant extract compared to the positive control insulin. The amplicons' differences in size were equated to differences in the mRNA levels of GLUT 4 expressed. The housekeeping gene (GAPDH) primers were used to normalize the cDNA loaded. Results are the average (\pm SD) of two assays (Each assay done in duplicate) $N=4$. GLUT4 translocation measurement using flow cytometry. The numbers of GLUT 4 on C_2Cl_2 myotubes plasma membrane surface were measured using flow cytometry.

The plant extract showed an increase in GLUT 4 cell surface transporters measured as 31.1 ± 11 MESF -FITC arbitrary units of molecules of equivalent soluble FITC fluorescence (figure 4). The untreated stained control had 4.0 ± 0.46 MESF -FITC. As a percentage of the untreated stained control the plant extract caused more increase in FITC levels compared to the positive control insulin. All the groups tested showed no significant differences of levels of surface GLUT 4 translocated to the surface membrane when compared amongst each other ($p > 0.14$) in all cases.

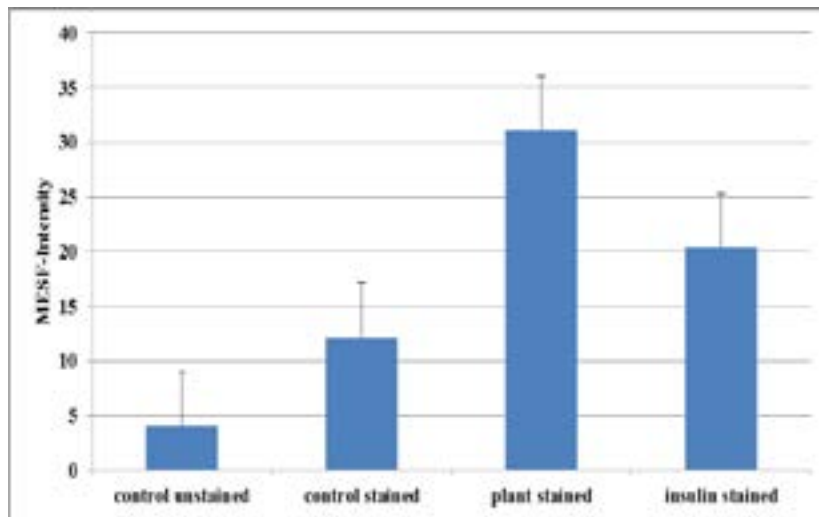


Figure 4: The effect of *A. stenophylla* aqueous extract on GLUT4 translocation in C2Cl2 myotubes.

The numbers of translocated GLUT 4 after treatment with plant and insulin standard control were quantified. Baseline fluorescence is shown in untreated stained cells (negative control). The intensities of MESF-FITC were equated to amounts of GLUT 4 translocated. Results are the average (\pm SD) of three assays (Each assay done in duplicate), (N=6).

Effect of wortmannin inhibitor on glucose uptake in C₂Cl₂ myotubes

Wortmannin inhibitor showed a decrease in the glucose

uptake when administered to C₂Cl₂ cells over 24 h (Figure 5). The 1.3 fold decrease in the glucose uptake was slightly reversed to a 1.0 fold decrease when wortmannin was incubated together with 250 μ g/ml of plant extract. The difference between the change in glucose uptake when treatment with inhibitor was compared to plant extract + inhibitor was, however, not significant ($p = 0.264$).

Effects of treatment with wortmannin alone, plant extract + wortmannin and plant extract alone was measured after 24 hours. Results are the average (\pm SD) of two assays done in duplicate (N=4).

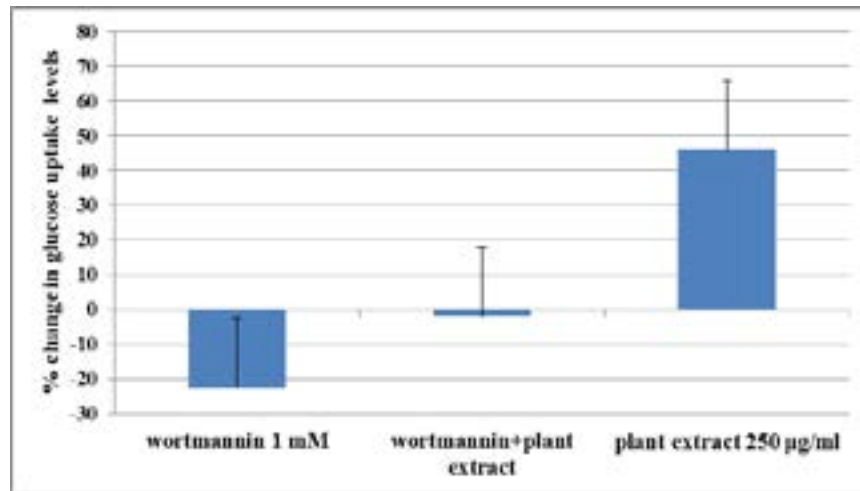


Figure 5 : Effects of wortmannin on glucose uptake in C2Cl2 cells.

Effect of wortmannin on GLUT 4 mRNA levels in C₂Cl₂ muscle cells

Wortmannin treatment on C₂Cl₂ muscle cells decreased

the levels of GLUT 4 mRNA expressed (Figure 6) when compared to plant extract treatment alone and combined wortmannin and plant extract treatment.

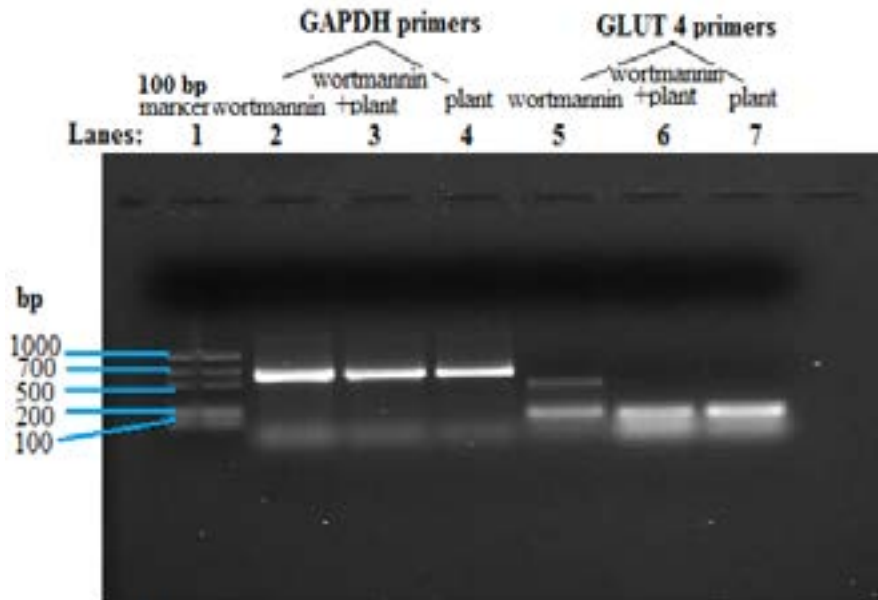


Figure 6: RT-PCR showing effect of wortmannin on GLUT 4 mRNA levels in C2Cl2 cells.

GLUT 4 primers were used to amplify the cDNA from cells treated with wortmannin compared to wortmannin + plant extract and plant extract alone. Differences in amplicon band sizes were equated to differences in the expressed mRNA levels of GLUT 4. Concentration of plant extract used was 250 µg/ml and wortmannin 1mM. Results are the average (± SD) of two assays (Each assay done in duplicate), N=4.

The differences in amplicon band sizes were equated to differences in the expressed mRNA levels of GLUT 4. Band densitometry analysis confirmed the decrease in

GLUT 4 mRNA levels, using GAPDH as an internal control (Figure 7).

Band intensities of the GLUT 4 amplicons resulting from cells treated with wortmannin compared to wortmannin + plant extract and extract alone were measured. Differences in amplicon band sizes were equated to differences in the mRNA levels of GLUT 4 expressed. The house-keeping gene (GAPDH) primers were used to normalize the cDNA loaded. Visual appreciation of the differences in band densities is shown. Results are the average (± SD) of two assays (Each assay done in duplicate) N=4.

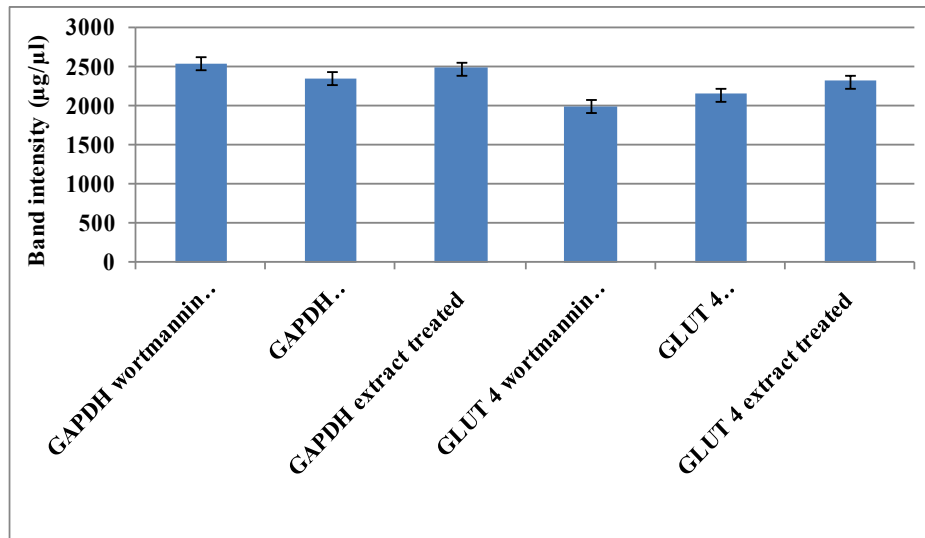


Figure 7: Band intensities of GLUT 4 mRNA levels in C2C12 myotubes treated with wortmannin.

Discussion

In previous studies the aqueous extract of *Annona stenophylla* demonstrated hypoglycaemic and insulinotropic effects in alloxan-induced diabetic rats¹⁸. Western diabetic drugs correct hypoglycemia by supplementing insulin, improving insulin sensitivity, increasing insulin secretion from the pancreas and/or glucose uptake by tissue cells²⁷. Certain herbs may lower blood glucose²⁸, however, their test results are subject to several factors and hence the present study was conducted to determine the potential mechanism of action of glucose-lowering effect shown by aqueous extract of *Annona stenophylla*.

Skeletal muscle is the principal site for postprandial glucose utilization and a major element in the maintenance of glucose homeostasis^{9,29}. A wide array of plant-derived molecules has been reported to be associated with beneficial effects on glucose transport and metabolism in skeletal muscle cells¹. In this study, plant extract treatment

increased glucose uptake in a concentration-dependent manner which shows that the more concentrated the drug the more the effect. The effect of extract treatment on GLUT 4 mRNA transcription was determined and proposed as a potential mechanism through which glucose uptake was increased. Gel electrophoresis showed a visual appreciation of how the different treatments affected glucose uptake in the C₂Cl₂ myotubes. The least uptake was shown in the untreated cells and amplification shown would possibly be due to the basal mRNA available. Extract and insulin treatment did show an increase in the transcription of GLUT 4 mRNA which would subsequently influence the amount of glucose taken up by the cells (Figures 1 & 2). Translocation of GLUT4 to the cell surface could be a part of the underlying molecular mechanism responsible for the insulin-mediated increased glucose transport³⁰. In cell cultures the delivery of glucose to the cell surface is not rate limiting like it

sometimes is in animals. Increased glucose entry depends on amount of GLUT 4 translocation and other facilitative transporters. The differences in expression of GLUT 4 in cell line experiments can, therefore, be attributed to different treatment responses.

GLUT4 gene expression is also subject to up or down regulation depending on the physiologic state of the organism. Changes in GLUT4 gene expression are observed in physiologic states of altered glucose homeostasis³⁰. Further to glucose uptake and mRNA assays, effect of *A. stenophylla* extract on translocation of GLUT 4 from the cytoplasm to the plasma membrane was assessed. GLUT 4 is only transiently expressed at the plasma membrane of cells and is endocytosed. The bound antibodies remain attached to the membrane, even during endocytosis, facilitating measurement of the total fluorescence signal of exposed GLUT 4 molecules³¹. The marginal increase in glucose uptake after treatment could be due to plants ability to enhance GLUT 4 translocation followed by increased gene transcription and expression⁴. Although the difference in the effects of untreated control compared to treatments were not significant, the influence of the extract can be considered for use in combination with other allopathic drugs or herbal formulations like proposed by Mohammad³².

Glucose uptake and GLUT 4 translocation has been shown to be mediated through two major pathways, insulin-mediated PI3K pathway and insulin-independent AMPK pathway. Wortmannin, a drug which inhibits insulin release and blocks many short term metabolic effects induced by insulin receptor activation, decreased glucose uptake in muscle cells. Decrease in glucose uptake was accompanied by decreased levels of GLUT 4 mRNA levels. Wortmannin did not completely abolish glucose uptake when incubated together with plant extract and this could be because *A. stenophylla* plant extract partly reversed the inhibitory effect of wortmannin. The role of PI3K in *A. stenophylla* plant extract induced glucose uptake and increased mRNA levels affected by coin-cubation with wortmannin suggest that uptake was partly PI3K dependent³³.

Conclusion

The major goal in treating diabetes is to minimize elevation of blood glucose without causing abnormally low levels of blood glucose. The action mechanisms for hy-

poglycemic herbs are multiple and *A. stenophylla* extract stimulates glucose transport in C₂Cl₂ myotubes by enhancing GLUT4 translocation from the internal membrane to plasma membrane accompanied with change in the total amount of GLUT4 or its gene expression.

Our findings show that *A. stenophylla* appear to have the ability to modulate glucose metabolism and provide the molecular basis of antihyperglycemic activity of the extract, which can be a promising candidate for the management of diabetes and associated metabolic disorders.

Acknowledgements

Many thanks go out to the Biomedical Department of Tshwane University of Technology through Dr L.J Shai and Professor D Katerere for the collaboration and all the assistance that made the work possible. The research work for the Doctoral research project was funded by the Southern African Consortium for Research Excellence (SACORE) grant number 087537/F/08/A. This work is part of a PhD project presented to the University of Zimbabwe in 2016. Supplementary research material was received from the University of Zimbabwe.

Conflict of interest

There is no conflict of interest from all the authors and the funders. I also affirm that all the authors have seen and agreed to the submission of the paper and their inclusion of name(s) as co-author(s).

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