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Research Article

Effects of *Cassia abbreviata* Oliv. and *Helinus integrifolius* (Lam.) Kuntze on Glucose Uptake, Glut-4 Expression and Translocation in Muscle (C2C12 Mouse Myoblasts) Cells

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ABSTRACT
Background: Herbal remedies have been used to successfully manage diabetes mellitus. However, the underlying mechanisms through which these remedies are able to manage diabetes mellitus are not well understood. Aim of the study: The aim of the study was to investigate the effects of *Cassia abbreviata* (stem bark) and *Helinus integrifolius* (leaves) water extracts on glucose absorption and expression of glucose transporters (GLUTs) 1 and 4 by muscle cell lines. Materials and Methods: Cells were treated with water extracts of both plants, and then incubated at 37 ºC and 5% CO_2 for 3 h and 24 h. Glucose uptake by cells was determined in the presence and absence of extracts of *Cassia abbreviata* and *Helinus integrifolius*. GLUT1 and GLUT4 mRNA levels were determined by PCR. GLUT4 translocation was determined using immunofluorescence microscopy and flow cytometry. Results and Discussion: A concentration-dependent increase in glucose uptake was observed in cells stimulated with *Cassia abbreviata* crude water extracts. Extracts of both plant species induced an increase in the translocation of GLUT4 in muscle cells, as evidenced by increased fluorescence intensity measured using flow cytometry. Treatment of cells with extracts of both plants did not affect GLUT1 and GLUT4 mRNA levels after a 3 h incubation period. However, 25 µg/ml led to down-regulation of GLUT1 mRNA levels after 24 h incubation. Conclusions: *Cassia abbreviata* increased the glucose uptake potential of cells, induced an increase the translocation of GLUT4 in muscle cells and also stimulated the expression of the GLUT1 gene. On the other `hand, *Helinus integrifolius* did not induce the absorption of glucose by muscle cells, but an increase in the translocation of GLUT4 was observed.

Keywords: Cassia abbreviata; Helinus integrifolius; Glucose uptake, GLUT4, GLUT1, diabetes

INTRODUCTION

Since ancient time, mankind has explored natural resources as sources of new drugs, and has largely depended on his environment for solutions to his health problems. The search for remedies, probably through trial and error, resulted in the use of large number of medicinal plants with curative properties to treat various diseases[1,2]. Plants are natural reservoirs of secondary compounds with therapeutic potential in primary healthcare, as supported by a large number of modern drugs derived from natural resources[3]. The consumption of plant-derived materials as alternative medicine persists, driven by the fact that they are cheap with minimal side effects and may significantly contribute to the improvement of human health in terms of cure and prevention of disease[2]. Chauke et al. (2014)[4] published an outline of plants used treat a variety of diseases, including diabetes mellitus, by traditional healers in the community of Mashishimale village, Phalaborwa.

Diabetes mellitus is a metabolic disease of epidemic proportions, with cases estimated at approximately 150 million worldwide. The number is projected to reach about 300 million in 30 years, driven by parallel epidemic of obesity[5]. The conservative South African estimate of the prevalence of diabetes mellitus is at 6.5% of the adult population aged between 20 and 79 years of age. Age-adjusted prevalences of up to 13% have been described in urban populations as far back as 1994. The effects of urbanisation and the prevailing unhealthy lifestyle among urban area dwellers are important contributors to the rising prevalence of obesity and type II diabetes mellitus. The 2003 Demographic and Health Survey reported that as many as 30% of South African populace is overweight or obese[6]. Type 2 diabetes is the predominant form of the diseases, which continues to rise in developing countries such as South Africa. Apart from microvascular complications, cardiovascular disease and associated

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morbidity and mortality are on the rise in people suffering type 2 diabetes mellitus[7,8]. Hyperglycaemia persists in patients suffering from diabetes mellitus, due to inability of target cells such as skeletal muscle cells, adipocytes and liver cells to effectively absorb glucose from blood circulation. Under normal circumstances, glucose transport activity in muscle cells increases 3-4 fold following insulin stimulation, and a large part of this effect is associated with a net translocation of the glucose transporter 4 (GLUT4) from intracellular compartment to the cell surface, where it facilitates the uptake and reduction of plasma glucose[9,10]. GLUT 4 is expressed primarily in muscle and fat cells, and usually located in complex intracellular tubule-vesicular network connected to the endosomal-trans-Golgi network (TGN) system[11]. A number of studies have indicated that GLUT4 has a crucial role to play during glucose homeostasis. Firstly, insulin stimulated glucose transport, mediated via GLUT4, is an important regulatory step for glucose metabolism in both muscle and fat cells. Secondly, the disruption of GLUT4 expression results in insulin resistance, setting the scene for development of type 2 diabetes mellitus[11]). Therefore, glucose transport is severely disrupted in type 2 diabetes. Analysing the molecular and cellular regulation of GLUT4 mediated-glucose transport may provide targets for therapeutic drugs in the management of diabetes mellitus[11]. Some of these anti-diabetic drugs may come from plant-derived remedies. The use of plant-derived remedies to treat and manage diabetes mellitus is widespread in South Africa. The mechanisms through which these remedies exert their anti-diabetic activities are not fully understood. The study was aimed at investigating the effects of extracts of two plant species, *Cassia abbreviata* Oliv. and *Helinus integrifolius* (Lam.) Kuntze, used to treat diabetes in South Africa[4] on glucose absorption, GLUT4 translocation as well as expression of GLUT4 and GLUT1 mRNA by C2C12 muscle cells. Chauke et al. (2014)[4] listed these plants as used in traditional preparations for treatment of diabetes and other diseased by healers and herbalists of Mashishimale Village, Phalaborwa.

MATERIAL AND METHODS

Plant collection

The stem bark of *Cassia abbreviata* (Molomana in Se-Phalaborwa; Sjambok pod in English) and the leaves of *Helinus integrifolius* (Morakane in Se-Phalaborwa; Soap creeper in English) were collected from Mashishimale village near Phalabowa, Limpopo Province of South Africa in March 2013. Identification was done with assistance of Magda Nel, a botanist at H.G.W.J. Schweickerdt Herbarium at the University of Pretoria, where voucher specimens were deposited. The unique identification numbers were PRU113819.0 for *Cassia abbreviata* and PRU117192 for *Helinus integrifolius.*

Preparation of the plant crude extracts

The plant material was ground to powder using a laboratory mill (Polymix PX-MFC 90D by Kinematica). This was followed by soaking 5 g of the respective powdered material in water, with intermittent shaking at room temperature for 24 hrs. The different plant crude extracts were then filtered using Whitman no. 1 filter papers and the filtrate was frozen at -80ºC for 24 hours and thereafter lyophilized (Advantage plus, from SP Scientific). The resulting extracts were dissolved in water to a concentration of 10 mg/ml.

Cell culture

The following cell lines were cultured: C2C12 muscle cells (ATCC, Manassas, VA, USA). They were grown and maintained in DMEM supplemented with 10% FBS (fetal bovine serum) and Penicillin-streptomycin (10,000U/ml penicillin G and10 mg/ml streptomycin) at 37^{O}C in 5% CO_2 incubator (Thermo electron corporation, USA)[12,13,14]. The culture medium was replaced with fresh medium every 2-3 days until cells were 80% confluent.

Treatment of cells with plant extracts

The cells were trypsinized and sub-cultured in 6 or 96 well plates at a density of 1×10^5 cells/ml[12,15]. After confluence, cells were treated with different concentrations of extracts of *Cassia abbreviata* and *Helinus integrifolius*, insulin and metformin. C2C12 cells were treated when fully differentiated into myotubules. Differentiation of C2C12 cells into myotubes was achieved by incubation of cells at 37ºC without a change of growth medium for 7 days.

Glucose uptake

About 1.0 x 10^5 cells/ml of C2C12 muscle were seeded in 96 well micro-titre plates and incubated at 37^{O}C in 5% CO_2. Cells were maintained in DMEM supplemented with 10% FBS and antibiotics. The cells were incubated for four days to allow time to reach confluency. C2C12 cells were only treated when fully differentiated into myotubules (after ± 7 days incubation without changing growth medium). The growth medium was removed by aspiration and replaced with fresh medium containing plant extracts at different concentrations. The cells were incubated for a further 3 and 24 h, followed by determination of glucose in the medium. Glucose in medium (25 µl) was determined using glucose oxidase-based assay kit (KAT Medicals). Insulin was used as a positive control, while untreated controls were used as negative controls. Extract-stimulated glucose uptake was determined by measuring glucose concentration in media in the presence and absence of plant extracts, and calculating the difference of the mean of triplicate of samples[16]. The results were expressed as amount of glucose (*mmol/L*) remaining in medium after incubation in presence of test drug (plant water extracts *Cassia abbreviata* and *Helinus integrifolius*). Glucose uptake was calculated using the formula:

$$\% \text{ Glucose uptake} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) x\ 100$$

GLUT4 Translocation

Flow cytometry

GLUT4 level at the cell surface of non-permeabilized C2C12 myotubes was measured through the use of a flow cytometer. Following the stimulation experiments the cells were washed in ice-cold PBS and then blocked with anti-mouse CD16/CD32 (clone 93) and placed on ice for 15 minutes. There after the cells were washed and dispensed in tubes at a concentration of 5 x 10^5 cells in 100 µl of FACS buffer containing the dyes. The cells were then

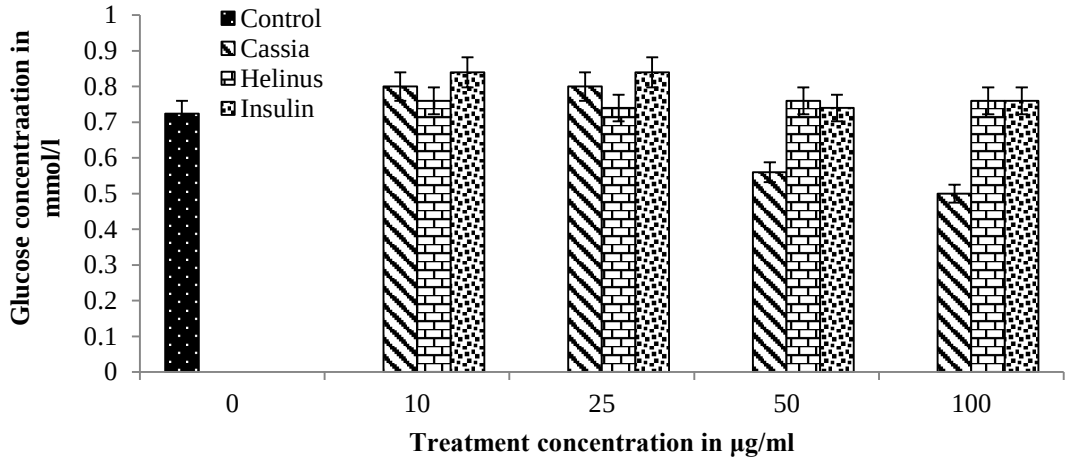


Figure 1: Glucose concentration in medium treated for 3 h with *Cassia abbreviata, Helinus integrifolius* extracts as well as insulin.

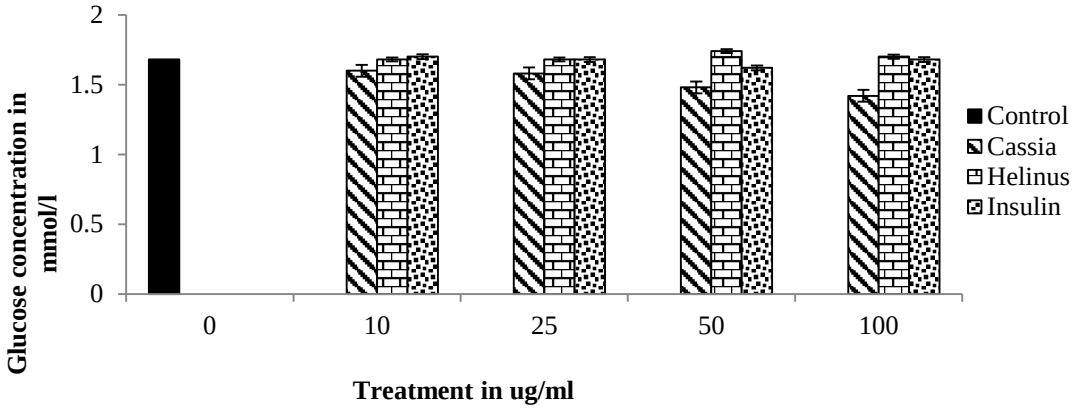


Figure 2: Glucose concentration in medium after a 24 h treatment with *Cassia abbreviata, Helinus integrifolius* and insulin.

stained with a FITC-conjugated rabbit anti-mouse GLUT4 antibody for 18 h at 4ºC. After staining, the cells were washed twice, re-suspended in 0.3ml of FACS buffer, and then subjected to analysis using a flow cytometer (MACSQuant Analyzer (MACS) Miltenyi Biotec, USA), in which 10,000 events where acquired for each setup. Positive staining was determined based on a biological comparison control consisting of stained untreated cells.

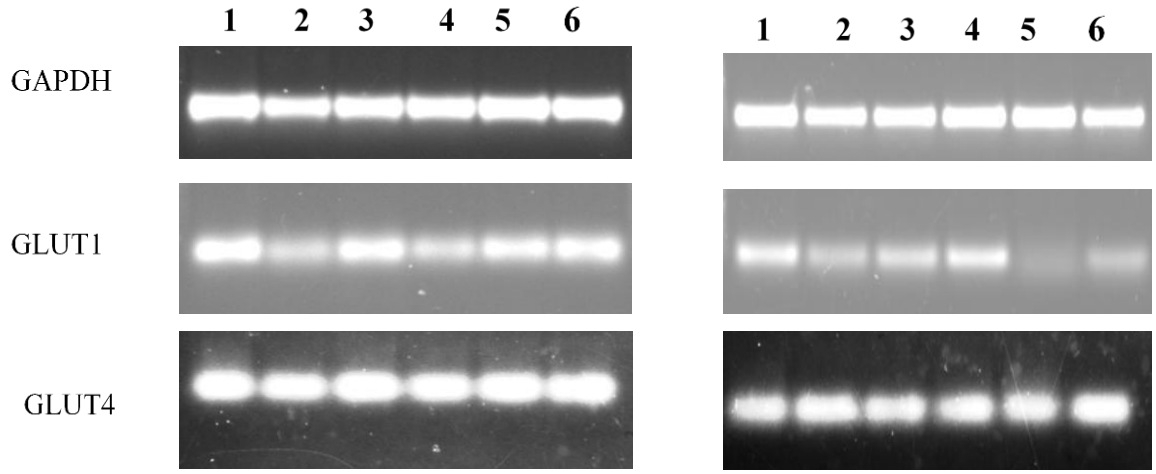
Fluorescence microscopy

C2C12 muscle cells were cultured for 5-7 days on microscope coverslips immersed in DMEM in 6-well plates. Cells were then fixed with 3% paraformaldehyde for 1 h, and then washed with ice cold PBS pH7.4. Cells were then for 1 h with 1% BSA (in TBS Tween) and thereafter washed with PBS, and then incubated with rabbit anti-mouse GLUT4 monoclonal antibody (1:500 dilution) for 1h at 4ºC in the dark[17]. The cells were subsequently incubated with FITC-conjugated goat anti-rabbit secondary antibody for 1.5 h at 4ºC. After washing, cells were mounted on microscope slides with a mounting solution and visualized under fluorescent microscope[18].

GLUT1 and GLUT4 expression

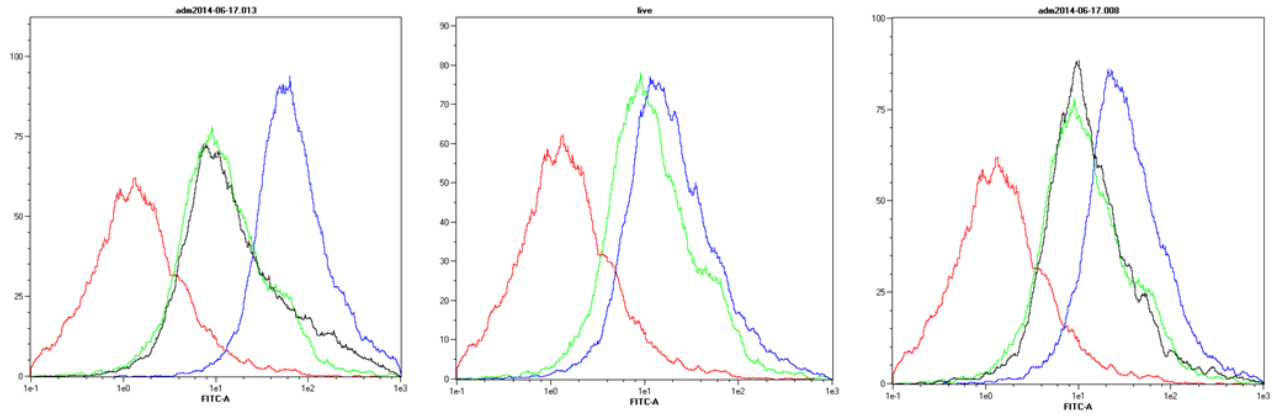
RNA extraction and cDNA synthesis

Isolation of total RNA from C2C12 cells was performed using an RNA extraction kit (ThermoFischer), according to the manufacturer's instructions. Cells were treated with C*assia abbreviata* and *Helinus integrifolius* extracts for 3 and 24 h before isolation of RNA. Cells were harvested by centrifugation (Beckman TJ-6) at 250 x g for 5 min. Thereafter the cell pellet was washed twice with ice-cold PBS, pH7.4. The cell pellet was resuspended in 600 µl lysis buffer supplemented with β-mercaptoethanol, followed by vortex mixing for 10 s. 360 µl of ethanol was added to cell lysate, and mixed by aspiration. The lysate (700 µl) was transferred onto the RNA purification column inserted in a collection tube. The column contents were subjected to centrifugation for 1 min at 12 000 x g. The flow-through was discarded. The purification column was washed with wash buffers 1 and 2, followed by centrifugation at 12 000 x g for 1 min, with the flow-through discarded. RNA was eluted with 50 µl nuclease-free sterile deionised water, followed by centrifugation at 12 000 x g for 1 min. The elute RNA was stored at -20 or -70ºC. RNA concentration was determined using Qubit 2.0 Fluorometer (Invitrogen life technologies, Singapore) according to the manufacturer instructions. 0.5 µg RNA was reverse transcribed to cDNA using the cDNA synthesis kit (Thermo Scientific), according to the manufacturer's instructions. The cDNA reaction mixture contained 10 µl template RNA, 2 µl oligo d(T) primer, 12 µl nuclease-free



(A) (B)

Figure 3: Agarose gel electrophoresis of PCR amplicons for GLUT1, GLUT4 and GAPDH cDNA, derived from total RNA isolated 3 and 24 h after treatment with insulin and extracts of *Cassia abbreviata* and *Helinus integrifolius.* Lane 1, 25 µg/ml insulin; lane 2, untreated control; lane 3, 50 µg/ml *Cassia abbreviata*; lane 4, 100 µg/ml *Cassia abbreviata;* lane 5, 25 µg/ml *Helinus integrifolius*; lane 6, 50 µg/ml *Helinus integrifolius*; MW, molecular weight markers.



(A) (B) (C)

Figure 4: Flow cytometry results showing GLUT-4 translocation in muscle cells after exposure to Cassia abbreviata, Helinus integrifolius and insulin. A: red, untreated unstained cells; untreated FITC-stained; black, 25 µg/ml Helinus integrifolius; blue, 50 µg/ml Helinus integrifolius. B: red, untreated unstained; green, untreated FITC-stained; blue, insulin-treated. C: Red, untreated unstained controls; green, untreated FITC-stained; black, 25 µg/ml Cassia abbreviata; blue, 50 µg/ml Cassia abbreviata.

deionised water, 4 µl 5x reaction buffer, 1 µl RibobLock RNase inhibitor and 1 µl MuLV reverse transcriptase. The reaction was performed at 42ºC for 60 min, and terminated by heating at 70ºC for 5 min. The resulting cDNA was stored at -70ºC until needed.

Polymerase Chain Reaction (PCR)

The PCR reaction mixture contained 2 µl of forward and reverse primers (0.4 µM each), 5 µl of template cDNA, 16 µl of nuclease-free sterile deionised water and 25 µl of 2x ReadyMix (Kapa Biosystems, USA). The PCR reaction consisted of 30 cycles of a 90 s denaturation at 95ºC, primer annealing at 59ºC for 30 s and elongation for 1 min at 72ºC. A final extension at 72ºC for 7 min was done. PCR products were analysed on 2% agarose gel electrophoresis at 75 V for at least 1 h at room temperature. The gel was viewed and band intensities estimated using Chemdoc (Bio-Rad). Forward and reverse primers complementary to GLUT 1, GLUT 4 and GAPDH are outlined in Table 1 below.

RESULTS AND DISCUSSION

Mechanisms of glucose control are varied, depending on the anti-diabetic drug used. Some of the actions of drugs include 1) stimulation of insulin secretion or synthesis by the pancreatic beta cells, 2) regeneration of damaged pancreatic beta cells, 3) improvement of insulin sensitivity (increase glucose uptake by fat and muscle cells), 4) mimicking the action of insulin (acting like insulin), 5) alteration of the activity of some of the enzymes involved in glucose metabolism and slowing down the absorption of sugars from the gut[19].

Glucose uptake

Cassia abbreviata facilitated glucose uptake in C2C12 muscle cells in a concentration-dependent manner. The

Table 1: Forward and reverse primer sequences used to amplify GLUT1, GLUT4 and GAPDH cDNA (Montel-Hegan et al., 2008).

Gene	Primer	PCR product size
GLUT1	Forward: GCTGTGCTTATGGGCTTCTC	114 bp
	Reverse: CACATACATGGGCACAAAGC	
GLUT 4	Forward: ACATACCTGACAGGGCAAGG	152 bp
	Reverse: CGCCCTTAGTTGGTCAGAAG	
GAPDH	Forward: ACTTTGGCATTGTGGAAGG	223 bp
	Reverse: ACACATTGGGGGTAGGAACA	

lowest amount of glucose remaining in growth medium was observed after incubation of C2C12 cells for 3 h in the presence of 100 µg/ml *Cassia abbreviata* extract, as indicated by the least amount of glucose in medium (Fig. 1). The calculation of % utilised glucose shows that *Cassia abbreviata* induced a dose -dependent increase in glucose utilisation by the muscle cells after three hours of incubation. In cells treated with 50 µg/ml and 100 µg/ml *Cassia abbreviata*, 23% and 31% increase in glucose utilisation was observed. Insulin (positive control) and *Helinus integrifolius* had no effect on glucose utilisation by the muscle cells after 3 h incubation period (Fig. 1). The trend persisted after a 24 h incubation period, with *Cassia abbreviata* enhancing glucose uptake by C2C12 cells in dose-depndent manner (Fig 2). The highest increase (15%) in glucose utilisation was observed in cells treated with 100 µg/ml *Cassia abbreviata. Helinus integrifolius* did not stimulate any increase in glucose uptake by C2C12 cells in culture. These findings suggest that the effectiveness of *Cassia abbreviata* as a traditional remedy for hyperglyacaemia may in part, be attributed to its ability to stimulate glucose uptake in target cells. An understanding of the chemical systems involved in the absorption of glucose is yet to be conclusively elucidated, and may involve the actions of glucose transporter proteins on cell surfaces. *Cassia abbreviata* and *Helinus integrifolius* are components of traditional preparations used to treat diabetes mellitus and other diseases. It was for this reason that combinations of the two plants were tested for effectiveness as glucose absorption stimulants in C2C12 cells. A 3 h incubation of cells in the presence of a combination of *Cassia abbreviata* and insulin (10 µg/ml each) led to an increase in glucose uptake of 12%. Insulin and *Helinus integrifolius* resulted in an increase in glucose uptake of 5.13%, while *Cassia abbreviata* and *Helinus integrifolius* managed a 12.82% increase in glucose absorption when compared with absorption in untreated controls. The observed values are far less than values achieved by single doses of *Cassia abbreviata* alone, suggesting that the two plants do not exert any synergistic effects, at least on glucose uptake. The findings further suggest that the two plants do not have an additive effect on insulin action, and may not interfere with the actions of insulin. However, the ability of the plant extracts to act downstream of insulin, and the activation of GLUT4 translocation in a pathway excluding insulin action, is not ruled out. Seven of eight ethnobotanically selected species were found to enhance glucose uptake in skeletal muscle cells in the absence of insulin by 10–45%, an effect similar to that of Metformin[20]. Similar results have been observed with *Cassia abbreviata*, where there is increase of glucose uptake by the liver cells, in the absence of insulin and or metformin. Although insulin effects on glucose utilization were minimal, the results of this study are still valid, especially when cells treated with plant extracts and untreated control cells are compared.

GLUT1 and GLUT4 mRNA levels

The effects of *Cassia abbreviata* and *Helinus integrifolius* on expression levels of GLUT1, GLUT4 and GAPDH in C2C12 cells were investigated. Insulin, 50 µg/ml *Cassia* abbreviata, 25 and 50 µg/ml *Helinus integrifolius* appeared to slightly upregulate GLUT1 mRNA levels after a 3 h incubation period. 100 µg/ml *Cassia abbreviata* slightly downregulated GLUT1 mRNA expression. The GLUT4 mRNA levels remained uniform when cells were incubated with different treatment regiments for 3 h (Figure 3A). The trend was different after a 24 h incubation period. *Helinus integrifolius* appeared to downregulate GLUT1 mRNA levels at the two tested, with pronounced downregulation at 25 µg/ml. The level of GLUT4 mRNA remained uniform in all treatments. The levels of the normalising GAPDH mRNA remained uniform throughout, indicating that the amounts of RNA used for cDNA synthesis were equal (Figure 3B). *Helinus integrifolius* failed to stimulate an increase in glucose uptake, only managing to impair glucose uptake in muscle cells. The failure to stimulate glucose uptake and the increase in glucose concentration in the medium might be linked to the inhibition of expression of GLUT1. The concentration of GLUT4 mRNA remained uniform throughout the treatments, suggesting that an increase in GLUT1 may account for additional glucose uptake in cells treated with *Cassia abbreviata.* Insulin, positive control did not result in any significant increase in glucose uptake by cells. The reasons for the observant are yet to be determined. Yu *et al*. (2001)[12] reported that increased glucose concentration results in decreased glucose uptake in the presence of insulin. The reason for these conflicting results is unknown, but the effects of insulin may be influenced by other bioactive factors in the culture medium. For instance, Yu *et al.* (2001)[12] demonstrated the requirement of dexamethasone for the upregulating effect on insulin, suggesting that the regulatory effect of insulin on GLUT4 gene requires adjuvant mechanisms. When grown in culture, the delivery of glucose to the cell surface of isolated myocytes is not limiting. The energy demands in culture are lower, so the overall rate of glucose transport is reduced, in turn, reducing stress on phosphorylation consequently, reduced glucose uptake[21]. Tamrakar *et al*. (2011)[13] showed that Pongamol stimulates glucose uptake

but had no effect on GLUT4 protein expression, or GLUT 4 mRNA level. It is suggested that the observed effect of Pongamol on glucose uptake might be due to increased translocation of GLUT 4 from internal compartments to plasma membrane, the PI-3-K/AKT-dependent pathway.

GLUT4 translocation

Insulin-regulated translocation of GLUT4 from the cytoplasm to the cell membrane is an important requirement for glucose absorption by target cells such as skeletal muscle, and adipocytes[9]. *Cassia abbreviata* (50 µg/ml) induced an increase in FITC fluorescence intensity compared to untreated stained control cells. *Helinus integrifolius* (25 µg/ml) stimulated a higher increase in GLUT4 translocation than 50 µg/ml a 2-fold increase was recorded. Insulin (100 µg/ml) also led to some increase in GLUT4 translocation (Figure 4). Translocation was also illustrated in the form of histograms. In panels A and B (Figure 5), amount of measurable FITC intensities in the presence of 50 µg/ml *Cassia abbreviata* and *Helinus integrifolius* (blue), respectively, were higher than measured on untreated unstained cells (red) and untreated FITC-stained cells (green). GLUT4 translocation was confirmed qualitatively using fluorescence microscopy of C2C12 cells stained with FITC-labelled anti-GLUT4 antibody. Fluorescence intensity was more pronounced in cells treated with *Cassia abbreviata* and *Helinus integrifolius.* Translocation efficiency was less in untreated controls and insulin-treated cells (Figure 5). This observation suggests that the observed increase in glucose absorption by cells treated with *Cassia abbreviata* extracts may be directly linked to the induction of GLUT4 translocation to the cell surface. *Helinus* stimulated an increase in GLUT4 translocation, but stimulated little increase in the absorption of glucose by C2C12 cells. It is not clear how this occurs, but may mean that the absorption of glucose is a complex process, requiring other mechanisms in addition to GLUT4 translocation. These results also correlate with those of immunofluorescence, with pronounced fluorescence on the membrane surfaces of cells treated with *Cassia abbreviata* and *Helinus integrifolius* extracts. Diabetes mellitus is one of the leading causes of death and have already reached an epidemic proportion globally. It is characterised by insulin resistance, and impaired pancreatic β cell function and hyperglycemia[22]. Decades after the discovery of the debilitating chronic nightmare diabetes mellitus, there is still lack of effective preventive measures and curative therapies to combat the global epidemic[23]. Despite significant breakthroughs with the available antidiabetic drugs as constituents which transform diabetes from a fatal into a manageable chronic disease, there remain considerable challenges. Antidiabetic drugs as a chronic suppressive therapy is limited by its cost, the requirement of life long adherence, side effects and the presently unknown effects of long term treatment[13,23]. Therapeutic choices for diabetic treatment are even more compromised in developing countries due to a persistent lack of access to antidiabetics, limited health care capacities and other socio-economic factors that counteract the benefits of treatment, such as malnutrition and poverty. There is therefore a perpetual need for new and more affordable anti-diabetic therapeutics. One strategy is to develop them from natural products (plants). In this study the effect of *Cassia abbreviata* and *Helinus integrifolius* on glucose uptake, on gene expression of GLUT4 and GLUT1 in C2C12 and liver cells, GLUT 4 translocation on muscle cells was investigated. Indeed there is a worldwide effort to discover new antidiabetic agents from plants[24]. *Helinus integrifolius*, down regulates or impair glucose uptake in the muscle cells, yet it stimulates the increased expression of mRNA levels for both GLUT1 and GLUT 4 in these cells. It also induces GLUT 4 translocation as shown by both flow cytometry and immunofluorescence, with increased surface GLUT4 at a concentration of 25 µg/ml. *Cassia abbreviata* was tested in another study by Shai et al. (2010)[25], and has indicated potential in *in vitro* studies for treatment of diabetes mellitus. The present data led us to conclude that up regulation of Glut4 (mRNA) may cause an increase of its protein level as a consequence of increased expression mRNA, previously reported by others. It is likely that the observed increase in glucose absorption by cells treated with *Cassia abbreviata* extracts is due to sustained expression of GLUT4 and GLUT 1, as well as stimulation of GLUT4 translocation to the cell surface.

Declaration of Interest

The authors report no declaration of interests. This study was supported by the National Research Fund (NRF) and Tshwane University of Technology

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