RESEARCH ARTICLE

Hydro-ethanolic extract of cashew tree (*Anacardium occidentale*) nut and its principal compound, anacardic acid, stimulate glucose uptake in C2C12 muscle cells

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Scope: Products of cashew tree (*Anacardium occidentale*) are used in traditional medicine for various ailments, including diabetes.

Methods and results: The anti-diabetic properties of cashew plant parts were studied using differentiated C2C12 myoblasts (myotubes) and rat liver mitochondria. Hydroethanolic extract of cashew seed (CSE) and its active component, anacardic acid (AA), stimulated glucose transport into C2C12 myotubes in a concentration-dependent manner. Extracts of other parts (leaves, bark and apple) of cashew plant were inactive. Significant synergistic effect on glucose uptake with insulin was noticed at 100 μg/mL CSE. CSE and AA caused activation of adenosine monophosphate-activated protein kinase in C2C12 myotubes after 6 h of incubation. No significant effect was noticed on Akt and insulin receptor phosphorylation. Both CSE and AA exerted significant uncoupling of succinate-stimulated respiration in rat liver mitochondria.

Conclusion: Activation of adenosine monophosphate-activated protein kinase by CSE and AA likely increases plasma membrane glucose transporters, resulting in elevated glucose uptake. In addition, the dysfunction of mitochondrial oxidative phosphorylation may enhance glycolysis and contribute to increased glucose uptake. These results collectively suggest that CSE may be a potential anti-diabetic nutraceutical.

Keywords:
Acetyl-CoA carboxylase / Adenosine monophosphate-activated protein kinase / Anacardic acid / Cashew seed extract / Diabetes

1 Introduction

Diabetes mellitus (DM) affects nearly 220 million people worldwide and its main complications, such as cardiovascular disease and renal failure are major causes of death [1]. Elevated blood glucose levels resulting from either insufficient circulating insulin, insulin resistance, or both, associated with the dysfunction and demise of pancreatic β-cells,

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Abbreviations: AA, anacardic acid; **ACC,** acetyl-CoA carboxylase; **AICAR,** 5-amino-imidazole-4-carboxamideribonucleoside; **AMP,** adenosine monophosphate; **AMPK,** AMP-activated protein kinase; **AOHE,** *Anacardiumoccidentale* hydroethanolic extract; **CC,** compound C; **CNSL,** cashew nut shell liquid; **CSE,** cashew seed extract; **DM,** diabetes mellitus; **DNP,** 2,4-dinitrophenol; **HS,** horse serum; **IR,** insulin receptor; **LDH,** lactate dehydrogenase

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is a characteristic of DM [2]. In addition, abnormalities in glucose and lipid metabolism are a common feature of DM [3, 4]. The most common therapeutic approaches for DM include sulphonylureas, which increase insulin release, metformin, which reduces hepatic glucose production, peroxisome proliferator-activated receptor-γ activators (thiazolidinediones), which increase insulin sensitivity, α-glucosidase inhibitors, which interfere with glucose absorption and insulin itself [5]. However, all these therapeutics suffer from the drawback of several side effects. Hence, the search for novel pharmaceuticals for the treatment of DM, with the least number of undesirable effects, remains pertinent and timely. Natural products and their derivatives have been extensively used as remedies for various diseases and a significant proportion of contemporary drugs are either natural compounds or their analogs [6]. Traditional approaches using extracts from different plant parts to treat DM have been known for a long time, although their mechanism of action has not always been studied in detail [7, 8].

Anacardium occidentale Linn, commonly known as the cashew tree, is a member of the family Anacardiaceae. This tropical tree native to northeastern Brazil is presently cultivated in many other countries. The uses of *A. occidentale* and of products derived from several parts of the tree cover a wide range of health benefits, including diabetes [9, 10].

In the south of Cameroon and other tropical countries like Brazil, the leaves and/or the bark of cashew tree have been shown to have anti-diabetic activities [11]. Cashew tree leaf extracts have been shown to cause hypoglycemia in normal individuals and normoglycaemic rats [12]. The chemical class of compounds present in cashew nut shell liquid (CNSL) is similar to that present in Ginkgo extracts. In the CNSL, they are called anacardic acids (AA) and in the Ginkgo, ginkgolic acids; but both are 6-alkylsalycylic acid or 2-hydroxy-6-alkyl-benzoic acids. CNSL is composed of AA (71%), cardol (18%), cardanol (4.7%) or a novel phenol (2.7%) (Fig. 1) [13]. Moreover, AAs present in CNSL have been demonstrated to have an uncoupling effect on isolated liver mitochondria [14].

Cashew tree parts are thus likely to have potential anti-diabetic properties, but the mechanism(s) of action of these extracts and associated active chemical components are poorly understood. In this study, we have screened several parts of the tree for potential anti-diabetic effects by assaying skeletal muscle cell glucose uptake. We observed that only cashew seed extract (CSE) and its principal component, AA, have significant stimulatory effect on glucose uptake in skeletal muscle cells possibly *via* the activation of AMP (AMP, adenosine monophosphate)-activated protein kinase (AMPK) with associated uncoupling effect, as assessed in rat liver mitochondria.

2 Materials and methods

2.1 Materials

DMEM and fetal bovine serum were purchased from Wisent (St. Bruno, Quebec, Canada), horse serum (HS), 2-deoxy-D-glucose, 2,4-dinitrophenol (DNP), compound C (CC), metformin and cytochalasin B were purchased from Sigma-Aldrich Chemicals (Oakville, Ontario, Canada). 5-Amino-imidazole-4-caboxyamide ribonuleoside (AICAR) was purchased from Toronto Research Chemicals (North York, Ontario, Canada) and 2-deoxy-D-[1-^{3}H] glucose was purchased from GE Healthcare (Baie D'Urfé, Quebec, Canada). All antibodies were from Cell Signaling Technology (Danvers, MA, USA). AA was purchased from Alexis Biochemicals (San Diego, CA, USA). Cytotoxicity Detection kit of lactate dehydrogenase (LDH) was obtained from Roche Diagnostics Gmbh (Laval, Quebec, Canada). C2C12 mouse myoblasts were obtained from ATCC (CRL-1772; Manassas, VA, USA).

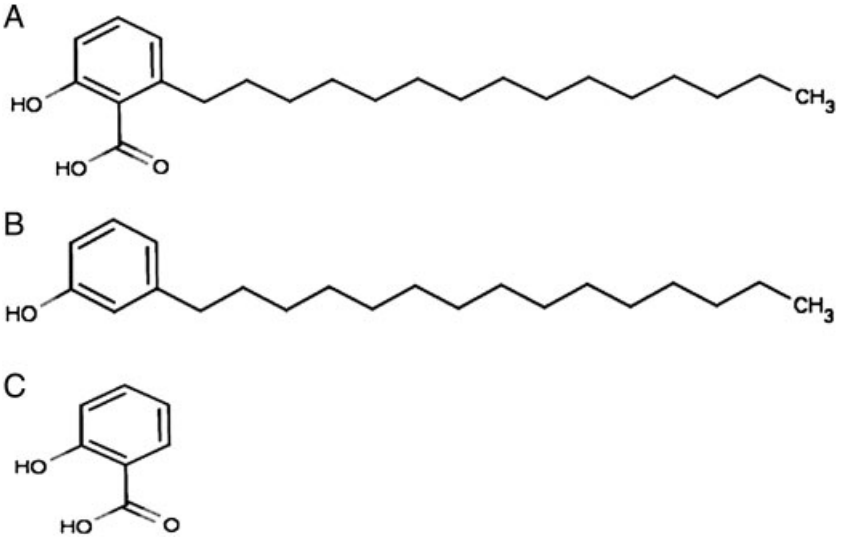

Figure 1. Chemical structures of (A) AA, (B) cardanol and (C) salicylic acid.

2.2 Plant harvest and extraction

The leaves, barks, seeds and cashew apple of *A. occidentale* were collected in May 2007 in Garoua (Northern Province), Cameroon. The plant samples were identified at the National Herbarium Cameroon (NHC), Obili-Yaounde, Cameroon. A voucher specimen of the collected plant sample was also deposited in the herbarium and given specimen number 65604/NHC. Plant samples were air-dried at room temperature. Extracts of various Cashew tree parts were prepared by the method described previously by Spoor *et al.* [15]. *A. occidentale* hydroethanolic extracts (AOHE) of leaves, bark, seeds and cashew apple were dried using a rotary evaporator followed by lyophilization. These lyophilized extracts were resuspended in 100% DMSO to a final concentration of 100 mg/mL and stored at −20°C. Frozen extracts were thawed at 37°C and diluted to required concentration in cell culture medium.

2.3 Cell culture

The C2C12 myoblasts were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum, 10% HS and penicillin (100 U/mL)–streptomycin (100 mg/mL) antibiotics, until 80% confluent in a humidified 5% CO_2 atmosphere at 37°C. Myoblasts were then differentiated in to myotubes over 6 days in DMEM containing 2% HS as described previously [16].

2.4 LDH cytotoxicity assay

Cells were cultured in 12-well plates in serum-free DMEM. After incubation with cashew tree crude extracts or AA (25, 50 and 100 μg/mL) for 18 h, LDH activity in the medium was assayed with the cytotoxicity detection kit (Roche Diagnostics).

2.5 Deoxyglucose uptake assay

Deoxy-D-glucose uptake assay was performed as described previously [15], with few modifications. Approximately 80% confluent and differentiated C2C12 myoblasts (in 12-well plates) were incubated for 18 h in differentiation medium (DMEM with 2% HS) containing different concentrations of cashew extracts (25, 50 and 100 μg/mL) or AA. DMSO (0.1%) and metformin (400 μM) were taken as negative and positive controls, respectively. In the experiments where the influence of CC on the effects of CSE and AA was studied, cells were incubated for the first 15 h with 10 μM CC followed by the addition of another 10 μM to a final concentration of 20 μM and the incubations continued for 3 h (total 18 h). After 18 h, cells were washed twice with prewarmed (37°C) Krebs phosphate buffer, pH 7.4 (136 mM NaCl, 20 mM HEPES, 4.7 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 4.05 mM Na_2HPO_4, 0.95 mM NaH_2PO_4) containing glucose (5 mM) and incubated in the same buffer for 30 min at 37°C. For the cells to be treated with insulin, Krebs phosphate buffer containing glucose (5 mM) and insulin (100 nM) were added. After 30 min, cells were rinsed three times with Krebs phosphate buffer of pH 7.4 (37°C) without glucose and glucose uptake was initiated by the addition of 500 μL (in each well) Krebs phosphate buffer (37°C) containing 10 μM 2-deoxy-D-[1-^{3}H] glucose (1 μCi/mL) and incubated for 10 min at 37°C. Some wells were treated with 10 μM cytochalasin B (an inhibitor of glucose transporters), to determine nonspecific glucose transport. After 10-min incubation, cells were rinsed three times with cold Krebs phosphate buffer (4°C) containing glucose (5 mM) and lysed with 500 μL of 0.1 M NaOH for 30 min at room temperature. Lysate from each well was added to 4 mL of liquid scintillation cocktail (Ready-gel, Beckman Coulte, Mississauga, Ontario, Canada) and amount of radioactivity from 2-deoxy-D-[1-^{3}H] glucose incorporated into cells was measured in a scintillation counter.

2.6 Mitochondrial respiration studies

The effects of plant extracts on oxygen consumption of isolated mitochondria were assessed using Clark-type oxygen microelectrode. Liver mitochondria were isolated from male Wistar rats [17]. Measurement of oxygen consumption was performed as described previously [18]. All animal manipulations were approved by the animal ethics committee of the University of Montreal and were carried out in respect of the principles set forth by the Canadian Council on the Care and Protection of Animals (CDEA 09–007).

Briefly, rats weighing between 200 and 225 g were anesthetized and subjected to laparotomy. The portal vein was cannulated, and liver was flushed with 100 mL of Krebs–Henseleit buffer (composed of 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 154 mM NaCl, 4.8 mM KCl, 2.1 mM $CaCl_2$, 1.2 mM $MgSO_4$, pH 7.4) at 22°C prior to excision. Two grams of liver tissue was homogenized on ice using a Teflon potter homogenizer in ice-cold medium composed of 250 mM sucrose, 10 mM Tris, 1 mM EGTA and pH 7.2. The homogenate was centrifuged at 600 × *g* for 10 min at 4°C in order to remove cellular debris. The supernatant was then centrifuged at 15 000 × *g* for 5 min at 4°C. The pellet was washed once with the same buffer, centrifuged and washed again with buffer without EGTA and then recentrifuged at 15 000 × *g*. The final pellet, containing intact mitochondria, was suspended in EGTA-free homogenizing buffer and kept on ice. Protein content of the mitochondrial preparation was determined by Lowry protein assay. Oxygen consumption was measured at 25°C in a Hansatech Oxygraph apparatus (Norfolk, UK) with a 1 mL reaction chamber. One milligram of mitochondrial protein was added to respiration buffer (composed of 5 mM

KH_2PO_4, pH 7.2, 250 mM sucrose (ultra pure), 5 mM $MgCl_2$, 1 mM EGTA and 2 μM complex I inhibitor rotenone) in the reaction chamber, in a final volume of 990 μL. Mitochondrial respiration was initiated by the addition of 6 mM complex-II substrate succinate, and basal succinate-supported rate of oxygen consumption (referred to as basal rate of O_2 consumption) was determined. CSE (25 or 50 μg/mL) was then added and its effect on basal rate of O_2 consumption was assessed. Following this, 200 μM ADP was added to induce oxidative phosphorylation and the rate of ADP-stimulated O_2 consumption was determined. DMSO-vehicle control experiments were conducted at the beginning and end of each experimental session in order to establish the session-normal rates of basal and ADP-stimulated O_2 consumption and to ensure no loss in mitochondrial function. It was ensured that DMSO, at the concentrations employed in this study, had no effect on the basal rate of O_2 consumption. Therefore, increase in the rate of basal O_2 consumption, an index of uncoupling, was calculated from each experiment by subtracting the rate measured in the absence of extract from the rate measured in the presence of extract and dividing the difference by the rate measured in the absence of extract. Decrease in the rate of ADP-stimulated O_2 consumption, an index of inhibition of oxidative phosphorylation, was calculated by subtracting the ratio of the rate of ADP-stimulated O_2 consumption to the rate of basal O_2 consumption in vehicle-treated mitochondria from the corresponding ratio obtained with extracts and dividing the difference by the ratio of the rate of ADP-stimulated O_2 consumption to the rate of basal O_2 consumption in vehicle-treated mitochondria.

2.7 Western blotting

C2C12 myotubes were treated with CSE (25, 50 and 100 μg/mL) and AA (100 μM) for 18 h. When studying the influence of CC on the effects of CSE and AA, cells were incubated for the first 15 h with 10 μM CC followed by the addition of another 10 μM to a final concentration of 20 μM and the incubations continued for 3 h (total 18 h). As controls, myotubes were treated for 20 min with AICAR (2 mM) or for 30 min with insulin (100 nM). The whole cell lysates were prepared in RIPA lysis buffer (0.1 M Hepes, 0.3 M NaCl, 10 mM EGTA, 4 mM $MgCl_2 \cdot 6H_2O$, 10% glycerol, 0.2% Triton X-100, 0.02% SDS, 0.2% Na-deoxycholate, 20 mM PMSF, 10 mM NaF, 100 μM Na-orthovanadate, 1 mM Na-pyrophosphate and protease inhibitor complete-mini (Roche Diagnostics), pH 7.4. The proteins were quantified by Bio-Rad protein assay. Proteins from each sample (20 μg) were boiled for 5 min in SDS sample buffer, separated in 10% SDS-PAGE for 90 min at 100 V, and transferred on to nitrocellulose membrane (Bio-Rad). Membranes were blocked in 5% milk in TBST (200 mM Tris base, 1.37 M NaCl and 0.1% Tween-20) for 1 h and were incubated with primary antibodies (Phospho-ACC (Ser79), Cell Signaling, ♯3661, 1//1000; Phospho-AMPKα (Thr172), Cell Signaling, ♯2531, 1/1000; Phospho-Akt (Ser473), Cell Signaling, ♯9271L; Phospho-Insulin Receptor β (Tyr1150/1151), Cell Signaling, ♯3024S.) overnight at 4°C and with the secondary antibody for 1 h at room temperature. The enhanced luminol reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) was used for detection. The membranes were reprobed with antibodies against acetyl-CoA carboxylase (ACC) (Cell Signaling, ♯3662, 1/1000), AMPKα (Cell Signaling, ♯2532,1/1000), Akt (Cell Signaling, ♯9272, 1/1000), IGF-I Receptorβ (Cell Signaling, ♯3027, 1/1000) and β-actin (Cell Signaling, ♯4967,1/1000) after stripping in 1 × Re-Blot Plus (CHEMICON International) for 15 min at room temperature. All of the experiments were conducted on three separate cell preparations. Image J 1.42q software (NIH, USA) was used to quantify the digitized Western blot signals.

2.8 Statistical analysis

All experiments were performed on at least three separate cell preparations. Data were analyzed using ANOVA and appropriate *post hoc* tests (StatView software, ver. 5.0.1, Cary, NC, USA). A level of significance of 5% was adopted for all comparisons ($p < 0.05$).

3 Results

3.1 Cytotoxicity of AOHE of leaves, bark, seeds and apples

Cytotoxicity assay based on LDH release revealed that none of the extracts of plant parts tested was toxic to the C2C12 cells even at a concentration of 100 μg/mL after 18 h exposure (data not shown).

3.2 Effect of *A. occidentale* extracts on 2-deoxy-D-[1-^{3}H] glucose uptake in C2C12 cells

C2C12 myotubes were incubated in the presence of various concentrations (25, 50 and 100 μg/mL) of hydroethanolic (AOHE) extracts of *A. occidentale* leaves, bark, CSE or cashew apple extract (CAE) for 18 h and then glucose transport was measured by following the uptake of 2-deoxy-D-[1-^{3}H] glucose into these cells. Among the plant part extracts tested, CSE significantly increased glucose transport into C2C12 myotubes, in a dose-dependent manner with the maximal effect (156%) being found at 100 μg/mL ($p < 0.0001$) as compared with vehicle (DMSO) control (set at 100%; Fig. 2). In comparison, a dose of 100 nM insulin yielded a maximal effect of 139% in this cellular model (Section 3.5). No effect was noticed with CAE, whereas leaf and bark extracts showed an inhibitory effect only at the higher concentration (100 μg/mL) (Fig. 2).

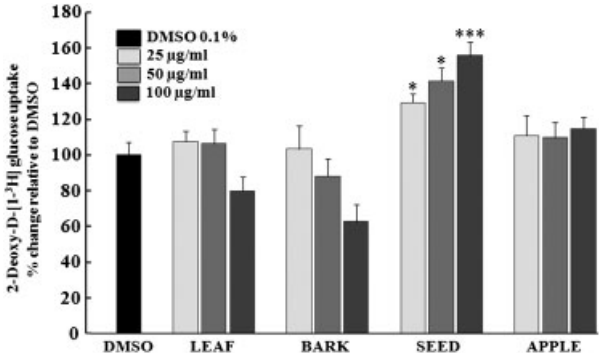

Figure 2. Effect of AOHE plant parts on glucose uptake in C2C12 myotubes. Differentiated C2C12 cells were incubated for 18 h with different plant part extracts at concentrations of 25, 50 and 100 µg/mL. Each value represents percentage increase or decrease in glucose uptake relative to DMSO (0.1%) control. (*$p<0.05$ *versus* DMSO, $n=3$).

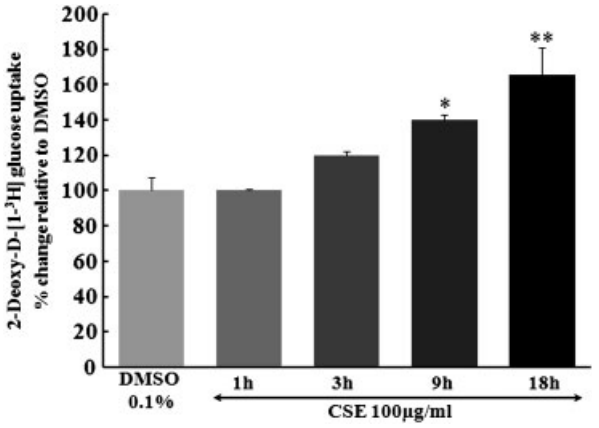

Figure 3. Time course of 2-deoxy-D-[1-3H] glucose uptake in C2C12 myotubes. Differentiated C2C12 cells were incubated for different times with 100 µg/mL of CSE. Each value represents the percentage of glucose uptake relative to DMSO (0.1%) control. (*$p<0.05$ *versus* DMSO, $n=3$).

3.3 Time course of 2-deoxy-D-[1-^{3}H] glucose uptake in C2C12 cells

Glucose transport in C2C12 myotubes was increased by CSE (100 µg/mL) in a time-dependent manner (Fig. 3). Stimulation of glucose uptake was statistically significant at 9 h ($p<0.0055$) and 18 h ($p<0.0002$).

3.4 Effect of AA as compared with CSE on glucose uptake in C2C12-cells

C2C12 myotubes were exposed for 18 h to various concentrations (10, 25 and 50 µM) of AA, CSE (100 µg/mL), metformin (400 µM) or an uncoupler of mitochondrial respiration, DNP (50 µM), before glucose transport was measured. There was a

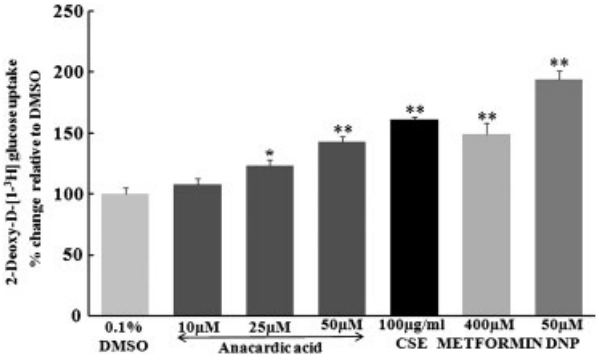

Figure 4. Comparative effect of AA, CSE, metformin and DNP on glucose uptake in C2C12 myotubes. Differentiated C2C12 cells were incubated for 18 h with 10, 25 and 50 µM AA, 100 µg/mL of CSE, 400 µM metformin or 50 µM DNP and glucose uptake was assayed. Each value represents percentage increase or decrease in glucose uptake relative to DMSO (0.1%) control. (*$p<0.05$ *versus* DMSO, $n=3$).

concentration-dependent increase in glucose uptake by AA with an approximate increase of 40% observed at 50 µM AA (Fig. 4). The anti-diabetic drug and known AMPK activator, metformin enhanced glucose transport significantly. Metformin is also known to act as an uncoupler of mitochondrial oxidative phosphorylation [19]. Therefore, in order to examine if other uncouplers also augment glucose transport, the effect of DNP was tested and the results showed marked elevation in glucose transport (Fig. 4).

3.5 Synergistic effect of CSE and AA with insulin on glucose uptake in C2C12 cells

In order to examine the combined effect of CSE or AA with insulin on glucose uptake, cells were incubated with 25, 50 and 100 µg/mL CSE or 50 µM AA (for 18 h) without or with 100 nM insulin (for the last 30 min). Incubation of cells with insulin alone increased glucose uptake significantly, as expected. Glucose uptake was significantly elevated in cells incubated with high concentration of CSE (100 µg/mL) plus insulin as compared with either CSE or insulin alone and similar results were obtained with AA plus insulin (Fig. 5). No such synergistic effect was noticed at lower concentration of CSE.

3.6 Effect of AMPK inhibitor, CC on glucose uptake in C2C12 cells

To examine whether the glucose uptake stimulatory effects of CSE and AA were mediated through the AMPK signaling pathway, we have tested the effect of CC, a specific inhibitor of AMPK. Un-stimulated glucose uptake in C2C12 cells was not significantly affected by CC. However, in the presence of CC, CSE (100 µg/mL) or AA (50 µM) failed to induce the stimulation

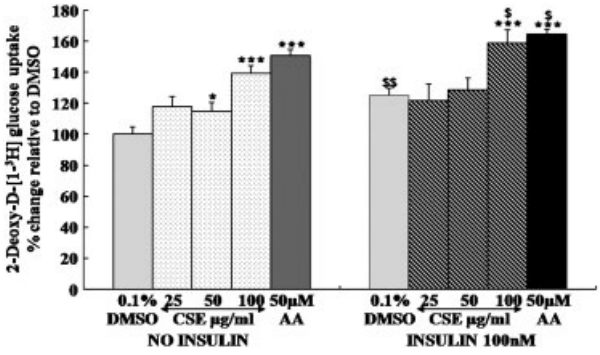

Figure 5. Synergistic effect of CSE and AA with insulin on 2-deoxy-D-[1-3H] glucose uptake in differentiated C2C12 muscle cells treated for 18 h. Differentiated C2C12 cells were incubated for 18 h with 0, 25, 50 and 100 μg/mL of CSE or 50 μM AA and the last 30 min incubation was with or without insulin. Each value represents percentage increase in glucose uptake relative to DMSO (0.1%) control (*$p<0.05$ *versus* DMSO, \$ $p<0.05$ *versus* DMSO with insulin, $n=6$).

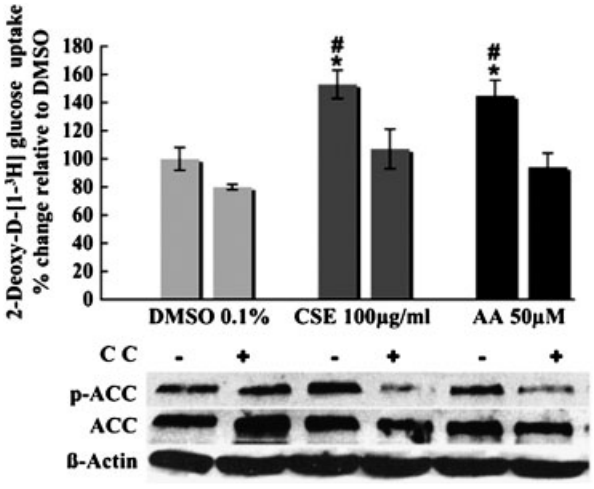

Figure 6. Effect of CC on CSE and AA stimulated 2-deoxy-D-[1-3H] glucose uptake and ACC phosphorylation in differentiated C2C12 muscle cells. Upper panel – Differentiated C2C12 cells were incubated for 18 h with 100 μg/mL of CSE or 50 μM AA, with or without CC. Each value represents the percentage increase in glucose uptake relative to the DMSO (0.1%) control (*$p<0.05$ *versus* DMSO, # $p<0.05$ *versus* corresponding CC-treated sample; $n=3$). Lower panel – C2C12 cells incubated as mentioned above (upper panel) were lysed and were subjected to immunoblotting analysis using antibodies against total or phospho-ACC. The blot presented is representative of two separate experiments with similar results.

of glucose uptake found in the absence of this AMPK inhibitor (Fig. 6).

3.7 Effect of CSE and AA on AMPK, Akt and insulin receptor phosphorylation

In order to determine the potential mechanisms of action underlying the increase in glucose uptake caused by CSE and AA in C2C12 myotubes, their effect on AMPK, IR and Akt activation (by phosphorylation) was examined. Marked increase in AMPK phosphorylation was noticed (Figs. 7A and C) in C2C12 myotubes within 6 h of incubation in the presence of CSE (25, 50 and 100 μg/mL). However, further incubation up to 18 h led to a significant decrease in AMPK phosphorylation (Figs. 7B and C). There was no change in total AMPK protein during this incubation (Fig. 7). One of the active ingredients of CSE, AA exerted a more sustained effect on AMPK phosphorylation, which could be found even after 18 h of incubation (Figs. 7B and C). In control incubations with AICAR and insulin, AMPK phosphorylation was enhanced and declined, respectively, as expected. As an index of AMPK activity, phosphorylation of its substrate ACC was monitored. Results indicated that ACC phosphorylation was sustained even after 18 h of incubation with CSE and AA (Figs. 7B and D).

Neither CSE nor AA showed any noticeable effect on IR and Akt phosphorylation, both of which were significantly elevated by insulin (Fig. 8) as expected in C2C12 cells.

3.8 Uncoupling effect of CSE and AA in rat liver mitochondria

Because of the similarity in the action of CSE, metformin and DNP, experiments were conducted to examine whether CSE and its active ingredient, AA exhibit mitochondrial uncoupling properties as well. Succinate-stimulated oxygen consumption by rat liver mitochondria in the absence of added ADP (to stimulate state-3 respiration) was markedly elevated by CSE (at both 25 and 50 μg/mL) (Table 1) and further addition of ADP had no effect on oxygen consumption. This suggested that CSE is exerting uncoupling effects on mitochondrial respiration. This was further examined by first inhibiting oxidative phosphorylation using atractyloside (which blocks ADP transport into mitochondria). Under these conditions, addition of ADP had no stimulatory effect on oxygen consumption. On the contrary, respiration was markedly increased by the addition of CSE (Fig. 9). As a positive control, DNP caused a strong uncoupling of ADP-stimulated respiration (Fig. 9). AA (25 and 50 μM) also exhibited a mild uncoupling effect on succinate-stimulated respiration (Table 1). The extracts from bark, fruit and leaf as well as other CSE phenolic compounds such as cardanol and salicylic acid failed to exert any noticeable uncoupling effect on respiration (Table 1).

4 Discussion

In order to investigate the anti-diabetic properties of *A. occidentale*, hydroethanolic extracts of its leaves, bark, seeds and apples were examined in this study for their action on skeletal muscle glucose metabolism. These extracts were first tested for cytotoxicity and were found to

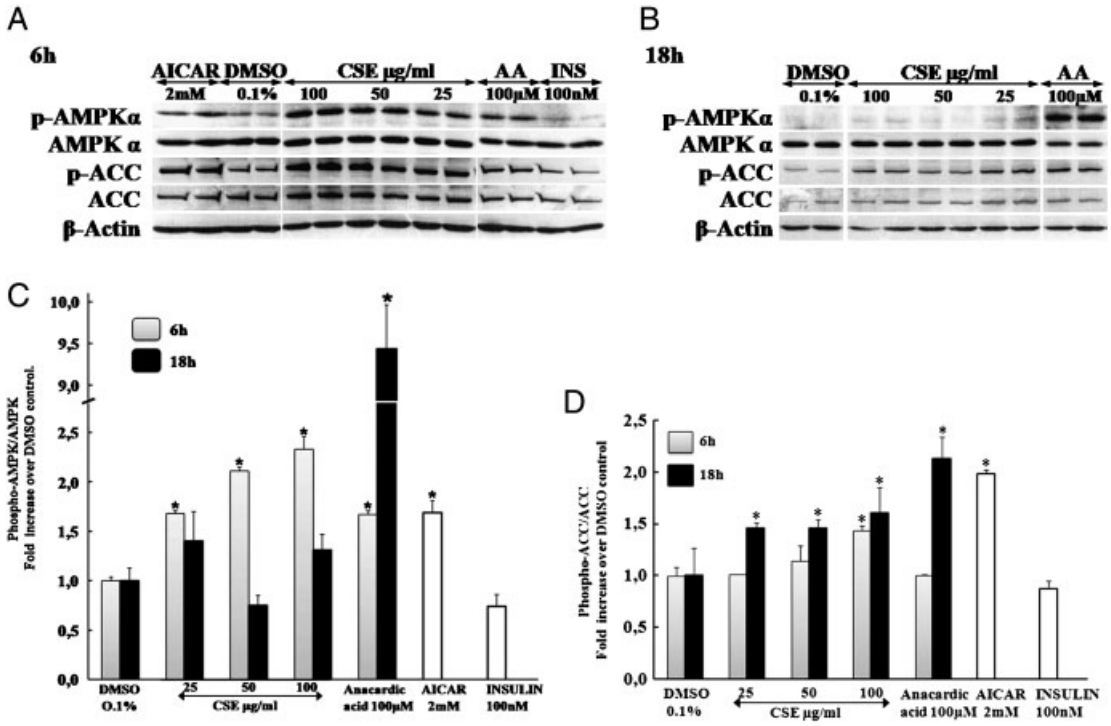

Figure 7. Effect of CSE and AA on AMPK signaling pathway. Total cell lysates of C2C12 myotubes (20 μg protein for each sample) were subjected to immunoblotting analysis using antibodies against total or phospho-AMPK, and total or phospho-ACC. (A) Myotubes were incubated with 2 mM AICAR for 20 min, 100 nM insulin for 30 min, 100 μM AA or 0, 25, 50 and 100 μg/mL CSE for 6 h. (B) Myotubes were incubated with 100 μM AA or 0, 25, 50 and 100 μg/mL CSE for 18 h. (C) The phosphorylation levels of AMPK were quantified using scanning densitometry and results are expressed as fold changes over DMSO (0.1%) control of the ratio of phospho-AMPK/total AMPK. All the values are normalized to corresponding β-actin values ($n = 3$). (D) The phosphorylation levels of ACC were quantified using scanning densitometry and results are expressed as fold changes over DMSO (0.1%) control of the ratio of phospho-ACC/total ACC. All the values are normalized to corresponding β-actin values ($n = 3$).

Table 1. Uncoupling and inhibition of oxidative phosphorylation in isolated mitochondria with extracts of different parts of cashew tree and their principal compounds

A. occidentale product	Δ basal O_2 consumption (%) (index of uncoupling)	ΔADP-stimulated O_2 consumption (%) (index of inhibition)	Relative percentage of glucose uptake
Seed extract (50 μg/mL)	+226/+275	−25/−43	41 ± 7
Seed extract (25 μg/mL)	+207/+240	−19/−30	29 ± 5
Bark extract (100 μg/mL)	−4/+11	−26/−31	−37 ± 9
Fruit extract (100 μg/mL)	+60/+65	−2/0	15 ± 6
Leaf extract (100 μg/mL)	−3/−4	−33/−37	−20 ± 8
AA (50 μM)	+119/+133	−51/−56	43 ± 4
AA (25 μM)	+116/+119	−33/−35	23 ± 5
Cardanol (50 μM)	+2/+17	−2/+2	3 ± 17
Salicylic acid (100 μM)	+15/+16	−2/−7	4 ± 7

Values given for basal oxygen consumption and ADP stimulated oxygen consumption are from two different experiments. Values given for percentage of glucose uptake are average ± SEM ($n = 3$).

have no significant effect on C2C12 myotube integrity during 18 h incubation, up to a concentration of 100 μg/mL.

One of the primary defects in type 2 DM is insulin resistance manifested as reduced uptake of blood glucose into skeletal muscle [20] and adipose tissues as well as increased liver glucose production. We therefore selected to assess whether the AOHE could modulate glucose uptake in C2C12 skeletal muscle cells as an initial approach; knowing that glucose uptake in insulin-responsive tissues is the rate-limiting step in glucose metabolism [21] and that, in view of the mass of skeletal muscle, glucose uptake in this tissue is of primary importance to systemic glucose homeostasis

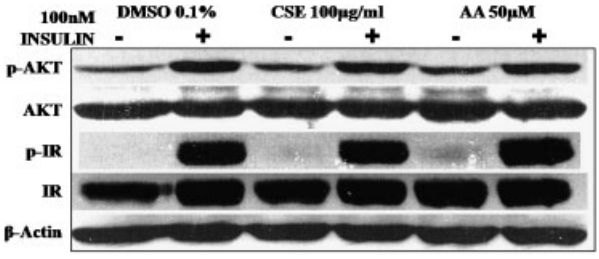

Figure 8. Effect of CSE and AA on IR/Akt signaling pathway. Myotubes were incubated with 100 µg/mL CSE or 50 µM AA for 18 h and the last 30 min incubation was with or without insulin (100 nM). Control incubations were done with DMSO (0.1%) vehicle with or without insulin. Total cell lysates of C2C12 myotubes (20 µg protein for each sample) were subjected to immunoblotting analysis using antibodies against total or phospho-Akt, and total or phospho-IR. The blot presented is representative of two separate experiments with similar results.

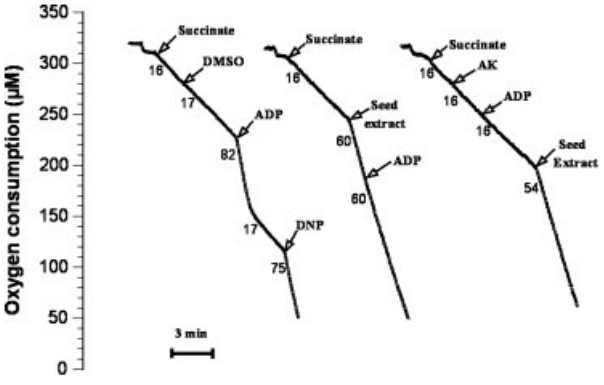

Figure 9. Effect of CSE on isolated rat liver mitochondria. Oxygen consumption by isolated rat liver mitochondria was measured with 6 mM succinate. State-3 respiration was measured in the presence of 200 µM ADP followed by 1 mM DNP. The second curve represents oxygen consumption by isolated rat liver mitochondria in the presence of 50 µg/mL CSE. An inhibitor of adenine nucleotide translocase, actralosydate potassium (AK) (2 mM) was added directly to the oxygen electrode chamber. The results represent the average of two experiments.

[22, 23]. Of all the extracts tested, only CSE showed significant stimulatory effect on glucose uptake. Extracts of other plant parts had no such effect, indicating that CSE likely contains active compounds, which can have potential anti-diabetic properties. The stimulation of glucose uptake by CSE was significant after 9 h of incubation, which further increased up to 18 h. This increase is likely due to either activation or increased number of plasma membrane glucose transporters. Further studies will be necessary to determine the exact mechanisms and implication of glucose transporters in the observed effects.

The deviation of our *in vitro* results on cashew tree leaf and bark extracts from the previous *in vivo* reports attributing anti-diabetic properties to these plant parts [9, 10] suggests that their anti-diabetic activity may be related to extra-muscular effects. Similarly, CSE may also have potential anti-diabetic actions on other insulin-responsive tissues or on pancreatic beta cells. Further studies will be necessary to address these issues.

AA is a major phenolic compound known to be present in the CSE and shown to have anti-oxidant and mitochondrial uncoupling effects [14]. It was therefore important to assess whether the observed glucose-uptake stimulating effect of CSE could be related to the presence of this phenolic compound in the CSE. The results showed that AA stimulates glucose uptake in C2C12 cells to an extent similar to CSE. Since AA is known to have mitochondrial uncoupling effects [14], its influence on glucose uptake was compared with a known uncoupler, DNP [24] and the oral hypoglycaemic drug, metformin [19]. Similar to AA, both DNP and metformin [25] enhanced glucose uptake. In addition, these compounds exhibited a strong correlation between their uncoupling capacity and glucose-uptake-stimulating effect (Table 1 and Fig. 4).

Our results have shown a synergistic effect on glucose uptake in the presence of AA and of high concentration of CSE (100 µg/mL) with insulin. Such effects were not noticed at lower CSE concentrations, possibly due to sub-optimal levels of the needed active compound(s) in the extract. It is known that derivatives of salicylic acids can inhibit protein tyrosine phosphatase-1B, which dephosphorylates and inactivates IRs and leptin receptors [26]. However, we have ruled out the possible involvement of this mechanism in our experimental conditions. Indeed, neither CSE nor AA were found to alter IR signaling pathway, as the insulin-stimulated phosphorylation of IR or Akt was not further enhanced in their presence (Fig. 8).

In addition, by virtue of their well-known anti-inflammatory action, salicylates can counter the effects of multiple proinflammatory pathways that are activated in obesity and participate in establishing the associated insulin resistance and type 2 diabetes. Indeed, salicylates can reduce insulin resistance in liver and peripheral tissues by modulating IKKβ and NFκB activity [27, 28] and were shown to increase insulin-stimulated glucose uptake by peripheral tissues [29, 30]. Whether AA- and CSE-stimulated glucose uptake involve such anti-inflammatory activity needs to be examined.

On the other hand, it has been suggested [31, 32] that the anti-diabetic effects of the thiazolidinedione class of compounds and of metformin are mediated in part *via* activation of AMPK probably by increasing the cellular AMP/adenosine triphosphate ratio through partial uncoupling of mitochondria and inhibition of respiratory complex I [19, 33]. Interestingly, in this study, it was observed that both CSE and AA cause AMPK activation in C2C12 cells and this is reflected in the phosphorylation of ACC, an AMPK substrate. CSE stimulation of AMPK phosphorylation was evident after 6 h of incubation and declined after 18 h, whereas ACC remained phosphorylated for a longer duration, as found previously in our laboratory

with extracts from several Canadian Boreal forest plants [25]. Interestingly, AA showed a stronger affect on ACC as well as AMPK phosphorylation at 18 h. Inasmuch as AA also acts as an uncoupler, its stimulation of glucose uptake could also be mediated by the activation of AMPK. This interpretation was confirmed for both CSE and AA through the use of the AMPK inhibitor, CC. Indeed, in the presence of CC, both CSE and AA were no longer able to increase glucose uptake.

It is well established that the activation of AMPK signaling leads to the increased synthesis and translocation of Glut4 transporters to the plasma membrane with the resultant increase in glucose uptake [24, 34, 35]. It is possible that activation of AMPK by CSE and AA in C2C12 cells in this study could have lead to similar effects and future studies should confirm this hypothesis. AMPK activation could also explain the synergistic effect of CSE or AA with insulin on the enhancement of glucose transport since IR and Akt phosphorylations were not affected by these plant-derived products. Alternatively, as it has been suggested earlier [33] for metformin, partial dysfunction of mitochondrial oxidative function by CSE may lead to accelerated glycolysis in order to compensate for reduced adenosine triphosphate production and this causes enhanced glucose uptake.

In summary, CSE was found to stimulate glucose uptake into skeletal muscle cells through the activation of AMPK, which can increase Glut4 synthesis as well as translocation to plasma membrane. Other possible mechanisms include increased glycolysis due to the dysfunction of mitochondrial oxidative phosphorylation. Our study validates the traditional use of cashew tree products in diabetes and points to some of its natural components that can serve to generate novel oral hypoglycaemic therapeutics.

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The authors have declared no conflict of interest.

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