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# Cassia Abbreviata Enhances Glucose Uptake and Glucose Transporter 4 Translocation in C2C12 Mouse Skeletal Muscle Cells

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## Abstract

*Background.* This study aim at assessing *C. abbreviata* aqueous extracts for its potential to exhibit anti-diabetic activity in skeletal muscle cells. In addition to the toxicological and glucose absorption studies, the action of *C. abbreviata* extracts on some major genes involved in the insulin signaling pathway was established. *Methods.* The *in vitro* cytotoxic effects *C. abbreviata* was evaluated on muscle cells using the MTT assay and the *in vitro* glucose uptake assay conducted using a modified glucose oxidase method described by Van de Venter et al. (2008). The amount of GLUT-4 on cell surfaces was estimated quantitatively using the flow cytometry technique. Real time quantitative PCR (RT-qPCR) was used to determine the expression of GLUT-4, IRS-1, PI3 K, Akt1, Akt2, PPAR-$\gamma$. *Results.* Cytotoxicity tests revealed that all extracts tested at various concentrations were non-toxic ($LC_{50}$ > 5000). Aqueous extracts of leaves, bark and seeds resulted in a dose-dependent increase in glucose absorption by cells, after 1 h, 3 h and 6 h incubation period. Extracts of all three plant parts had the best effect after 3 h incubation, with the leaf extract showing the best activity across time (Glucose uptake of 29%, 56% and 42% higher than untreated control cells after treatment with 1 mg/ml extract at 1 h, 3 h and 6 h, respectively). All extracts, with the exception 500 $\mu$g/ml seed extract, induced a two-fold increase in GLUT-4 translocation while marginally inducing GLUT-10 translocation in the muscle cells. The indirect immunofluorescence confirmed that GLUT-4 translocation indeed occurred. There was an increased expression of GLUT-4, IRS1 and PI3 K in cells treated with insulin and bark extract as determined by the RT-qPCR. *Conclusion.* The study reveals that glucose uptake involves GLUT-4 translocation through a mechanism that is likely to involve the upstream effectors of the PI3-K/Akt pathway.

## Keywords

glucose absorption, *Cassia abbreviata*, translocation, gene expression, GLUT4

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## Introduction

Diabetes mellitus is a long-lasting non-communicable condition that has significantly increased globally in recent years.[1] About 171 million people worldwide are affected by this scourge, almost five times more than the global estimates ten years ago. This number may probably double (366 million) by the year 2030 if no meaningful interventions are introduced.[2] Goldenberg and Punthakee (2013) define diabetes mellitus as a multifactorial condition, characterized by hyperglycaemia resulting from insulin deficiency or impaired insulin efficacy or the action of both.[3] Diabetes mellitus has been associated with various complications some of which include peripheral vascular disease, coronary artery disease and death, if not managed adequately.[4] In South Africa, it is estimated that 2 million people aged 30 years or above were affected by diabetes mellitus in 2009.[5]

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The use of plants for the management and treatment of diabetes mellitus dates back many years.[6] More than 30000 higher plant species are found in South Africa and 3000 of them are used in traditional medicine across the country.[7] In an ethnobotanical survey conducted by Erasto et al. (2005) in the richest flora regions in South Africa, 32 plant species used to manage and control diabetes in South Africa were identified.[8]

### *Cassia Abbreviata Oliv*

(Caesalpiniaceae), also known in English as "sjambok or long pod cassia", "sambokpeul" in Afrikaans or "Monepenepe" by certain tribes, is used in traditional medicine.[9] It is a tropical plant which occurs in bushland, woodland and grassland across Africa.[10] *Cassia abbreviata* was selected for this study because it is commonly used for the treatment of numerous ailments such as constipation, headache, diarrhea, fever, infertility, cough, bilharzia, syphilis, malaria, jaundice and skin disease and diabetes mellitus in many parts of Africa.[11-14] Furthermore, it is readily available and has received little scientific attention relating to its role as a hypoglycaemic plant

It has been established that plants exert their hypoglycaemic activities through different mechanisms. Some plants are insulin mimetic,[15] others have been recognized to replicate the mechanism of action of synthetic drugs such as sulfonylureas and biguanides.[16] GLUT4 translocation to the cell surface is a critical determinant of the amount of insulin-stimulated glucose transport in muscle cells.[17] During rest, GLUT-4 is almost absent on the plasma membrane. However, when stimulated by exercise or insulin, GLUT-4 is transferred from its intracellular location to the cell membrane.[18] The translocation of GLUT-4 is instigated by the action of insulin on the cells membrane in presence of high blood glucose level.[19] Plants with hypoglycaemic potential have been shown to increase glucose uptake through the up regulation of the translocation of glucose transporter GLUT4 from the intra cellular pool to the plasma membrane.[20] The translocation of GLUT4 is influenced by two important and distinct signaling pathways. One pathway involves the activation of phosphatidylinositol 3-kinase (PI3 K), which regulates glucose uptake and glycogen synthesis.[21] The other pathway involves the activation of AMP-activated protein kinase (AMPK).[22] These two physiological mechanisms initiate a series of events that culminate in the enhancement of GLUT-4 translocation and hence glucose uptake.

Insulin signaling pathway is triggered by activation of the insulin receptor tyrosine kinase leading to tyrosine phosphorylation of insulin receptor substrate and the recruitment of PI3 K. PI3 K then catalyses the conversion of phosphatidylinositol (4,5) P2 to phosphatidylinositol (3,4,5) P3 (PIP3). The second messenger, PIP3 in turn, triggers the activation of the protein kinase Akt through the actions of two intermediate protein kinases, PDK1 and Rictor/Mtor.[23,24] Muscle contraction increases AS 160 phosphorylation which may be done through PI 3-kinase independent mechanism and mediated by AMPK pathway, providing a potential convergence of insulin and exercise-mediate signaling to GLUT-4.[25] In order to validate the traditional use of the selected plant, this present study aimed to assess the toxicity and possible mechanisms associated with the hypoglycaemic effects of the aqueous extract of *C. abbreviata* leaf, bark and seeds, using mouse skeletal cells model.

## Materials and Methods

### *Plant Material*

Two collections of *Cassia abbreviata* leaves, seeds and bark were obtained at the South African National Biodiversity Institute (SANBI) in summer 2013. The plant materials were collected in woven sacks to allow ventilation during transportation. The leaves, seeds and bark were allowed to air dry at room temperature.

### *Preparation of Plant Extracts*

After drying at room temperature, the leaves, seeds and bark were ground using a pestle and mortar, then a laboratory mill (Polymix-Kinematica, Swizerland), into a fine powder before extraction for 24 h with distilled water, followed by filtering using a Whatman No.1 filter paper. The extraction process for each material was repeated three times. The filtrates were pooled together and frozen at -70°C before freeze drying for 3-5 days using a freeze dryer (Virtis SP Scientific, UK).

### *Cell Culture*

Mouse C2C12 cells were used for the toxicity assay, glucose uptake, GLUT-4 translocation and gene expression assays. The C2C12 cells were grown in RPMI 1640 (Highveld Biological, South Africa) supplemented with 10% fetal bovine serum (FBS) and 5% penicillin-streptomycin antibiotic cocktail. The cells were incubated in a humidified atmosphere with 5% $CO_2$ at 37°C. The cultured cells were used for experimental assays once they reached 80% confluency.

### *MTT Toxicity Assay*

The cytotoxic effects of the aqueous crude extracts of *C. abbreviata* on C2C12 were evaluated using the 3-(4,5-dimethyl-2-Thiazyl)-2,5-diphenyl-2H-tetrazoluim bromide (MTT) assay as developed by Mossman, 2003.[26] The muscle cells were seeded into a 96 well plate at a concentration of 1 x $10^5$ cells per well and incubated for 72 h at 37°C in 5% $CO_2$ to allow cells to attach and them to adhere to the surface of the culture plate become confluent. Cells were then treated with the crude water extract at 0.625, 1.25, 2.5 and 5 mg/ml prepared in complete growth media. Hydrogen peroxide (10%) was used as a positive control. The cells were then incubated for 24 h at 37°C in 5% $CO_2$. A volume of 10 µl of 1:10 dilution of MTT (5 mg/ml in phosphate-buffered saline, pH 7.2) was added and the plates further incubated for 4 h at 37°C for MTT reduction, followed by three washes in PBS (154 mM NaCl, 5.6 mM $Na_2HPO_4$, 1.1 mM $KH_2PO_4$, pH 7.4). Thereafter, the resulting formazan was solubilized in DMSO. Then, optical density, at 540 nm, was measured using a plate reader (ThermoFisher Scientific, Finland). Untreated cells and insulin were used as a negative control. Cell viability was estimated using the formula:

$$\%\ Cell\ viabilty = \left(\frac{A_{sample}}{A_{control}}\right)x100$$

## Glucose Uptake Assay

The *in vitro* glucose uptake assay was carried out by using a modified methods described by Van de Venter et al. (2008).[27] The C2C12 cells were grown to 80-90% confluency and then dislodged by a brief exposure to 0.25% Trypsin in PBS, counted (25,000 cells/ml) using a T20 cell counter (Bio-Rad) and suspended in complete growth medium. The cells were then seeded in a 96 well plate in a total volume of 200 µl/well and incubated at 37°C until they were 80% confluent. The cells were then treated with the crude plant extracts to reach a final concentration of 500 µg/ml and 1 mg/ml. The cells were incubated for 1, 3 and 6 h at 37 °C and at each time point, 50 µl of the supernatant was transferred from each well to a new 96 well plate, where 100 µl of glucose oxidase reagent (Sigma) was added. The mixture was then incubated at 37°C for 15 min and the absorbance measured at 540 nm using a microplate spectrophotometer. Cells treated with insulin at concentrations of 100 nM were used as positive control for the measurement of glucose uptake, while the untreated cells were used as negative controls. Glucose uptake was calculated as in the formula below, and expressed as increase in glucose uptake compared to untreated controls %.

$$\%Glucose\ uptake = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right)x100$$

## GLUT-4 and GLUT-10 Translocation (Flow Cytometry)

GLUT-4 and GLUT-10 translocation was conducted using a modified method carried out by Divya et al. (2015).[28] Confluent cultures of C2C12 skeletal muscle cells grown in a 6 well-plates were treated with 500 µg/ml and 1 mg/ml of specific extracts of *C. abbreviata* and insulin solution (100 nM) for 3 h. Then, the cells were washed three times with ice cold PBS and fixed with 3% formaldehyde for 30 min at room temperature. Fixed cells were then rinsed 3 times with PBS and then blocked with 1:10 dilution of goat serum and 3% bovine serum albumin (BSA) (W/V) in PBS at 4°C for 15 min in the dark. After blocking, the cells were then washed 3 times with PBS and incubated with rabbit anti-mouse GLUT-4 or GLUT-10 antibodies (conjugated with FITC) for 18 h at 4°C in the dark. The cells were subsequently washed 4 times with ice-cold PBS and re-suspended in 300 µl of PBS. At this point, cells were wrapped in foil and stored in the fridge (4-8°C) or taken to the flow cytometer (MilliporeSigma, USA) for immediate data acquisition. Cells treated with insulin were used as positive controls whereas stained untreated cells were used as negative control. The untreated unstained cells were used to normalize and validate the technique.

## 2.7. Indirect Immunofluorescence Microscopy

Approximately 5 000 - 10 000 cells were seeded on ethanol-sterilized cover slips placed in six-well plates. The cells were allowed to grow to confluency on coverslips. The cells were treated with a concentration of 1 mg/ml of water extracts of *C. abbreviata* or insulin (100 nM) for 3 h. Cells were then washed three times in ice-cold PBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.4). Cells were then fixed in 3% (v/v) *p*-formaldehyde for 30 min and quenched in 100 mM glycine for 10 min at 4°C. The fixed cells were blocked with 1:10 dilution of goat serum and 3% bovine serum albumin (BSA) (W/V) in PBS at 4°C for 15 min in the dark and then incubated with rabbit anti-mouse GLUT-4 and GLUT10 antibody solution (antibodies in 1.0 µg/ml in PBS) for 1 h at 4°C in a dark room. Excess antibodies were removed by extensive washing (three times with shaking) in ice-cold PBS. Cell surface-bound GLUT-4 and GLUT10 antibodies were probed by FITC-conjugated secondary antibodies. Cells were visualized under a fluorescent microscope (Olympus, Shinjuku-ku, Tokyo, Japan). Cells treated with insulin were used as positive control and untreated cells as negative controls.

## Reverse Transcriptase Polymerase Chain Reaction

*RNA extraction.* Muscle cells were pelleted up to 1 x $10^7$ cells in an appropriate centrifuge tube by spinning for 5 min at 2500 rpm, following treatment with extracts of *C. abbreviata* leaves, seeds and bark. Total RNA was extracted from the muscle cells according to the method described by the manufacturer (BioFlux) instructions and the purified RNA use immediately or stored at -20°C or at -70°C until use.

cDNA *synthesis.* Complementary DNA (cDNA) was synthesized from each RNA sample (1 µg), using a Revert Aid First Strand cDNA synthesis kit, following the recommendation of the manufacturer (Thermoscientific, Lithuania). The composition of the reaction mixtures for preparation of cDNA included 10 µl total RNA template and 2 µl of Oligo(dT) primer. To this mixture, 4 µl of 5X reaction buffer, 1 µl of Riboblock RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix and 1 µl RevertAid M-MULV reverse transcriptase (200 U/µl) were added. The above mixture was gently mixed, centrifuged and incubated for 60 min at 42°C. The reaction was then terminated by heating at 70°C for 5 min.

*Quantitative real-time polymerase chain reaction (IRS1, PI3 K, Akt1, Akt2, PPAR- γ, GAPDH and GLUT-4).* The cDNA derived from reverse transcription of mRNA was analyzed using a thermal cycler (Applied Biosystems) PCR, by using RNA-direct SYBR Green real time PCR master mix (Accuris qMax Green qPCR Master Mix 2X). The primers used for the amplification of genes are provided in Table 1.

qPCR was set up as follows. Template-cDNA (6.8 µl); 2 X SYBR Green Mix (10 µl), Forward primers (0.8 µl), Reverse primers (0.8 µl), H2O (1.6 µl) to make a total volume of 20 µl. The amplification was initiated by denaturation of cDNA at 95°C for 3 min followed by 30 cycles of 30 s at 95°C, 30 sec at 65°C and 1 min at 72 °C. A 7 min extension step at 72°C completed the PCR run. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

## Enzyme-Linked Immunosorbent Assay for the Measurement of IRS-1 and pAkt Total Proteins

*pAkt.* Phosphorylated Akt (Akt-pS473) was determined using an Enzyme-linked immunosorbent assay (ELISA) kit, following the recommendation of the manufacturer (Elabscience, China).

Confluent cultures of C2C12 skeletal muscle cells grown in a 6 well-plates were treated with 1 mg/ml of specific extracts and insulin solution (100 nM) for 3 h. The cells were repeatedly (3X) washed with pre-warmed PBS and lysed by the addition of cell lysis buffer in which protease and phosphate inhibitors were added. The lysates were

**Table 1.** The Primers Sequences used for the Amplification of IRS1, PI3 K, Akt2, PPAR- γ, GLUT-4, Akt1 and GAPDH.

| Genes | Primers |
|---|---|
| IRS1 | Forward:5'-TATCTGCATGGGTGGCAAGG-3' |
| | Reverse:5'-GGGTAGGCAGGCATCATCTC-3' |
| PI3K | Forward:5'-TGACGCTTTCAAACGCTATC-3' |
| | Reverse:5'-CAGAGAGTACTCTTGCATTC-3' |
| Akt2 | Sense:5'-CGCCCTCTCGGTCGGTCTTCATCAG-3' |
| | Antisense:5'-TTCCAGCCATGAGCTACGTC-3' |
| PPAR-γ | Forward:5'-GGTGAAACTCTGGGATTC-3' |
| | Reverse:5'-CAACCATTGGGTCAGCTCTCTT-3' |
| GLUT-4 | Forward:5'-CAACTGGACCTGTAACTTCATTGT-3' |
| | Reverse:5'-ACGGCAAATAGAAGGAAGACGTA-3' |
| Akt1 | Sense:5'-AAGGCCACAGGCCGCTACTA-3' |
| | Antisense:5'-AAGAAGAGCTCGCCCCGTT-3' |
| GAPDH | Forward:5'-ACCACAGTCCATGCCATCAC-3' |
| | Reverse:5'-TCCACCACCCTGTTGCTGTA-3' |

suspended and incubated with shaking at 8 °C for 30 min and then centrifuged at 13000 rpm for 10 min at 8 °C. The supernatant transferred to clean test tube and used immediately or stored at -70 °C. A volume of 100 µl of each sample or positive control was added into appropriate wells, which were covered with a plate holder and incubated overnight at 4 °C with shaking. The solution was subsequently discarded and the wells washed 4 times with 1 x wash solution. The plate was inverted and blotted against a clean paper towels. An amount of 100 µl of prepared 1000 dilution of rabbit anti-phospho-Akt (Ser473) antibody was added to each well, followed by incubation for 1 h at room temperature with shaking. The solution was then discarded and the wells washed 4 times with wash buffer, followed by an addition of a volume of 100 µl of prepared 1 x horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG to each well and incubated for 1 h at room temperature with shaking. The solution was again discarded and the wells washed 4 times with washed buffer. A volume of 100 µl of TMB one–step substrate reagent was subsequently added to each well and incubated for 30 minutes at room temperature with shaking in the dark. Then, 50 µl of stop solution was added to each well and the plate was read immediately at 450 nm using a microplate reader.

*Insulin receptor subtrate-1*. The expression of insulin receptor substrate-1 was determined using an ELISA-based kit, following the recommendation of the manufacturer (Elabscience, China). Confluent cultures of C2C12 skeletal muscle cells grown in a 6 well-plates were treated with 1 mg/ml of specific extracts or insulin solution (100 nM) for 3 h. The cells were washed 3 x with PBS and lysed by the addition of cell lysis buffer in which protease and phosphate inhibitors were added. The lysates were suspended and incubated with shaking at 8 °C for 30 min and then centrifuged at 13000 rpm for 10 min at 8 °C. The supernatant transferred to clean test tube and used immediately or stored at -70 °C. A volume of 100 µl of standard working solution was added to the first two columns of the plate. Samples (100 µl) were added to the others wells and the plate covered with a sealer and incubated for 90 min at 37 °C. The liquid (samples) were removed from each well and 100 µl of Biotinylated detection Ab working solution added to each well. The plate was again covered with the plate sealer, gently mixed on an orbital shaker for 1 h at 37 °C. The solution from each well was decanted and 350 µl of wash buffer added to each well and solution discarded from each well followed by inversion and blotting against a clean absorbent paper. A volume of 100 µl of HRP conjugate working solution was then added to each well and the plate covered with the plate sealer and incubated for 30 min at 37 °C. The solution was then removed from each well and 350 µl of wash buffer added to each well. This last step was repeated 5 times. Then, 90 µl of substrate reagent was added to each well and covered with a new plate sealer, followed by a further 15 min incubation at 37 °C. Finally, 50 µl of stop solution was added to each well and and $A_{450}$ of each well was read using a microplate reader.

### *Statistical Analysis*

All experiments were conducted in triplicates. All numeric values are represented as the mean ± SD. The statistical significance of differences between data was determined using Tukey's test. The results accepted level of significance was $p < 0.05$.

## Results and Discussion

### *Toxicity Assay*

The toxicity of plant materials must be determined before drug development or consumption.[29] The cytotoxic effect of crude aqueous extracts prepared from the leaves, bark and seeds of *C. abbreviata* were assessed on C2C12 cells using the MTT assay. The results revealed that the aqueous crude extracts from *C. abbreviata* were not toxic to the C2C12 at concentrations used except for the bark extract, which exhibited a slight toxicity (7% toxicity compared to the untreated control cells) at 5 mg/ml. A significant increase in cells viability was observed after treatment with 2.5 mg/ml leave extracts compared to the untreated cells. The aqueous extract of the seeds and bark also maintained cell viability levels higher than the untreated cells in a concentration-dependent manner increasing cells viability to a maximum of 236% and 127%, respectively compared to the untreated cells. The values indicated that the intensity of the formazan in cells treated with extracts of leaves, seeds and bark were 92, 136 and 27% higher than in untreated controls. The positive control, hydrogen peroxide, reduced the cells number to 68% while insulin had no effect on the cell viability (Figure 1). A toxicity study by Cholendra et al. (2014) showed no toxicity in rats after the administration of up to 3200 mg/kg of *Cassia tora*.[30] The same results were obtained in a comparative toxicity study between *Cassia fistula* and *Cassia acutifolia* by Akanmu et al. (2004).[31] The authors found no or very low toxicity on the isolated guinea-pig ileum after treatments with both plant species extracts at a concentration of 6600 mg/kg. Furthermore, Jothy et al. (2011) reported that a dose of 5000 mg/kg of *Cassia fistula* resulted in no mortalities or evidence of adverse effects, implying that *C. fistula* is nontoxic.[32] It is well established that toxicity is concentration dependant.[29] This study however, interestingly found that all plants extracts under study resulted in higher absorbance intensity of the formazan, suggesting that cell viability was maintained higher than that of untreated of cells. Similar pattern was observed with the treatment of 3T3 cells treated with *Kalanchoe brasiliensis* leaf extract (10 µg /ml - 5000 µg/ml).[33]

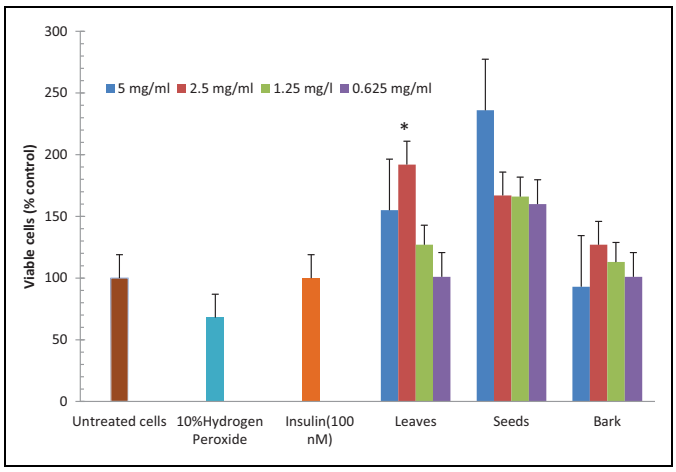


**Figure 1.** Toxicity studies of muscle cells after 24 h exposure to aqueous extracts of leaves, seeds and bark of C. abbreviata. The concentrations of plant extracts used were 5, 2.5, 1.25 and 0.625 mg/ml. Hydrogen peroxide (10%) was used as positive control, while insulin (100 nM) and untreated cells served as negative controls.

This proliferation of cells at low concentrations may be an indication of a possible mitogenic effect or induction of expression of growth-stimulating substances. The 50% cytotoxic concentration (CC50) needed to kill 50% of the cells by all plant extracts exceeded 5000 µg/ml, the highest concentration used. This high CC50 value indicates the relative safety of the plant extract understudy.

The hypoglycaemic activity of *C. abbreviata* leaves, bark and seeds on muscle cells was also studied after the plant extracts were incubated with different concentrations (500 and 1000 µg/ml) for 1, 3 and 6 h. In this part of study, we explored the ability of extracts of selected plant parts to stimulate glucose absorption in muscle (C2C12) cells.

## One Hour Glucose Uptake

*Cassia abbreviata* leaf extract led to the highest stimulation of glucose absorption after 1 h incubation, inducing an uptake of 16% and a significant glucose absorption of 29% (**$p < 0.01$) higher than untreated controls at concentration 500 µg/ml and 1 mg/ml, respectively. *Cassia abbreviata* bark extract was the least performing at 1 h incubation, inducing glucose absorption of 14% higher than controls with 1 mg/ml treatment and failing to induce any glucose uptake at 500 µg/ml concentration. Both concentrations of the seed extract increased glucose absorption by 15% compared to control cells although not significant (Figure 2A). Insulin, on the other hand, stimulated a significant glucose absorption of 46% (**$p < 0.01$) compared to the untreated cells. In a study by Silawat et al. (2009), the assessment of the hypoglycaemic activity of *Cassia fistula* leaf extract on diabetic rats revealed a reduction in blood glucose level at 200 mg/ml to 9.82% compared to 5.43% of acarbose treated rats and 17.55% of normal rats.[34]

## Three Hour Glucose Uptake

We also investigated the ability of the selected plant extracts to stimulate glucose uptake by muscle cells after 3 h treatments. A similar trend, as after 1 h treatment, was observed after 3 h treatment period, with the highest recorded glucose absorption increase of 56% (**$p < 0.01$) above untreated controls resulting from treatment of cells with leaf extract of *C. abbreviata*. *Cassia abbreviata* bark extract, as was the case after 1 h incubation, exerted the lowest effect on glucose uptake although significant (**$p < 0.01$) at both concentrations tested, stimulating glucose uptake of 44% and 47% higher than controls at 500 µg/ml and 1 mg/ml, while the seed extract came a close second after the leaf extract. The higher concentration of bark extract led to 51% significant increase in glucose uptake, while 500 µg/ml resulted in a 46% increase. Insulin, on the other hand, stimulated a significant increase (**$p < 0.01$) of glucose absorption of up to 51% compared to the untreated cells (Figure 2B).

## Six Hour Glucose Uptake

Figure 2C shows glucose consumption by the muscle cells after 6 h exposure to *C. abbreviata* extracts. Extracts of leaves, seeds and bark enhanced glucose uptake in a dose-dependent manner, with treatment with 1 mg/ml inducing more glucose utilization than 500 µg/ml, although both had a diminished effect than that observed at 3 h. The extract of leaves was still the best performing with a significant (**$p < 0.01$) glucose absorption of 42% and 41% higher than controls after treatment with 500 µg/ml and 1 mg/ml of the plant extract, followed by the seed extract with a significant (**$p < 0.01$) glucose uptake of 33% and 29% higher than controls at 500 µg/ml and 1 mg/ml treatment, respectively. Insulin increased glucose uptake by 37% (**$p < 0.01$) compared to the untreated cells. Similar glucose absorption patterns were demonstrated by a study conducted by Seabi et al. (2016) on the extracts of *C. abbreviata* bark, which revealed a dose-dependent glucose uptake by C2C12 cells, with increase by up to 23% and 31% of glucose absorption at 50 µg/ml and 100 µg/ml, respectively after 3 hour incubation.[14] Furthermore, a recent study by Bati et al. (2017) confirmed the anti-diabetic effects of *C. abbreviata* ethanolic bark extract on diabetic rats.[35] Moreover, Shalaby et al. (2014) indicated that the administration of cinnamomum cassia extract in alloxan induced obese diabetic Sprague Dawley rats induced a significant reduction in blood glucose level. The authors attributed the hypoglycaemic effect of the plant to compounds identified as trimers and tetramers of the flavonoids, catechin and epicatechin.[36] Results indicate that all extracts effectively stimulated glucose uptake at all times studied. Observations from this study may suggest that the peak activity of plant extracts under study was reached after a 3 h exposure of skeletal muscle cells. The diminished glucose consumption observed after 6 h incubation may most probably be due to the fact that active ingredients lose their activity over time.

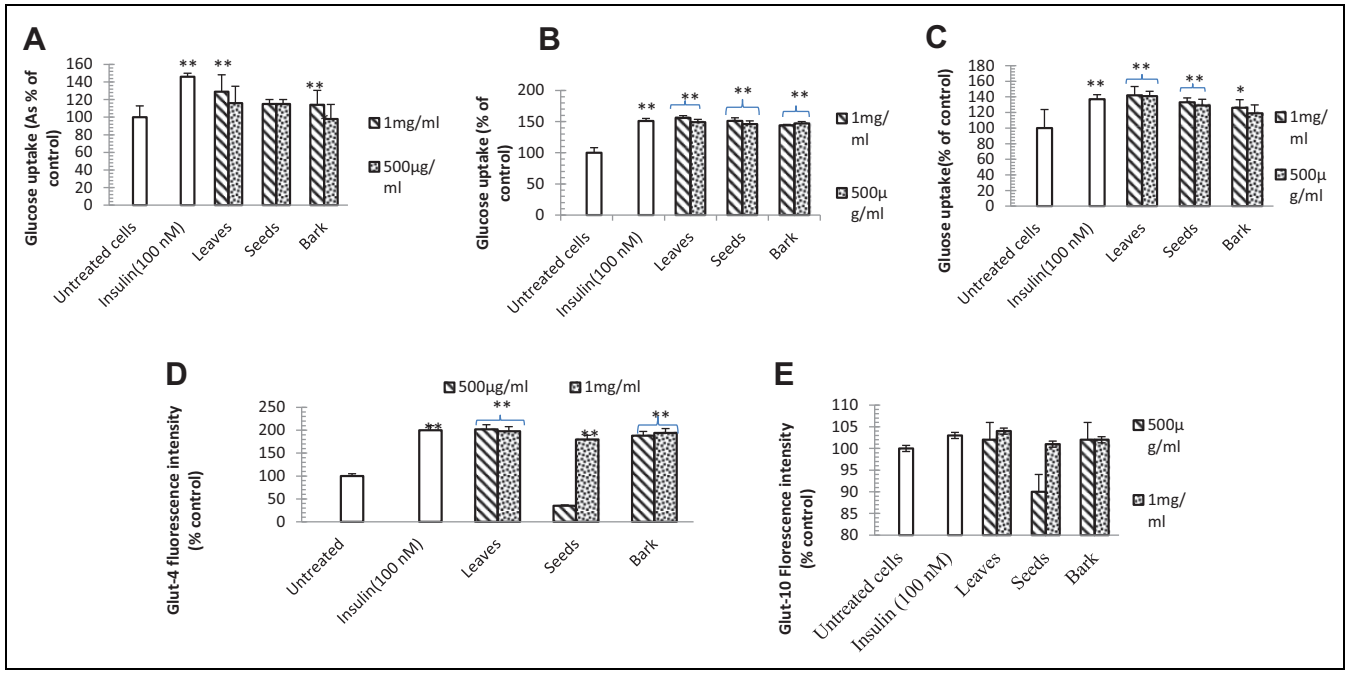


**Figure 2.** Hypoglycemic activity of C. abbreviata leaves, bark and seeds on muscle cells after 1 h (A), 3 h (B) and 6 h (C) incubation and the quantitative analysis of GLUT-4 (D) and GLUT-10 (E) membrane content in muscle cells after treatment with aqueous extracts of Cassia abbreviata. Note: Insulin (100 nM) was used as positive control while untreated unstained cells were used as negative controls. C2C12 were treated with indicated concentrations.

## *Quantitative GLUT-4 and GLUT10 Translocation*

It has been established that glucose absorption in the muscle cells depends largely on GLUT-4 translocation from the cytoplasm to the cell membrane.[37,38] Therefore, we explored the ability of leaf, bark and seed extracts of *C. abbreviata* to stimulate the translocation of GLUT-4 in the muscle cells, in order to explain part of the mechanism involved in the observed glucose uptake stimulation by extracts of *C. abbreviata*. Flow cytometry data demonstrated that all tested extracts at the exception of the seeds extract (500 µg/ml) stimulated GLUT-4 translocation to at least two-fold (**$p < 0.01$) higher than the negative controls. Insulin also induced a significant (**$p < 0.01$) increase (2-fold) in GLUT-4 translocation in comparison to the untreated controls. These results denote a steady contribution of GLUT-4 translocation in the mechanism of glucose absorption and that glucose uptake induced by extracts of *C. abbreviata* may primarily depend of GLUT-4 translocation (Figure 2D). The results may imply that the extracts of the selected plants have active ingredients targeting the upstream events controlling GLUT-4 translocation, thereby contributing to the hypoglycaemic effects.[39] This finding is in line with a study conducted by Kadan et al. (2018), who reported that *Teucrium polium* enhanced translocation of GLUT-4 in muscle cells (L6) in a concentration and time dependent manner when compared to the negative control and insulin-treated controls.[20] Another study reported that *Salacia oblonga* stimulated GLUT-4 translocation in L6 myocytes and 3T3 adipocytes.[40] A separate study by Shen et al. (2014) revealed that cinnamon cassia extract improves type 2 diabetes by inducing GLUT4 translocation via the AMPK signaling pathway in C2C12 myocytes.[41] This study therefore concludes that the observed increase in glucose uptake was associated with GLUT-4 translocation in C2C12 cells.

A previous study by Dawson et al. (2001) established the ability of GLUT-10 to transport glucose in the liver, muscle and pancreas.[42] Recent work by Evans et al. (2019) in the plantaris muscle of both wild-type and muscle-specific GLUT-4 knockout mice demonstrated an increase of GLUT-10 expression following a functional overload-induced muscle glucose transport.[43] The contribution of GLUT-10 to the overall increase in glucose was studied. All plant extracts at both concentrations (500 µg/ml and 1 mg/ml) showed a marginal increase in translocation in the muscle cells varying from 1 to 4% except for *C. abbreviata* seeds which did not stimulate any translocation at 500 µg/ml. Insulin also induced a negligible enhancement of translocation (3%) compared to the untreated controls (Figure 2E). These observations confirm that GLUT-10, although present in the muscle cells, has a lesser involvement in the mechanism of glucose transport in the muscle cells. These results may also suggest a reduced number of GLUT-10 in the muscle cells.

## *Qualitative GLUT4 Translocation*

The indirect immunofluorescence technique was used to confirm the flow cytometry data and determined the localization of GLUT-4 in the cells treated with the extracts of *C. abbreviata*

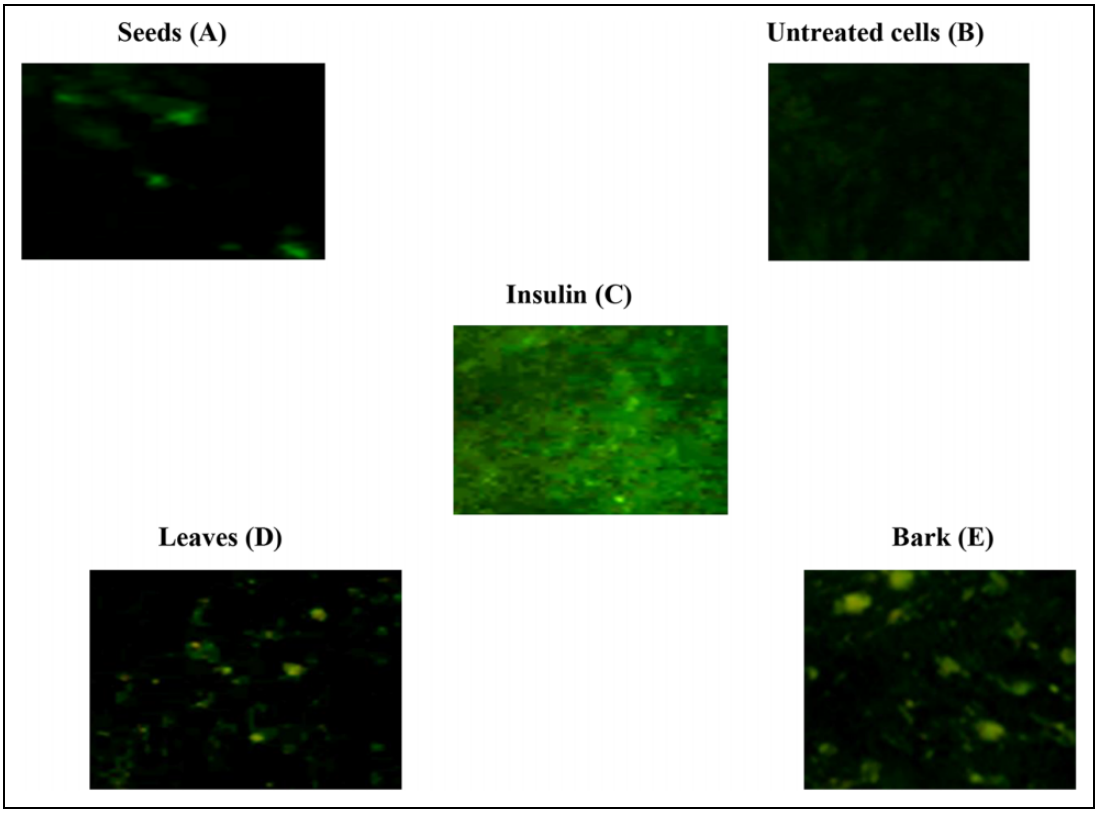


**Figure 3.** The confocal images of C2C12 muscle cells incubated with aqueous extracts (1 mg/ml) of Cassia abbreviata or insulin (100 nM).

and insulin. This technique uses a primary antibody specific for GLUT4 molecule, then staining with a FITC-conjugated secondary antibody or a primary antibody tagged with FITC-conjugated secondary antibodies to detect the areas to which the primary antibody had bound.[44,45] The green fluorescence indicated the interaction of the surface protein (GLUT-4) and the FITC-linked anti-GLUT-4 antibody. Fluorescence microscopy revealed pronounced GLUT-4 translocation in cells treated with *C. abbreviata* extracts and insulin than untreated cells (Figure 3), and thus confirms the flow cytometric results.

## *Gene expression of IRS1, PI3 K, Akt1, Akt2, PPAR- γ and GLUT-4*

The translocation of GLUT-4 is preceded by events that occur as a result of insulin binding to its specific receptor. The translocation of GLUT-4 requires the activation of insulin receptor substrate (IRS1), phosphoinositide 3-kinase (PI3 K), protein kinase B (PKB), also known as Akt. To ascertain whether the observed requires either the enhancement of expression of these key genes or activation of already existing protein, we determined the expression of these genes at mRNA level. We found downregulation of GLUT-4, IRS1, and PI3 K by the extracts of *C. abbreviata* leaves and seeds. On the other hand, the levels of GLUT-4 mRNA were significantly upregulated by the action of insulin (**$p < 0.01$) and bark extract (**$p < 0.01$). IRS1 and PI3 K mRNA levels were also increased by the action of insulin and bark extract. The same conclusion was reached by Zhao et al. (2019) who reported an increase mRNA expression level of GLUT-4, following a treatment of L6 with neferine, an alkaloid derived from lotus seeds and insulin.[46] There was an upregulation of Akt1, Akt2 and peroxisome proliferator–activated receptor γ (PPAR-γ) in C2C12 cells treated with leaf and seed extracts (Figure 4). The expression of the house keeping gene GAPDH remained uniform in all treatments. Recent studies by Cai et al. (2018) established that a concoction composed of *Folium mori*, *Momordica charantia* and *Rhizoma dioscorea* increased the expression of IRS1, IRS2, PI3 K, Akt1 and GLUT-4 in the liver cells of diabetic rats, suggesting the activation of the PI3K/Akt pathway.[47] Yoshitomi et al. (2017) indicated that the extracts of *Cyclocarya paliurus* stimulated glucose absorption and GLUT-4 translocation by activating the IRS, PI3 K, Akt and AS160 in an insulin-independent manner.[48] Prabhakar and Doble (2011) established that *Berberis aristata* enhances the translocation of GLUT-4 through the AMPK pathways.[49] Many plant extracts exert their hypoglycaemic effects by mimicking insulin on glucose metabolism.[50,16] Based on the results obtained, we suggest that the hypoglycaemic activity of *C. abbreviata* leaf and seed extracts involve the enhancement of glucose uptake in cells via GLUT-4 translocation without the need for synthesis of new proteins in the PI3K/Akt pathway. The mechanism may involve the activation of already existing proteins in the pathway or an alternative method involving enhancement GLUT-4

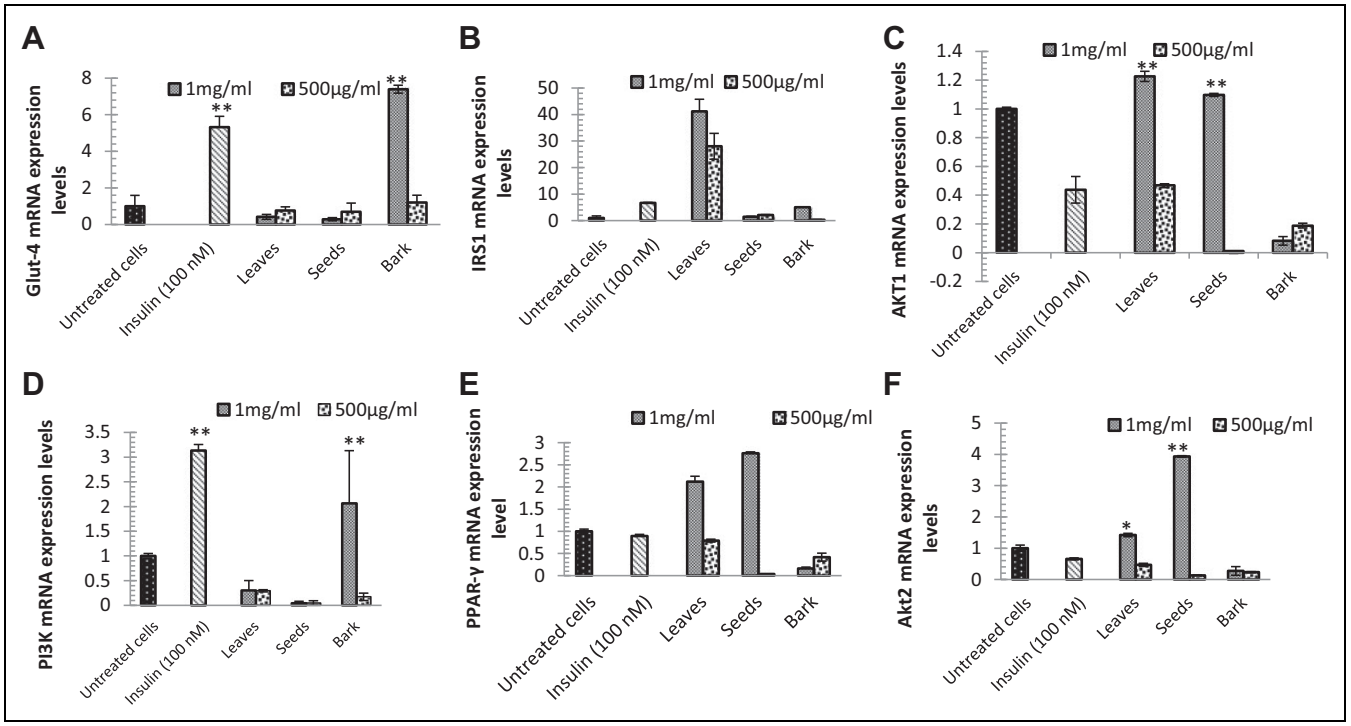


**Figure 4.** Expression levels of IRS1, PI3K, Akt1, Akt2, PPAR- γ and GLUT-4 after treatment of C2C12 cells with the extracts of Cassia abbreviata leaves, seeds and stem bark.

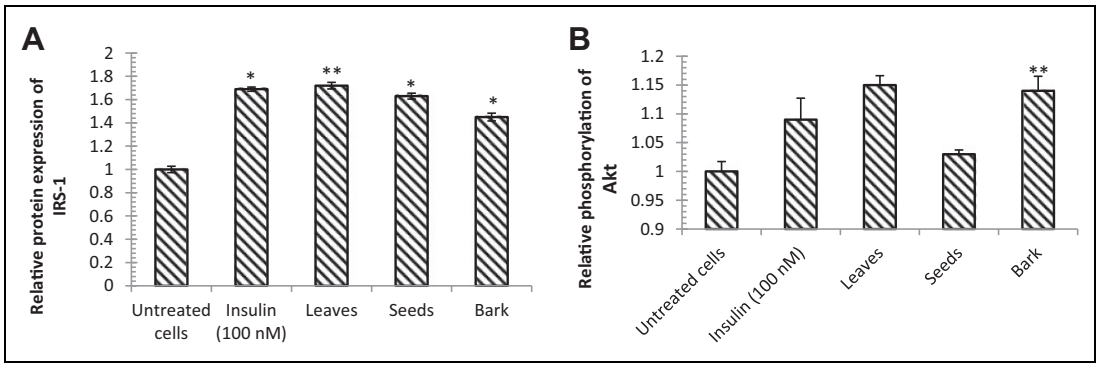


**Figure 5.** The effects of extracts of C. abbreviata and insulin on the level of insulin receptor substrate 1 (IRS1) (A) and the amount of phosphorylated Akt (pAkt-S473) (B) in cells treated with aqueous extracts of leaves, seeds and stem bark of C. abbreviata, as well as 100 nM insulin.

translocation. It was also interesting to notice that the extract of the leaves, like insulin, led to a concentration-dependent upregulation of IRS-1, suggesting that the extract may exert its hypoglycaemic effects through enhancing expression of IRS-1. The involvement of the AMPK pathways in plant extract-stimulated GLUT-4 translocation cannot be ruled out, and thus will be the focus of future studies.

We further examined the mechanism of the extracts of *C. abbreviata* in the regulation of insulin signal transduction. As shown in Figure 5A, the expression of IRS-1 was significantly increased (**$p < 0.01$) following treatment with leaf, seed and bark extracts. Insulin, on the other hand, also significantly (*$p < 0.05$) increased the protein expression of IRS-1 although to a lesser extent.

The amount of phosphorylated AKT (pS473) was upregulated by insulin and all selected extracts of *C. abbreviata* with the extract of C. *abbreviata* bark showing a significant (**$p < 0.01$) increase of phosphorylated AKT (pS473). The extract of the seeds was the least active in increasing the amount of phosphorylated AKT (Figure 5B).

These results correlate with findings by Tian et al. (2017), which established that IRS-1, Akt and GLUT4 proteins were significantly reduced in T2DM rats compared to the control group. However, following treatment of the rats with

Wushenziye, a Chinese medicinal cocktail used in the treatment of T2DM, the protein expression of the insulin signaling molecules, viz, IRS-1, Akt and GLUT4 was increased, resulting in improved fasting blood glucose in the animals.[51] Moreover, a study by Li et al. (2016) demonstrated that Simvastatin, a 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor, inhibited glucose absorption and GLUT4 translocation in C2C12.[52] This reduction of glucose uptake was attributed to the suppression of the phosphorylation of IRS-1, Akt and total expression of IRS-1 although no change was observed in Akt at protein level. This highlights the importance of IRS protein expression in carbohydrate metabolism.

Akt or protein kinase B (PKB) plays a vital role in glucose and oxidative metabolism downstream of the insulin signaling pathways and exhibit altered responses among insulin-sensitive targets such as skeletal muscle cells and adipocytes.[53,54] Its activation is influenced by the activation of IRS and PI3 K.

The activation of Akt is directly linked to the increase translocation of GLUT4 from the intracellular pool to the cell membrane in muscle cells and adipocytes.[55] All selected *C. abbreviata* extracts stimulated an increase in cellular concentrations of IRS-1 and phosphorylated Akt. It is therefore not surprising that treatment of the cells with selected plant extracts resulted in increase in glucose consumption by the muscle cells. These results also suggest that the hypoglycaemic effect of C. leaves, bark and seeds may be mediated through the regulation of IRS-1/PI3K/Akt pathway. Whether the translocation of GLUT-4, the increase in the levels of IRS-1 or the phosphorylation of Akt on serine-473 as a result of treatment of cells with extracts of *Cassia abbreviata* involves an insulin mimetic mechanism involving the insulin receptor or the AMPK pathway is the subject of future research. Some evidence-based reports suggest that agonists of AMPK can also stimulate the activation of IRS-1, PI3 K and Akt similarly to the activation of these proteins in a mechanism that involves both insulin and insulin receptor.[56-58]

## Conclusions

This study revealed the hypoglycaemic property of aqueous extracts of leaves, bark and seeds of *Cassia abbreviata.* all plant extracts stimulated glucose uptake across time at both concentrations tested. Cytotoxicity tests revealed that all extracts tested at various concentrations were non-toxic ($LC_{50} > 5000$) indicating the relative safety of the plant studied. The use of both quantitative and qualitative methods confirmed that GLUT-4 translocation was enhanced by treatment of cells with extracts of the plant species. GLUT-10 contributes in a lesser extend in the mechanism of glucose transport in the muscle cells. The study revealed that, at gene expression level, the extracts of *C. abbreviata* bark displayed the same pattern as insulin implying it is insulin mimetic, as confirmed by the enhancement of glucose uptake by cells. Further studies are necessary to determine the effects of the extracts on proteins playing a significant role in glucose homeostasis. Our findings require further detailed studies which will allow for an unequivocal pronouncement conclusion on the actual mechanism of action of these plant extracts. We confirm the plant parts understudy can serve as therapeutic agent and can be used as potential sources of new bioactive compounds for the treatment and management of type 2 diabetes mellitus.

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### Author Contribution and Role

Conceptualization: LJ Shai and FDY Kamga-Simo-III Methodology: FDY Kamga-Simo-III. Software: LJ Shai. Validation: LJ Shai FDY Kamga-Simo-II and GP Kamatou. Formal Analysis: FDY Kamga-Simo-III. Investigation: FDY Kamga-Simo-III. Resources: LJ Shai and GP Kamatou, C Ssemakalu. Data Curation: LJ Shai. Writing – Original Draft: FDY Kamga-Simo-III. Writing – Review & Editing: FDY Kamga-Simo-III, GP Kamatou and LJ Shai C Ssemakalu. Visualization: C Ssemakalu and FDY Kamga-Simo-III. Supervision: LJ Shai and GP Kamatou. Project Administration: LJ Shai. Funding Acquisition: LJ Shai.

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### Supplemental Material

Supplemental material for this article is available online.

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