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Phytochemical Analyses, Glucose Stimulatory Effects and Cytotoxicity of *Cassia abbreviata* and *Helinus integrifolius* Leaf Extracts in *in vitro* Cell Cultures

O.O. Olaokun[1,*] and V.P. Ntini[1,2]

[1]Department of Biology and Environmental Science, School of Science and Technology, Sefako Makgatho Health Science University, 0208 Pretoria, South Africa

[2]Department of Biomedical Sciences, Tshwane University of Technology, 0001 Pretoria, South Africa

*Corresponding author: E-mail: oyinolaokun@yahoo.com

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Numerous phytochemical constituents are found in plants and have been used either as single entities or extracts to treat various ailments. This study investigated the extracts of *Cassia abbreviata* and *Helinus integrifolius in vitro* for phytochemicals and its effect on glucose utilization of C2C12 muscle and H-4-II-E liver cells and for cytotoxicity against raw 264.7 cells using the real-time xCelligence system method. The ethyl acetate and acetone extracts of phenolic rich *C. abbreviata* stimulated greater C2C12 muscle cells glucose utilization at concentrations of 0.065 mg/mL and 0.125 mg/mL and acetone extract of *C. abbreviata* enhanced a concentration dependent H-4-II-E cells glucose utilization. For cytotoxicity, all extracts in concentration dependent manner decreased the viability of the raw 264.7 cells in real time assay with concentrations of 0.05 mg/mL the least cytotoxic. The extracts of *C. abbreviata* in *in vitro* assay demonstrated glucose stimulatory potential but further investigation of the cytotoxicity is required.

Keywords: *Cassia abbreviate*, *Helinus integrifolius*, Glucose utilization, Real-time xCelligence system, Cytotoxicity.

INTRODUCTION

In humans, carbohydrates are broken down into simpler monomers such as glucose, which enters in bloodstream and serves as rapid source of energy for cells. The presence of glucose in the blood stimulates the secretion of insulin by pancreatic β-cells. The hormone, insulin is responsible for stimulating glucose transporter 4 (GLUT 4) and maintaining glucose homeostasis through the absorption of glucose by various cells, particularly, the liver, muscle and adipocytes [1]. The liver is the first organ that insulin reaches after secretion. During fasting and postprandial states, it helps the body to maintain a normal glucose concentration [2]. Skeletal muscles store glucose in the form of glycogen while adipose tissues stores additional glucose in form of triglycerides, which are oxidized to produce energy when required [3]. The most effective system that lowers blood glucose levels is through storage in adipose tissues.

Without adequate insulin secretion, absorption of glucose by target cells is impaired, leading to elevated levels of glucose in the blood (hyperglycaemia). Over time, potentially fatal complications that damages organs like the heart, blood vessels, nerves and the eyes, can result from hyperglycemia [4]. To align with other insulin resistance in the hepatic and peripheral tissues (adipose tissue and muscle) is thought to be the cause of type 2 diabetes mellitus [5]. A complex network of adipokines and free fatty acids that make cells less sensitive to insulin exacerbates insulin resistance. To address these problems, commercially available therapeutics such as metformin and nateglinide are administered to ameliorate this condition [6].

Indigenous people in various parts of the world utilize the numerous phytochemicals of medicinal plants extracts to treat various ailments due to their affordability, ease of access and less undesirable effects [7]. Several medicinal plants are used without knowing their safety or efficacy due to the misconception that being natural, medicinal plants are harmless including medicinal plants like *Cassia abbreviata* and *Helinus integrifolius*, which were selected based on their common use in traditional medicine. While traditionally in South Africa, *C. abbreviata* is used for various ailments including treatment of diabetes mellitus, sexually-transmitted diseases, symptoms associated with AIDS, infertility and abdominal pains; in Botswana it is used for treatment of snake bite [8]. The root decoction has been used for disorders such as pneumonia, malaria and as purgative [9,10]. Also the leaf, bark and roots

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are used to alleviate ailments such as for diarrhoea, headache, cough, vomiting, epilepsy and infertility [10,11]. *H. integrifolius* is used in traditional medicine for the treatment of hair loss [12]. This plant in combination with other medicinal plants is used for preparation of remedies for infertility, erectile dysfunction and sexually transmitted diseases [8]. A study showed that some plants utilized for treating diseases could produce adverse effects [13]. While another study [14] reported that 15% of the cases of acute poisoning in hospitals were due to the administration of traditional medicines. These reactions can be associated with different types of interactions that can occur depending on the concentration of certain herbs [15]. While some interactions are beneficial, others can be deleterious to people. The objective of this work is to evaluate *Cassia abbreviata* and *Helinus integrifolius* extracts, commonly used medicinal plants in the community, for potential to stimulate the glucose utilization *in vitro* in the liver and muscle cells while the second was to evaluate their cytotoxic effects on raw 264.7 cells.

EXPERIMENTAL

The plants were procured from the Mashishile region in Limpopo Province, South Africa. The names and voucher numbers for the plants were *Helinus integrifolius* (PRU117192) and *Cassia abbreviata* (PRU113819). Voucher specimens were deposited at the H.G.W.J. Scheickerdt Herbarium at University of Pretoria, South Africa. The leaves after collection were dried at room temperature, pulverised and kept in dark bottles.

Plant extraction: A 5 g each of ground material in 20 mL solvent either ethyl acetate or acetone in tightly closed bottles were left to extract after 24 h. A Whatman No. 1 filter paper was used to filter the supernatant before it was dried in a stream of air into pre-weighed glass vials. All extracts were dissolved to obtain 100 mg/mL stock solution in DMSO, Sigma-Aldrich prior to the assay.

Qualitative phytochemical screening: All plant extracts were qualitatively tested for the presence of bioactive compounds using the reported method [16].

Test for carbohydrates (iodine test): Each plant extract (2 mL) was mixed with 2 mL of iodine solution. A dark blue or purple colour indicated the presence of carbohydrate.

Test for glycosides (Salkowski's test): Briefly 100 µL of each plant extract was mixed with 2 mL of chloroform and 2 mL of conc. H_2SO_4 and gently shaken. A reddish brown colour indicates the presence of steroidal ring, which represents the glycone portion of glycoside.

Test for steroids: About 2 mL of chloroform was added to 1 mL of each plant extract followed by adding 1 mL of conc. H_2SO_4. The presence of red colour is a indicative of presence of steroids.

Test for phenols: Each plant extracts (2 mL) was mixed with 2 mL of 2% solution of $FeCl_3$. The presence of blue/black colouration indicates the presence of phenols.

Test for flavonoids (Shinoda test): About 2 mL of each crude extract was mixed with 2 mL of 2% solution of NaOH. The presence of flavonoids is indicative when the yellow colour formed by the mixture turns colourless following the addition of 5-8 drops of dil. HCl.

Total polyphenolic assay: The total phenolic content of the extracts was determined using the 96-well plate method [17]. The extract (20 µL) was added into 96-well plate well containing 100 µL of 20% Folin-Ciocalteu reagent and 80 µL of 7.5% Na_2CO_3 solution. The mixture was shaken and incubated for 60 min in dark at room temperature and then absorbance was measured at 760 nm with Anthos 2010 micro plate reader. The total phenolic content was calculated from the linear regression curve of gallic acid and results are expressed as mg gallic acid equivalent (GAE/mg).

Total flavonoid content assay: The total flavonoid content of the extracts was determined using the 96-well plate method [16]. The extracts (100 µL) were dispensed into the wells of 96-well plate and 100 µL 2% aluminum chloride was added. The final mixture was shaken, incubated for 15 min and the absorbance was read at 430 nm with Anthos 2010 micro plate reader. A yellow colour indicates the presence of flavonoids. Total flavonoid content was calculated from the linear regression curve of quercetin and results are expressed as mg quercetin equivalent (QE/mg).

Cell culture maintenance: The cells (C2C12 muscle and H-4-II-E liver) were gifted by Phytomedicine Lab, Faculty of Veterinary Science, University of Pretoria, South Africa. The C2C12 muscle cells were maintained in Dulbecco's Minimum-Eagle Medium (DMEM, Gibco, New Zealand) while the H-4-II-E liver in Minimum Essential Medium Eagle (MEME, Sigma) containing 10% foetal bovine serum (FBS, Gibco, New Zealand) and 1 mL of gentamycin at 37 ºC in a humidified 5% CO_2 incubator.

Glucose utilization assay: The muscle and liver cells maintained at logarithmic growth phase were evaluated for glucose utilization after exposure to *C. abbreviata* and *H. integrifolius* acetone and ethyl acetate extracts as previously described [18]. Briefly, the C2C12 (2.5×10^3 cells/mL) and H-4-II-E (1.5×10^3 cells/mL) cells in 96-well plates were treated with extracts (100 mg/mL) in serial dilutions from 0.015-0.25 mg/mL. After 24 h incubation at 37 ºC, 50 µL of medium from cells in new plates were mixed with 100 mL of glucose oxidase reagent (Sigma) and incubated at 37 ºC for another 30 min. Insulin was used as the positive control for muscle cells, metformin as positive control for liver cells while DMSO was used as the solvent control. Thereafter, 50 µL of H_2SO_4 was added and the absorbance at 540 nm using an Anthos 2010 micro plate spectrophotometer reader. The glucose utilized was determined from the plot of the standard glucose linear graph.

Cytotoxicity test using real-time xCelligence system

Cell culture maintenance: Raw 267.4 macrophage cells was maintained at the logarithmic growth rate in DMEM containing a mixture of penicillin, streptomycin and neomycin (PSN) antibiotics and 10% foetal bovine serum (FBS, Gibco, New Zealand) at 37 ºC in a humidified 5% CO_2 incubator.

Cytotoxicity test: The cytotoxicity of acetone and ethyl acetate extracts of *C. Abbreviata* and *H. Integrifolius* were evaluated on the raw 264.7 cells at a logarithmic growth phase

in real-time xCelligence system (Roche) and according to manufacturer's instruction. Prior to the assay, DMEM (100 µL) was dispensed into E-plate (96-well) in the xCelligence system and incubated in a 5% CO_2 and 95% humidity incubator at 37 ºC for 24 h with readings taken every 15 min intervals. Following this, raw 264.7 cells (100 µL) in suspension (2.5 × 10^3 cells) were dispensed into the wells of E-plate and exposed to the extracts (100 µL) at three concentrations of 0.05, 0.01 and 0.25 mg/mL in the xCelligence system and incubated overnight. DMSO was the negative control while Actinomycin D was the positive control. The cytotoxicity value was calculated as cell index initiated after 10 h and readings taken every 15 min for another 10 h.

RESULTS AND DISCUSSION

The primary disposal locations for glucose are peripheral tissues. About 80% of insulin-mediated glucose utilization occurs in skeletal muscles. These play a big part in keeping the body's glucose levels normal. Several factors, including insulin resistance, resulting in hyperglycaemia, cause these cells to use less glucose [19]. In addition to the recommended diet and exercise for the management of type 2 diabetes mellitus, hyperglycaemia can be managed with a variety of oral medications that can reduce blood glucose levels in a variety of ways. The study explored the potential of the phytochemicals of the medicinal plants extracts in serial dilution to stimulate the glucose utilization *in vitro* in cell culture model.

Phytochemical analyses: The results of qualitative and quantitative phytochemical analyses of the acetone and ethyl acetate extracts of *C. abbreviata* and *H. integrifolius* are presented in Tables 1 and 2. The qualitative phytochemical screening analysis showed that only starch was absent (Table-1). The total phenolic content (Table-2) of *C. abbreviata* extracts is higher than those of *H. integrifolius* with the ethyl acetate extract of *C. abbreviata* being the highest (400 GAE/mg). However, the total flavonoid content (Table-2) of the extracts of *H. integrifolius* is higher than those of the extracts of *C. abbreviata* with acetone extract of *H. integrifolius* possessing the highest (4.13 QE/mg).

TABLE-1
QUALITATIVE PHYTOCHEMICAL ANALYSIS OF EXTRACTS OF *C. abbreviata* AND *H. integrifolius*

Phytochemical group	*C. abbreviate* extract		*H. integrifolius* extract	
	Acetone	Ethyl acetate	Acetone	Ethyl acetate
Phenols	Present	Present	Present	Present
Flavonoids	Present	Present	Present	Present
Glycosides	Present	Present	Present	Present
Steroids	Present	Present	Present	Present
Starch	Absent	Absent	Absent	Absent

TABLE-2
TOTAL PHENOLIC CONTENT AND TOTAL FLAVONOID CONTENT OF EXTRACTS OF *C. abbreviata* AND *H. integrifolius*

Plant extracts	Total phenolic content (GAE/mg)		Total flavonoid content (QE/mg)	
	Acetone	Ethyl acetate	Acetone	Ethyl acetate
C. abbreviata	392	400	1.23	0.89
H. integrifolius	79	27	4.13	5.57

Glucose utilization assay: The results of glucose utilization showed that the acetone and ethyl acetate of extracts of *C. abbreviata* stimulated higher glucose utilization of C2C12 muscle cells at 0.065 mg/mL and 0.125 mg/mL (Fig. 1) than *H. integrifolius* extracts. Insulin (positive control) also stimulated the glucose utilization of the C2C12 cells. For the glucose utilization of H-4-II-E liver cells (Fig. 2), the *C. abbreviata* extracts enhanced greater glucose utilization than *H. integrifolius* extracts and the positive control (metformin). The acetone and ethyl acetate extracts of *C. abbreviata* at the highest concentration (0.25 mg/mL) enhanced greater glucose utilization of the liver cells.

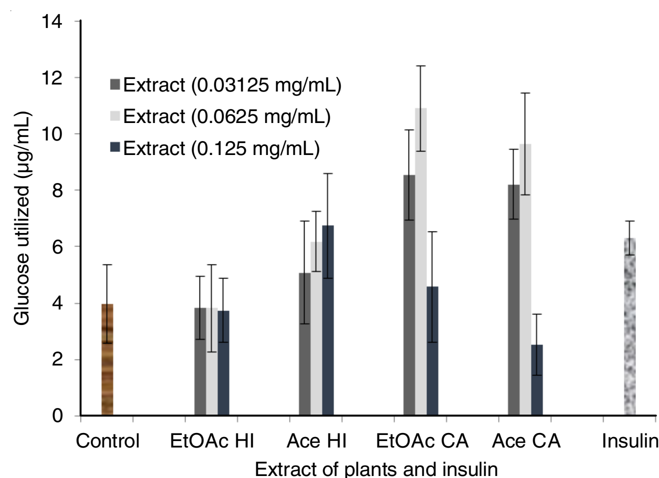

Fig. 1. The glucose utilization activity of C2C12 muscle cells (µg/mL ± SE) when treated with acetone (Ace) and ethyl acetate (ETOAc) extracts of *H. integrifolius* (HI) and *C. abbreviata* (CA) at various concentrations. Insulin was used as a positive control (0.001 mg/mL)

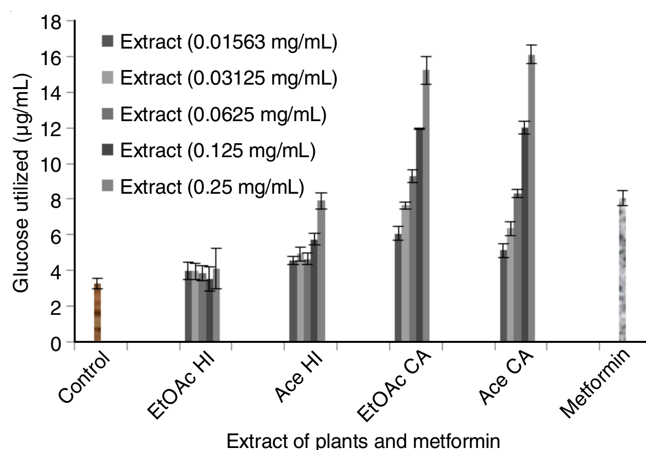

Fig. 2. The glucose utilization activity of H-4-II-E liver cells (µg/mL ± SE) when treated with acetone (Ace) and ethyl acetate (ETOAc) extracts of *H. integrifolius* (HI) and *C. abbreviata* (CA) at various concentrations. Metformin was used as a positive control (0.001 mg/mL)

According to the findings of this study, the phytochemicals and probably the phenolic of the plants extracts increased the cells' utilization of glucose in various ways at different concentrations. The effects of insulin and untreated control cells were compared to that of the two plants extracts. At 0.065 mg/mL and 0.125 mg/mL, the phenolic rich extracts of *C. abbreviata*

stimulated muscle cells glucose utilization. The utilization by muscle cells treated with *H. integrifolius* extracts on the other hand, did not appear to be effective, particularly at lower concentrations. This could be because *H. integrifolius* phytochemicals is low in phenolic and was unable to stimulate glucose utilization at lower concentrations. As a result, increasing the extract's concentration may result in toxic effects on other cell functions, raising concerns about the extracts' toxic effects at higher concentrations. Compared to muscle cells, liver cells treated with either *C. abbreviata* and *H. integrifolius* extracts stimulated glucose utilization. However, only cells treated with *C. abbreviata* extracts at 0.125 mg/mL stimulated glucose utilization in muscle and liver cells in a similar manner. The extracts may have enabled the utilization by temporarily disrupting the mitochondria of the cell and activating the AMPK pathway, which may be the cause of the observed improvement, though the mechanism is unknown [20]. Acetyl-CoA carboxylase is one of the several pathways regulated by the phosphorylation and regulation of downstream targets that occurs when AMPK is active. Mitogen-activitase protein kinase (MAPK) activity and phosphorylation are upregulated as a result of these pathways, triggering Glut4 translocation to initiate insulin-stimulated glucose utilization [21]. Due to drug biotransformation, *in vivo* and *in vitro* studies may not be directly comparable, but the mechanism of glucose utilization may be comparable to metformin, a well-known anti-hyperglycaemic agent [22]. The plant extracts may have contained compounds with insulin-like action, resulting in enhanced glucose absorption and cell entry or their signalling cascade might have played a role [23]. The majority of plant extracts are said to mimic insulin by triggering the tyrosine phosphorylation of insulin receptors, IRS1 and PI3K, which in turn causes liver cells and adipocytes to use glucose [24].

Cytotoxicity studies: The real-time xCelligence system generated a variety of cell indexes after exposing the raw 264.7 cells to extracts of the plants at varying concentrations. The cell index, which is concentration dependent, increased as concentration of extract exposed to cells decreased [25]. The highest cell index of about 0.4 was recorded for all the cells exposed to plant extracts at the lowest concentration of 0.05 mg/mL (Figs. 3 and 4) indicating low cytotoxic effect against the cells. Conversely, the cell index of about 0.2 was recorded for cells exposed to extracts at 0.25 mg/mL and actinomycin D (positive control), with actinomycin D causing the most cytotoxic effect (Figs. 3 and 4). The cells exposed to the acetone extracts of *C. abbreviata* and *H. integrifolius* at the concentration of 0.1 mg/mL (Figs. 3a and 4a) recorded a higher cell index than those of exposed to ethyl acetate extracts of *C. abbreviata* and *H. integrifolius* (Figs. 3b and 4b) at the same concentration indicating acetone extracts exert less cytotoxic effects on the cells than the ethyl acetate extracts. The cell indexes of all cells treated with either plant extract were higher than that of actinomycin D, which is used to stop cells from growing.

The ethyl acetate extracts of the plants were relatively more cytotoxic at 0.1 mg/mL than the acetone extracts indicating the presence of more cytotoxic phytochemical compounds in the former. This disparity in toxicity between the acetone and ethyl acetate extracts may be caused by different compounds

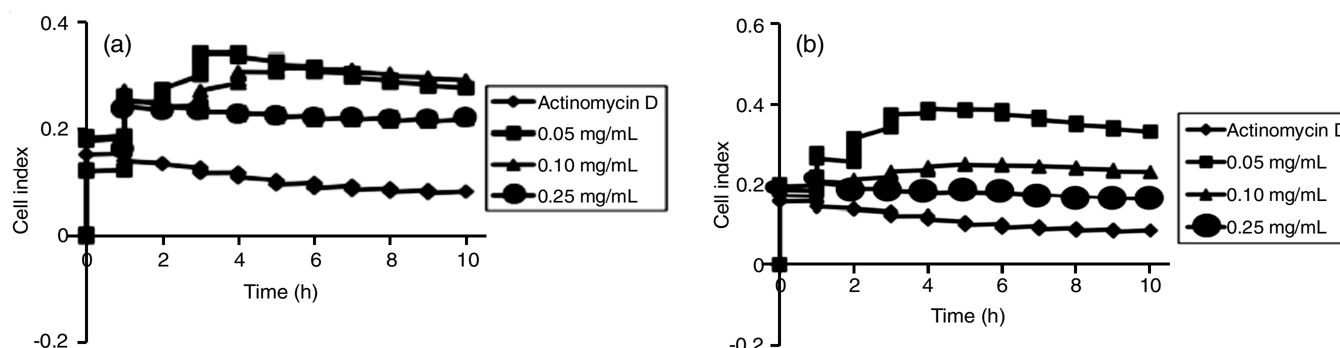

Fig. 3. Different cell index obtained when raw 264.7 cells were treated with 0.05, 0.1 and 0.25 mg/mL of (a) acetone extracts of *H. integrifolius*; and (b) ethyl acetate extracts of *H. integrifolius* for 10 h. Actinomycin D (0.001 mg/mL) used as positive control

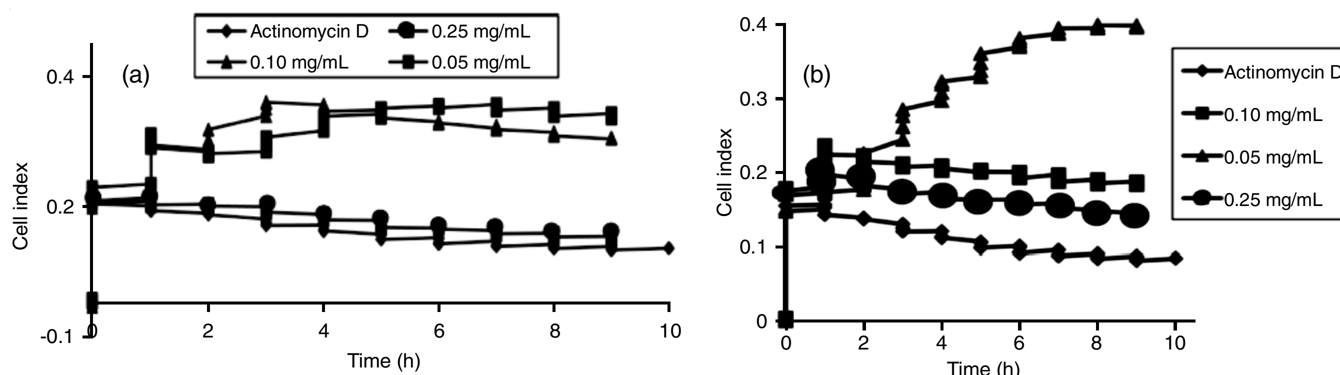

Fig. 4. Different cell index obtained when raw 264.7 cells were treated with 0.05, 0.1 and 0.25 mg/mL of (a) acetone extracts of *C. abbreviata*; and (b) ethyl acetate extracts of *C. abbreviata* for 10 h. Actinomycin D (0.001 mg/mL) used as positive control

extracted by the solvents as the acetone solvent is of intermediate polarity in comparison to the more polar ethyl acetate solvent. Some workers *et al*. [26,27] also reported that some triterpenes are harmful to humans and other mammals.

Conclusion

The muscle and liver cells exposed to acetone extract of *C. abbreviata* at the concentration of 0.125 mg/mL were to some extent stimulated to utilize glucose. The acetone extract of *C. abbreviata* at the concentration of 0.1 mg/mL was relatively less cytotoxic against the viability of raw 264.7 cells indicating the potential of *C. abbreviata* as a target for antidiabetic compound. Since the plant extract at low doses was less hazardous in the *in vitro* assay, more thorough toxicity tests utilizing an *in vivo* assay are essential to compare these results.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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