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Journal of Ethnopharmacology

A *Citrullus colocynthis* fruit extract acutely enhances insulin-induced GLUT4 translocation and glucose uptake in adipocytes by increasing PKB phosphorylation --Manuscript Draft--

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First Author:	Farah Drissi
Order of Authors:	Farah Drissi Farid Lahfa Teresa Gonzalez Franck Peiretti Jean-Francois Tanti Mohamed Haddad Nicolas Fabre Roland Govers
Abstract:	<p>Ethnopharmacological relevance: <i>Citrullus colocynthis</i> (L.) Schrad is a common fruit in traditional medicine and used as remedy against various diseases, especially diabetes. Up to now, its anti-diabetic effects have been fully attributed to its enhancement of pancreatic insulin secretion. However, whether it also ameliorates insulin action in peripheral tissues has been unaddressed.</p> <p>Aim of the study: In the present study, using 3T3-L1 adipocytes as cell model, we have investigated whether colocynth fruit extracts affect insulin action.</p> <p>Materials and Methods: Various extracts were prepared from the <i>C. colocynthis</i> fruit and screened using a cell-based 96 well plate GLUT4 translocation assay. Promising extracts were further studied for their effects on glucose uptake and cell viability. The effect on insulin signal transduction was determined by Western blot and the molecular composition was established by LC-MS.</p> <p>Results: The ethyl acetate fractions of aqueous non-defatted extracts of seed and pulp, designated Sna1 and Pna1, acutely enhanced insulin-induced GLUT4 translocation. In accordance, both extracts increased insulin-stimulated cellular glucose uptake. Pna1, which displayed greater effects on GLUT4 and glucose uptake than Sna1, was further investigated and was demonstrated to increase GLUT4 translocation without changing the half-maximum dose (ED50) of insulin, nor changing GLUT4 translocation kinetics. At the molecular level, Pna1 was found to enhance insulin-induced PKB phosphorylation without changing phosphorylation of the insulin receptor. Pna1 appeared not to be toxic to cells and, like insulin, restored cell viability during serum starvation. By investigating the molecular composition of Pna1, nine compounds were identified that made up 87% of the mass of the extract, one of which is likely to be responsible for the insulin-enhancing effects of Pna1.</p> <p>Conclusions: The <i>C. colocynthis</i> fruit possesses insulin-enhancing activity. This activity may explain in part its anti-diabetic effects in traditional medicine. It also identifies the <i>C. colocynthis</i> as a source of a potential novel insulin enhancer that may prove to be useful to reduce hyperglycemia in type 2 diabetes.</p>

Journal of Ethnopharmacology

September 17th, 2020

Dear Editor,

I hereby electronically submit our manuscript, entitled " A *Citrullus colocynthis* fruit extract acutely enhances insulin-induced GLUT4 translocation and glucose uptake in adipocytes by increasing PKB phosphorylation" by Farah Drissi, Farid Lahfa, Teresa Gonzalez, Franck Peiretti, Jean-François Tanti, Mohamed Haddad, Nicolas Fabre, and Roland Govers.

This manuscript reports on the insulin-enhancing activity that we have found in extracts of the *Citrullus colocynthis* fruit. This fruit has been intensively used in traditional medicine for the treatment of various diseases, especially diabetes. Laboratory animal studies have demonstrated its long-term effects on pancreatic insulin secretion. Our study is the first one to investigate the acute effects of *Citrullus colocynthis* fruit extracts on an insulin-dependent physiological process, in our case the intracellular traffic of GLUT4 and the related cellular glucose uptake (both key elements in glucose homeostasis and diabetes) and to put this in perspective of the mechanism involved. Basically, we show that certain extracts specifically enhance insulin action, by acting on the insulin signaling cascade. As far as we know, this is the first time that such an activity is described for a plant extract or a plant metabolite. Moreover, by providing insight into the molecular composition of the extracts, we reveal which metabolites could be implicated in this activity. Since compounds that display such an insulin-enhancing activity may have clinical potential for the treatment of type 2 diabetes, we feel that our manuscript is of considerable interest to many scientists working in ethnopharmacology, phytomedicine and diabetes research, in particular those studying the colocynth and/or insulin action and those developing novel antidiabetic therapeutics.

We hope that this manuscript is suitable for publication in your journal.

Yours sincerely,
Roland Govers

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JOURNAL OF ETHNOPHARMACOLOGY CHECKLIST

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- In case of mixtures, are all the ingredients listed by their full scientific names with authorities?
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-

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- Are the conclusions critical and specific defining the current state of the art? YES NO
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- For reviews of individual species: Have you included a detailed review of local and traditional uses based on primary sources? YES NO NA

A *Citrullus colocynthis* fruit extract acutely enhances insulin-induced GLUT4 translocation and glucose uptake in adipocytes by increasing PKB phosphorylation

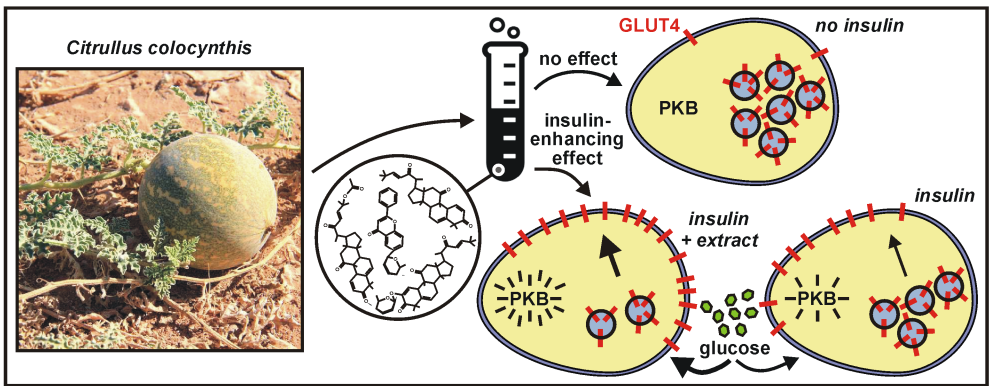
by

Farah Drissi, Farid Lahfa, Teresa Gonzalez, Franck Peiretti, Jean-François Tanti, Mohamed Haddad, Nicolas Fabre, Roland Govers

Highlights

- In traditional medicine, the colocynth is known for its anti-diabetic properties
- Colocynth fruit extract Pna1 acutely enhances insulin-induced GLUT4 translocation
- Pna1 acutely increases cellular glucose uptake in insulin-stimulated cells
- Pna1 enhances insulin action by increasing protein kinase B (PKB) phosphorylation
- Our study may explain the anti-diabetic properties of the colocynth

GRAPHICAL ABSTRACT



**A *Citrullus colocynthis* fruit extract acutely enhances
insulin-induced GLUT4 translocation and glucose uptake in adipocytes
by increasing PKB phosphorylation**

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Abstract

Ethnopharmacological relevance: *Citrullus colocynthis* (L.) Schrad is a common fruit in traditional medicine and used as remedy against various diseases, especially diabetes. Up to now, its anti-diabetic effects have been fully attributed to its enhancement of pancreatic insulin secretion. However, whether it also ameliorates insulin action in peripheral tissues has been unaddressed.

Aim of the study: In the present study, using 3T3-L1 adipocytes as cell model, we have investigated whether colocynth fruit extracts affect insulin action.

Materials and Methods: Various extracts were prepared from the *C. colocynthis* fruit and screened using a cell-based 96 well plate GLUT4 translocation assay. Promising extracts were further studied for their effects on glucose uptake and cell viability. The effect on insulin signal transduction was determined by Western blot and the molecular composition was established by LC-MS.

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Conclusions: The *C. colocynthis* fruit possesses insulin-enhancing activity. This activity may explain in part its anti-diabetic effects in traditional medicine. It also identifies the *C. colocynthis* as a source of a potential novel insulin enhancer that may prove to be useful to reduce hyperglycemia in type 2 diabetes.

Keywords

Type 2 Diabetes; Metabolism; Signal transduction; Chromatography; *Citrullus colocynthis*; GLUT4; Insulin enhancers; Traditional medicine

1. Introduction

Type 2 diabetes (T2D) prevalence is rapidly increasing around the world and associated with obesity, unhealthy diets, and a sedentary lifestyle (Lam and LeRoith, 2012). The onset of T2D is closely related to the desensitization of skeletal muscle, adipose tissue, and liver for insulin, also known as insulin resistance (Fujimoto, 2000; Thule, 2012). Insulin resistance is characterized by a reduced response of skeletal muscle and adipose tissue to regular insulin levels, in particular concerning the uptake of glucose from the blood during the postprandial phase. To compensate for this insulin resistance, the pancreas secretes high amounts of insulin, just until its β cells cannot further sustain the increased production of insulin, resulting in hyperglycemia. Glucose transporter GLUT4 is responsible for the insulin-regulated uptake of glucose from the blood during the postprandial phase (Fukumoto et al., 1989; James et al., 1988; James et al., 1989). In between postprandial phases, insulin levels are low and GLUT4 is retained within intracellular compartments in muscle and adipose tissue. Once insulin levels rise during the postprandial phase, intracellular signaling cascades are activated, leading to the translocation of GLUT4 from the intracellular compartments towards the cell surface, allowing these tissues to take up glucose (Bogan, 2012; Govers, 2014).

Despite the use of numerous drugs for the treatment of T2D, the search for novel anti-diabetic compounds is not over, mainly due to unsatisfactory results at long term and the serious side effects of certain treatments, for example those involving the insulin-sensitizing thiazolidinediones (TZDs) (Fonseca, 2013; Kumar et al., 2017). Plants have been scientifically studied for over half a century for their effects on diabetes and have appeared to be useful sources for the identification of novel bioactive compounds (e.g. metformin predecessors (Bailey and Day, 1989) and acarbose (Shu, 1998)). Moreover, plant extracts have been used as anti-diabetic remedy for even much longer, especially in traditional (herbal) medicine. Plants that have been intensely studied for their anti-diabetic properties include *Allium sativum* (garlic), *Gymnema sylvestre* (gurmar), *Trigonella foenum graecum* (fenugreek), *Momordica charantia* (bitter melon, ampalaya), *Ficus benghalensis* (banyan fig), *Artemisia absinthium* (wormwood) and *Citrullus colocynthis* (colocynth, bitter apple, bitter cucumber) (Bailey and Day, 1989; Li et al., 2015; Patel et al., 2012).

The *Citrullus colocynthis* (L.) Schrad is a member of the Cucurbitaceae family. Plants of this family are tolerant to drought, but intolerant to wet and poorly drained soil and frost (Hussain et al., 2014). The *C. colocynthis*, of which the appearance resembles that of watermelon, is a perennial herbaceous vine that possesses yellow flowers, producing 15-30 round fruits that are 7-10 cm in size (Shi et al., 2014). It is widely distributed in desert areas around the world, especially in the Sahara desert and the South-West part of Asia, including the Arabian desert, Pakistan, Iran, and India, but also in China (Hussain et al., 2014). In all these places, parts of this plant have been used in traditional medicine, especially for the treatment of constipation. More generally, its seeds, pulp, rind, leaves, and roots have been reported to be used to treat diabetes, intestinal disorders (constipation, indigestion, ulcer, dysentery, gastroenteritis, colic pain), bacterial infections, cancer, hypertension, rheumatism, common cold, cough, toothache, wounds, jaundice, asthma, and bronchitis (Dhakad et al., 2017; Hussain et al., 2014). At present, the plant (especially its fruit) is the topic of pharmacological investigations, mainly

concerning its anti-diabetic and (cucurbitacin-related) anti-cancer effects (Hussain et al., 2014). Other current areas of investigation include those concerning its anti-hyperlipidemic (cholesterol- and triglyceride-lowering), anti-inflammatory, analgesic, insecticidal, and anti-microbial activities (Dhakad et al., 2017; Gurudeeban et al., 2010; Hussain et al., 2014). In addition to its medicinal value, the *Citrullus colocynthis* is also well-appreciated for its marked nutritional value, due to its high protein amount and the elevated concentration of essential amino acids (Gurudeeban et al., 2010; Hussain et al., 2014).

In clinical studies, the powder of dried colocynth fruits appeared to reduce fasting blood glucose (FBG) and glycosylated hemoglobin (HbA1c) levels in T2D patients (Barghamdi et al., 2016; Huseini et al., 2009). In accordance, in animal studies, seed, pulp and rind from the colocynth fruit but even the plant roots and leaves have been demonstrated to possess anti-diabetic activity (Abdel-Hassan et al., 2000; Agarwal et al., 2012; Amin et al., 2017; Benariba et al., 2012; Dallak et al., 2009; Ebrahimi et al., 2016; Huseini et al., 2009; Ostovar et al., 2020; Shi et al., 2014). Long-term treatments with colocynth extracts have been demonstrated to improve hyperglycemia through preservation and restoration of β cell mass (Amin et al., 2017; Sebbagh et al., 2009) and stimulation of synthesis and secretion of insulin (Dallak et al., 2009). Interestingly, four animal studies have suggested that extracts of this plant also have acute blood glucose lowering effects. First, in normoglycemic rats, the administration of an aqueous root extract 30 minutes before an oral glucose tolerance test (OGTT) reduced glycemia already within the first 30 minutes of the OGTT (Agarwal et al., 2012). Second, seed and rind extracts reduced glycemia in overnight food-starved normal and streptozotocin (STZ)/alloxan-induced diabetic rats and rabbits within 60-90 minutes of administration (Abdel-Hassan et al., 2000; Azzi et al., 2015; Lahfa et al., 2017). For the moment, these acute effects remain unexplored. Hypothetically, these extracts might acutely affect glycemia via insulinotropic, insulin-mimetic, or insulin-enhancing/sensitizing actions. In favor of an insulinotropic action is the finding that colocynth extracts acutely induce the release of insulin from isolated pancreatic islets (Benariba et al., 2013; Ebrahimi et al., 2016; Nmila et al., 2000). However, their acute effects in STZ/alloxan-treated animals rather provides evidence for an insulin-mimetic action (Abdel-Hassan et al., 2000; Azzi et al., 2015; Lahfa et al., 2017). At present, there are no indications that support the presence of insulin-enhancing or insulin-sensitizing activity in colocynth extracts.

In the current study, the acute anti-diabetic effects of the colocynth were investigated at the cell biological level. Various colocynth extracts were prepared and tested for their acute effects on insulin-induced GLUT4 translocation in adipocytes. The results of the present study highlight two promising non-toxic ethyl acetate extracts from the colocynth fruit, Sna1 from seed and Pna1 from pulp, which enhance the effect of insulin towards both GLUT4 translocation and cellular glucose uptake. The molecular composition of these extracts was determined in order to provide insight into the nature of the compound responsible for this insulin-enhancing activity.

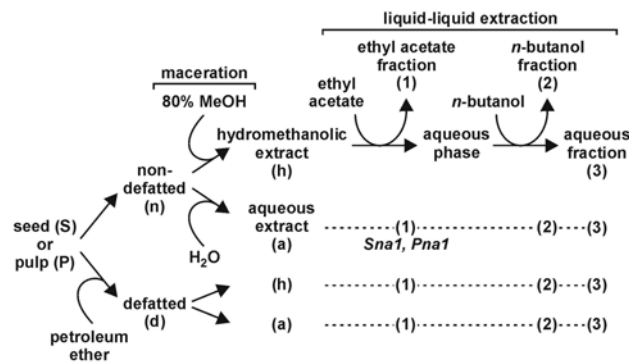
2. Material and Methods

2.1. Materials

3T3-L1 preadipocytes were obtained from ATCC/LGC Standards (Molsheim, France). Fetal bovine serum was from D. Dutscher (Brumath, France). Media, Hepes, and Alexa488-conjugated goat-anti-mouse secondary antibody were from Invitrogen (Cergy Pontoise, France). Insulin was from Lilly (Suresnes, France), monoclonal anti-HA (haemagglutinin) antibody (ascites) was from Covance (Emeryville, CA), and HRP-conjugated secondary antibodies were from Jackson ImmunoResearch (Interchim; Montlucon, France). Antibodies against ERK, phospho-ERK, PKB, phospho-PKB (T308 and S473), AMPK, phospho-AMPK, and anti-ACC were from Cell Signaling Technology (Ozyme; Saint-Cyr-l'École, France), anti-insulin receptor β (C19) and anti-phospho-insulin receptor (pY1162/1163) from Santa Cruz (Heidelberg, Germany), anti-tubulin from Sigma-Aldrich (Souffelweyersheim, France), and anti-phospho-ACC (S79) from Millipore (Merck; Molsheim, France). Methanol, acetonitrile and formic acid were HPLC grade. These and all other chemicals were obtained from Sigma-Aldrich (Souffelweyersheim, France). Black clear-bottom well plates were from Greiner Bio-One (Courtaboeuf, France).

2.2. Extraction and fractionation of plant material

Fruits from *Citrullus colocynthis* (L.) Schrad were harvested at maturity in Ain Sefra (Algeria; GPS coordinates 32°44'23.3"N 0°34'43.9"E) and dried for six weeks in a temperate and dark place. The plant was formally identified in the Antibiotics Antifungal Laboratory: Physical-Chemistry, Synthesis and Biological Activity (Department of Biology, Faculty of Sciences, Tlemcen University, Algeria) and its name verified at <http://www.theplantlist.org>. The extraction protocol outlined below was repeated twice with fruits from the same harvest (2016) and another time with fruits from another harvest (2017). For degreasing, crushed seed and pulp were treated with petroleum ether in a Soxhlet for 4 hours at 60°C, followed by evaporation in a Buchi R 114 rotavapor at 45°C. Defatted and untreated seed and pulp were macerated for 2 h at 50°C and subsequently for 24 h at room temperature in either water or 80% methanol. The 80% methanol extracts (after evaporation of the methanol) and the crude aqueous extracts were partitioned by two successive liquid-liquid extractions involving ethyl acetate and *n*-butanol, respectively, resulting in ethyl acetate, *n*-butanol, and remaining aqueous fractions (fractions 1, 2, and 3 in Fig. 1). Upon lyophilization, crude aqueous extracts and final aqueous fractions were dissolved in cell culture medium DMEM (Dulbecco's modified Eagle's medium), crude methanol extracts were dissolved in a mixture of DMEM and DMSO (1:1), and ethyl acetate and *n*-butanol fractions were dissolved in DMSO.



< **Figure 1.** Schematic outline of the preparation of *C. colocynthis* fruit extracts. Seed and pulp extracts were prepared as indicated. Further details are described in *Materials and Methods*. The two major insulin-enhancing extracts, Sna1 and Pna1, are indicated within the diagram. >

2.3. Cell culture

3T3-L1 preadipocytes were cultured and differentiated as described previously (Talantikite et al., 2016). The cDNA encoding HA-GLUT4 (GLUT4 with an HA epitope tag in its first luminal domain (Quon et al., 1994)) inserted into the retroviral pBABE-puro vector has been described previously (Govers et al., 2004). To express HA-GLUT4 in adipocytes, preadipocytes were infected with retrovirus (Zaarour et al., 2012). Adipocytes were used for experiments 9–10 days after the onset of differentiation, with medium renewed every day. Cells were starved for 2 h in serum-free DMEM supplemented with 2 g/l BSA before stimulation.

2.4. Fluorescence-based techniques

The fluorescence-based assay for the detection of GLUT4 at the cell surface has been described in detail previously (Govers et al., 2008; Zaarour et al., 2012). Briefly, HA-GLUT4-expressing cells grown in 96 well plates were fixed and immunolabelled using an anti-HA antibody and a fluorescent Alexa488-conjugated goat-anti-mouse antibody, after the cells had been incubated for 40 minutes at 37°C with or without insulin in the absence or presence of extracts at a concentration of 4, 20, or 100 µg/ml. The final concentration of DMSO in all experiments involving living cells was 0.5% (v/v). At the end of the experiments, the 96 well plates were analyzed using the bottom-reading mode in a fluorescence microtiter plate reader (FLUOstar Optima, BMG Labtechnologies; Offenburg, Germany; excitation 485 nm, emission 520 nm). An epifluorescence microscope was also used to analyze the plates to ensure that changes in fluorescence truly represented changes in cell-surface GLUT4 amounts. Non-immune mouse IgG, titrated along with anti-HA antibody on control cells not expressing HA-GLUT4, was used in the GLUT4 assays to determine non-specific signal.

Cell viability assays were performed using Alamar Blue compound resazurin. Adipocytes, cultured in black clear-bottom 96 well plates, were treated for 1 h (in the absence of serum and preceded by a 2 h serum deprivation) or 24 h (in the presence of 2% serum) with various concentrations of extracts Sna1 and Pna1, followed by a 60 min

incubation with 100 μM resazurin in serum- and phenol red-free DMEM, supplemented with 2 g/l BSA. As a control for toxicity, cells were incubated with various concentrations of the hydrogen peroxide-generating enzyme glucose oxidase. For long-term serum-deprivation experiments, cells were washed once with serum-free DMEM supplemented with 2 g/l BSA and incubated for 1 to 4 days in serum-free DMEM/BSA, with medium renewed every day. Fluorescence (excitation 544 nm, emission 590 nm) was measured using a fluorescence microtiter plate reader (FLUOstar Optima, BMG Labtechnologies; Offenburg, Germany).

2.5. Glucose uptake

Glucose uptake was measured as described previously (Martinez et al., 2010). Briefly, adipocytes grown in 12 well plates were incubated for 2 h in DMEM, supplemented with 2 g/l BSA and for 5 min in Krebs Ringer Phosphate buffer (KRP; 12.5 mM Hepes (pH 7.4), 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO₄, 1 mM CaCl₂, 0.4 mM NaH₂PO₄, 0.6 mM Na₂HPO₄, 2 g/l BSA). Subsequently, the cells were incubated for 40 min at 37°C in KRP in the absence or presence of 1 or 100 nM insulin and extracts Sna1 (20 $\mu\text{g}/\text{ml}$) or Pna1 (100 $\mu\text{g}/\text{ml}$). To control cells, 50 μM cytochalasin B was added 1 min before the addition of 0.1 mM 2-[³H]deoxyglucose (2-DOG; 0.28 $\mu\text{Ci}/\text{well}$; PerkinElmer; Courtaboeuf, France) to determine assay background. After a 3 min 2-DOG incubation, cells were extensively washed with ice-cold PBS, and lysed in 1% Triton X-100. Radioactivity was measured by scintillation counting.

2.6. Western blot analyses

Adipocytes were incubated for 2 h in DMEM supplemented with 2 g/l BSA and for 5 or 15 min in the absence or presence of 1 or 100 nM insulin and 100 $\mu\text{g}/\text{ml}$ Pna1 or 0.5% DMSO. Cells were lysed in ice-cold buffer containing 20 mM Tris pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and protease inhibitor cocktail Complete (Roche; Meylan, France). Nuclei and insoluble material were removed by centrifuging for 10 min at 20.000 g at 4°C. Equal amounts of protein were subjected to SDS-PAGE and transferred to PVDF membrane. Membranes were incubated with the indicated antibodies. HRP-conjugated secondary antibodies were visualized using chemiluminescence reagent (PerkinElmer; Courtaboeuf, France) and a CCD camera-based imager (LAS-4000 mini, GE Healthcare, Vélizy-Villacoublay, France). Relative intensities were quantified using MultiGauge software (Fujifilm).

2.7. Extract chromatography

Ultra-high-performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) analyses were performed on a UHPLC-PDA-CAD-LTQ Orbitrap XL instrument (Ultimate 3000, Thermo Fisher Scientific, Hemel Hempstead, UK). Separations were performed using an Acquity UHPLC® BEH C18 1.7 μm column (150 mm \times 2.1 mm, Waters, Milford, MA, USA) preceded by a VanGuard™ BEH C18 1.7 μm (Waters) guard column using an UHPLC Ultimate 3000 system (Dionex, Sunnyvale,

CA, USA) controlled by Chromeleon Xpress 6.8 software (Dionex). The column compartment temperature was set at 40°C and the injected sample volume was 4 µl. The mobile phase consisted of milliQ water (solvent A) and CH₃CN (solvent B) each containing 0.1% formic acid. Flow rate was set at 400 µl/min. The column was equilibrated in 10% B and elution was carried out with the following steps: 10% B to 90% B from 0 to 28 min, followed by a decrease to initial conditions 10% B for 2 min and kept at 10% B for 2.5 min before the next injection. High Resolution MS analyses were carried out under positive and negative modes at 30,000 resolving power (full width at half maximum (FWHM) at 400 m/z) using an ESI probe. The mass scanning range was m/z 100–2000, the capillary temperature was 275 °C and the ionization spray voltage was 4.2 kV (positive mode) and 3.0 kV (negative mode). Mass measurement was externally calibrated just before starting the experiment. Each full MS scan was followed by data dependent MS/MS on the most intense ion using the collision-induced dissociation (CID) fragmentation mode at 35% normalized collision energy, with an isolation width of 2 Da and activation Q set at 0.250. The obtained spectra were compared with those from literature (Chawech et al., 2015; Dhakad et al., 2017; Gurudeeban et al., 2010; Hussain et al., 2014), in order to determine their identity, based on the molecular formula deduced from Tr, M+HCOO⁻ and M+NH₄⁺ values (see Table 1). The CAD profile was used to quantify the relative presence of the compounds in Pna1 (normalized to 100%).

2.8. Statistics

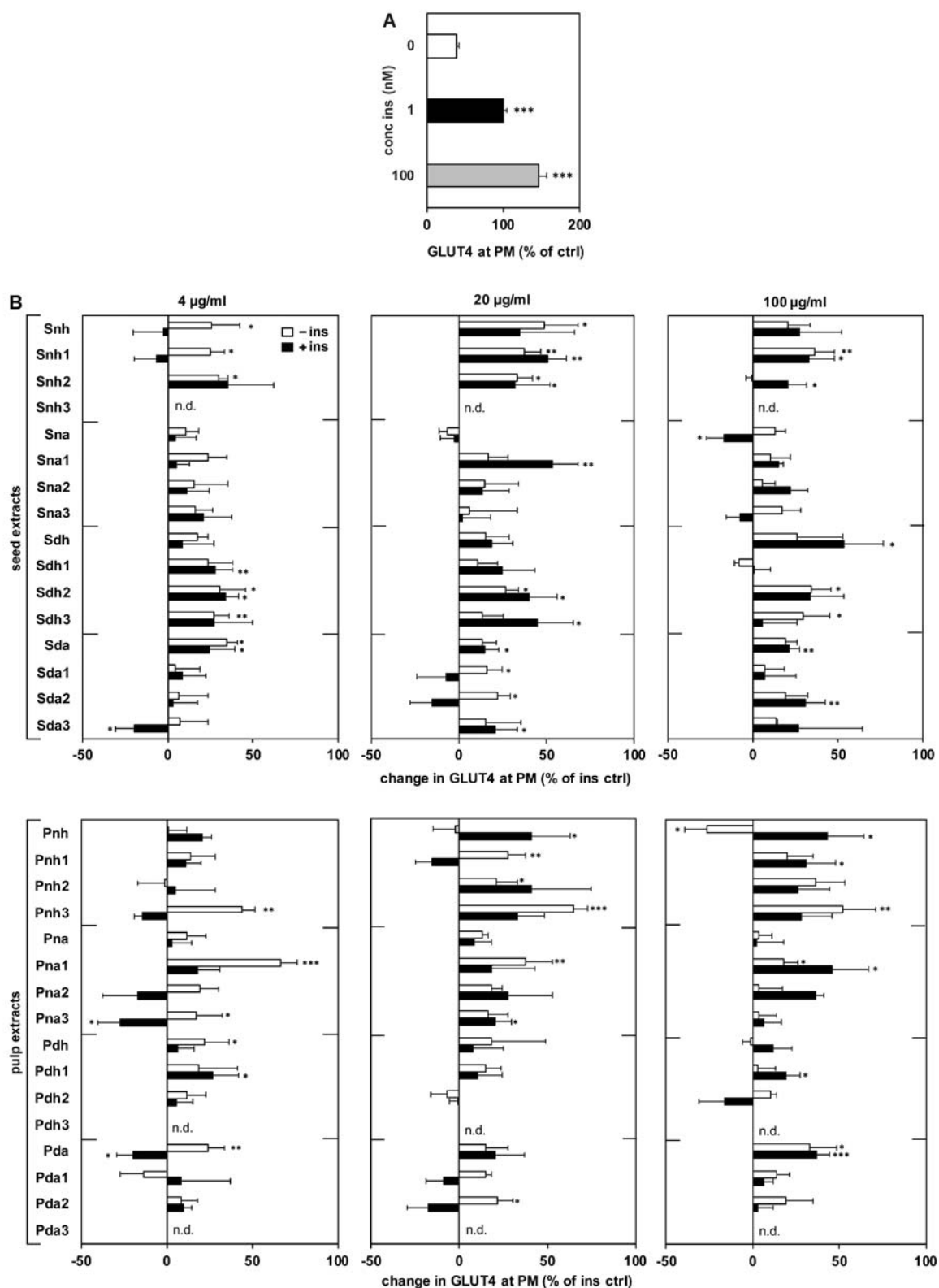
All data are presented as means ± S.D. All experiments were repeated at least three times. Representative experiments are shown. For fluorescence measurements in 96 well plates, four wells were used for each condition. Statistical significance between individual data points was analyzed by two-tailed *t*-test. Differences between insulin dose-response curves were analyzed by extra-sum-of-squares *F*-tests using sigmoidal log(dose)-response curve fitting (GraphPad Prism). Differences between curves of GLUT4 translocation kinetics were analyzed by extra-sum-of-squares *F*-tests using Two Phase Association curve fitting (GraphPad Prism). Differences between data sets were considered statistically different when $P < 0.05$.

3. Results

3.1. Identification of *Citrullus colocynthis* fruit extracts that enhance the insulin-induced appearance of GLUT4 at the cell surface of 3T3-L1 adipocytes

Extracts were prepared as outlined in Figure 1 from defatted ('d') or non-defatted ('n') seed ('S') and pulp ('P') from the *Citrullus colocynthis* fruit by maceration in water ('a') or in 80% methanol ('h'), followed by extractions using ethyl acetate and *n*-butanol, respectively, resulting in ethyl acetate fractions ('1'), *n*-butanol fractions ('2') and the remaining aqueous fractions ('3'). The resulting extracts were subjected to a cell-based GLUT4 screen in 96 well plates, in which the effects of the extracts on cell surface GLUT4 levels were measured, both in the absence as well as in the presence of insulin (Fig. 2). Treatment of the cells in the absence of extracts revealed an insulin dose-dependent increase in cell surface GLUT4 levels, with a 2.5- and 4-fold increase at 1 and 100 nM insulin, respectively (Fig. 2A). To ascertain that certain extracts would be able to enhance the effect of insulin on GLUT4 translocation, extracts were screened in the absence and presence of the sub-maximal dose of 1 nM insulin. The 3T3-L1 adipocytes, expressing GLUT4 with an HA epitope tag within its first extracellular domain (known to behave identically as endogenous GLUT4 (Quon et al., 1994; Sano et al., 2007; Shewan et al., 2000)), were cultured in 96 well plates and incubated for 40 minutes at 37°C with or without insulin in the absence or presence of extracts at a concentration of 4, 20, or 100 µg/ml. Subsequently, cell surface amounts of HA-GLUT4 were measured by quantitative indirect immunofluorescence immunolabeling of the intact adipocytes using an anti-HA antibody.

Screening the *Citrullus colocynthis* seed and pulp extracts revealed that certain concentrations of several extracts, in particular the methanolic extracts of non-defatted and defatted seed (Snh1-2 and Sdh1-3), increased cell surface GLUT4 levels in adipocytes in the absence of insulin. This increase was maintained when the cells were co-stimulated with 1 nM insulin. In sharp contrast, 20 µg/ml of seed extracts Sna1 and Sdh3 and 100 µg/ml of pulp extract Pna1 increased cell surface GLUT4 considerably more in the presence than in the absence of insulin. Remarkably, degreasing of seed and pulp prior to aqueous maceration abolished the insulin-enhancing activity of these two ethyl acetate fractions (i.e. Sna1 vs Sda1, and Pna1 vs Pda1).

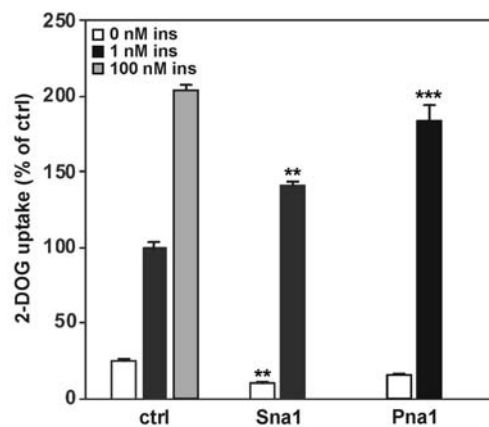


< **Figure 2.** Screening of *C. colocynthis* extracts for their effects on cell surface GLUT4 levels. (A) Adipocytes were incubated for 40 min at 37°C with the indicated concentrations of insulin in the absence of extracts. Relative amounts of GLUT4 at the plasma membrane (PM) were measured and expressed as percentage of cell surface

GLUT4 levels in the presence of 1 nM insulin. **(B)** Adipocytes were incubated for 40 min in the absence or presence of 1 nM insulin and 4, 20, or 100 $\mu\text{g}/\text{ml}$ of extracts. Relative amounts of GLUT4 at the plasma membrane were measured. Extract-induced changes in cell surface GLUT4 levels in the absence or presence of insulin were calculated and expressed as percentage of GLUT4 PM levels in the presence of 1 nM insulin without extract. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. n.d., not determined. >

3.2. *Citrullus colocynthis* extracts Sna1 and Pna1 increase insulin-induced glucose uptake

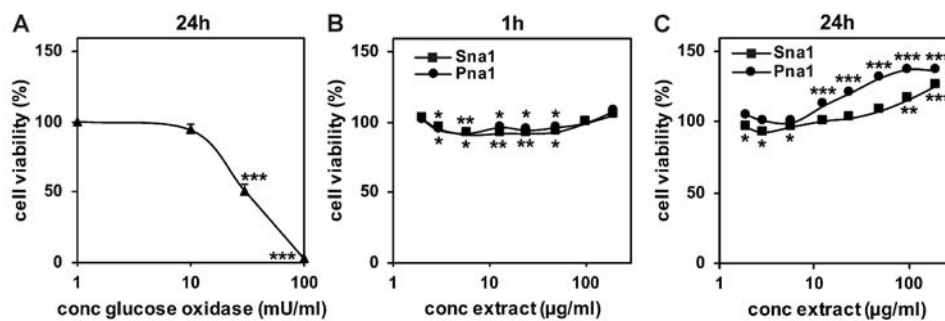
Since the Sna1 ethyl acetate extract of non-defatted water-macerated seed and its pulp homologue Pna1 both enhanced insulin-induced GLUT4 translocation (albeit at different concentrations) while only marginally increasing cell surface GLUT4 levels in non-stimulated adipocytes, these two extracts were tested for their effect on cellular glucose uptake, both in the absence as well as in the presence of 1 nM insulin (Fig. 3). In the absence of extracts, 100 nM insulin doubled the amount of glucose taken up when compared with 1 nM insulin, in accordance with its effects on GLUT4 (Fig. 2A). Interestingly, at the concentrations that enhanced insulin-induced GLUT4 translocation, Sna1 and Pna1 significantly enhanced insulin-stimulated glucose uptake by 55 and 110%, respectively, without increasing basal glucose uptake. Importantly, when these two extracts were prepared again from other fruits from the same harvest and from fruits from the harvest from the next year, similar results were obtained for both glucose uptake and GLUT4 translocation (data not shown).



< **Figure 3.** *C. colocynthis* extracts Sna1 and Pna1 enhance insulin-induced glucose uptake. 3T3-L1 adipocytes were incubated for 40 min at 37°C in the absence or presence of 1 or 100 nM insulin, 20 $\mu\text{g}/\text{ml}$ Sna1, and 100 $\mu\text{g}/\text{ml}$ Pna1, followed by a 3 min incubation with radiolabelled 2-DOG. Amounts of cellular 2-DOG were determined and expressed as percentage of 2-DOG uptake at 1 nM insulin in the absence of extracts. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, vs control ('ctrl') incubations without extracts. >

3.3. Extracts *Sna1* and *Pna1* do not impair cell viability

Any compound or extract that enhances insulin action has potential for a possible therapeutic use in insulin-resistance and type 2 diabetes, but only if there are no major toxicity issues. Therefore, these two insulin-enhancing extracts were analyzed for their effect on adipocyte viability using the Alamar Blue compound resazurin (Fig. 4). In control incubations, the hydrogen peroxide-generating enzyme glucose oxidase dose-dependently decreased adipocyte viability (Fig. 4A). In sharp contrast, *Sna1* nor *Pna1* substantially reduced cellular viability, when present in the extracellular medium for 1 hour (Fig. 4B) or 24 hours (Fig. 4C). A marginal 10% reduction in cell viability was seen at low/intermediate concentrations, but not at high concentrations (up to 200 $\mu\text{g/ml}$). Moreover, when present for 24 hours, high concentrations of these two extracts enhanced cellular viability (or at least improved the cellular redox balance).

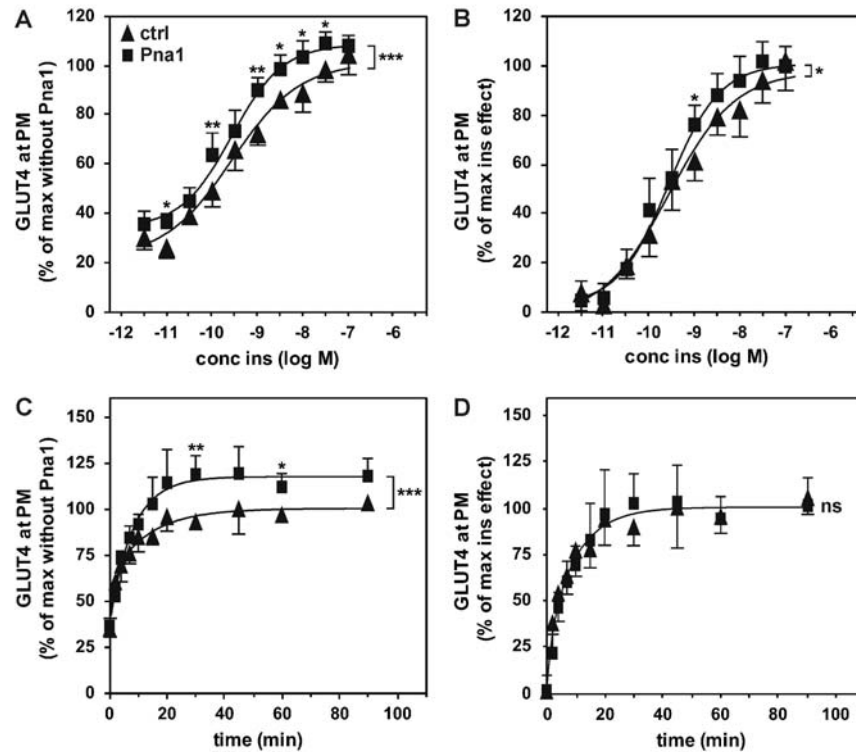


< **Figure 4.** Extracts *Sna1* and *Pna1* do not impair cell viability. (A-C) Adipocytes were incubated for 24 h with various concentrations of the hydrogen peroxide-generating enzyme glucose oxidase (A), or for 1 h (B) or 24 h (C) with various concentrations of *Sna1* and *Pna1*. Subsequently, viability was measured using resazurin and expressed as percentage of viability of untreated cells. >

3.4. Extract *Pna1* enhances *GLUT4* translocation without altering insulin sensitivity and *GLUT4* translocation kinetics

Since *Pna1* increased insulin-induced glucose uptake more than *Sna1* without displaying any sign of toxicity, this extract was studied in further detail. In order to determine whether, in addition to its insulin-enhancing action, *Pna1* also sensitized the cells for insulin regarding *GLUT4* translocation, cells were incubated for 40 minutes with various concentrations of insulin in the absence or presence of 100 $\mu\text{g/ml}$ *Pna1*, followed by the analysis of cell surface *GLUT4* levels and expression of these levels as percentage of maximum cell surface *GLUT4* levels in the absence of *Pna1* (Fig 5A). This revealed that *Pna1* increased cell surface *GLUT4* at submaximal insulin concentrations. This was accompanied by a non-significant shift in insulin's ED50 from 0.383 nM in the absence to 0.216 nM in the presence of *Pna1* (P 0.0538). The absence of insulin-sensitization was also evident when these data were used to calculate the increase in cell surface *GLUT4* above background for the various concentrations of insulin and expressed as percentage of the maximum increase in cell surface *GLUT4* levels, in the absence or presence of

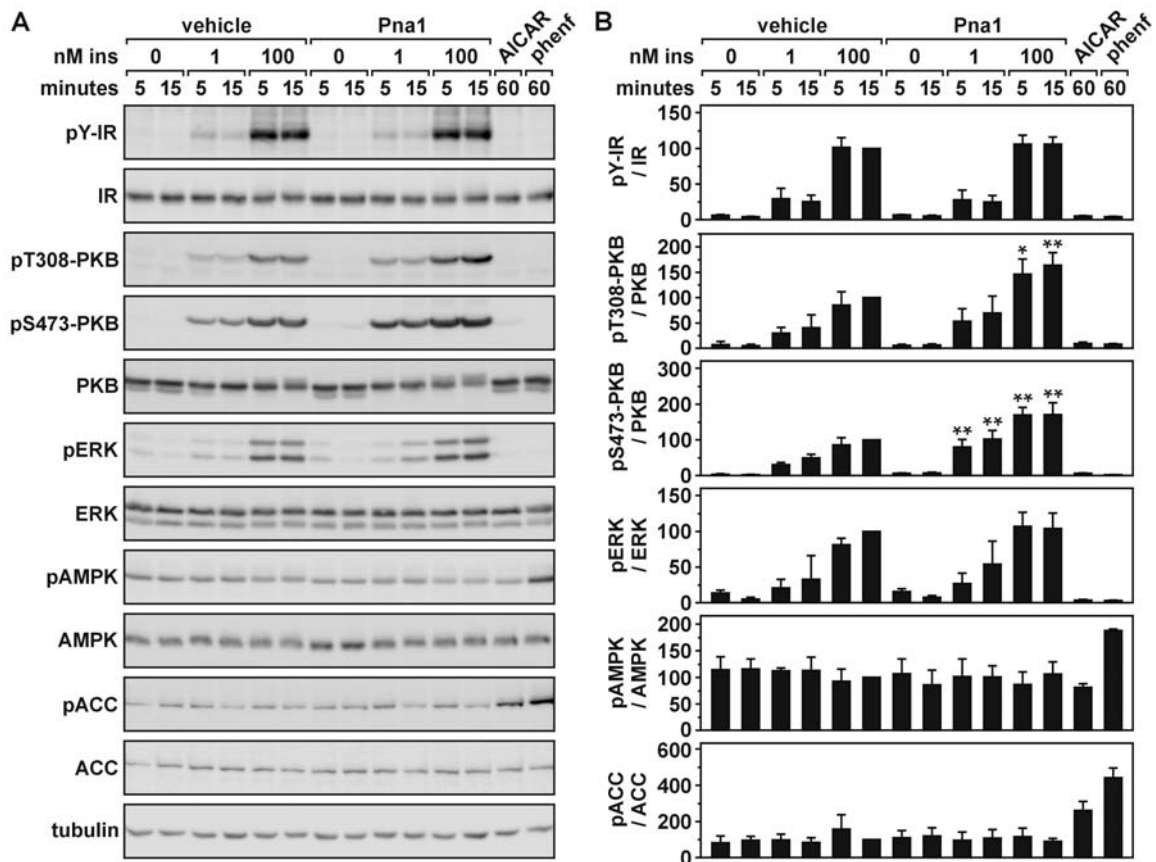
Pna1 (Fig. 5B). In order to investigate whether Pna1 changed the kinetics of insulin-induced GLUT4 translocation, adipocytes were incubated for different time periods with 1 nM insulin in the absence or presence of 100 μ g/ml Pna1, followed by the analysis of the amount of GLUT4 at the plasma membrane. While the resulting curves were significantly different (Fig. 5C), expressing the increases as percentage of the maximum insulin effect in the absence and presence of Pna1 demonstrated that Pna1 did not alter relative translocation kinetics (Fig. 5D).



< **Figure 5.** Extract Pna1 does not alter adipocyte insulin sensitivity nor GLUT4 translocation kinetics. **(A)** Adipocytes were incubated for 40 min with various concentrations of insulin in the absence or presence of 100 μ g/ml Pna1. The amount of GLUT4 at the plasma membrane (PM) was determined and expressed as percentage of maximum GLUT4 PM levels in the absence of Pna1. ED50 of ctrl and Pna1 is 0.383 nM and 0.216 nM, respectively ($P = 0.0538$). **(B)** Data from (A) were transformed in dose-response curves in which the increases induced by the various insulin concentrations were related to the maximum increases in the absence and presence of Pna1. **(C)** Adipocytes were treated for up to 90 min with 1 nM insulin in the absence or presence of 100 μ g/ml Pna1. Plasma membrane GLUT4 levels were determined and expressed as percentage of maximum PM levels without Pna1. **(D)** Data from (C) were transformed into increases in cell surface GLUT4 levels above GLUT4 PM levels in non-stimulated adipocytes and expressed as percentage of maximum increases in the absence and presence of Pna1. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. n.s., non-significant. Asterisks above symbols indicate point-wise comparisons. Asterisks beside graphs indicate graph-wise comparisons. >

3.5. Extract Pna1 enhances insulin action by increasing PKB phosphorylation

To determine how extract Pna1 enhanced insulin action and GLUT4 translocation, the effect of Pna1 on insulin signaling was investigated (Fig. 6). Adipocytes were incubated for 5 or 15 minutes in the absence or presence of 1 or 100 nM insulin and 100 $\mu\text{g}/\text{ml}$ Pna1. Subsequently, cell lysates were subjected to immunoblotting using various antibodies. This demonstrated that insulin dose-dependently induced the phosphorylation of the insulin receptor ('IR'), serine/threonine kinase PKB (Protein Kinase B, also known as Akt), and serine/threonine Extracellular signal-Regulated Kinases ERK1 and ERK2, two mitogen-activated protein kinases (MAPK) that are downstream effectors of the Shc/GRB2/SOS pathway. As expected, insulin treatment did not result in activation of the serine/threonine AMP-activated kinase (AMPK), as measured by phosphorylation of AMPK and its substrate acetyl-CoA carboxylase (ACC), in contrast to AICAR and phenformin, two known activators of AMPK. Pna1 by itself did not induce phosphorylation of any of these proteins. However, when co-incubated with insulin for 5 or 15 minutes, Pna1 enhanced the phosphorylation of PKB on residues Thr308 and Ser473 without altering the phosphorylation of the insulin receptor, nor enhancing the insulin-induced phosphorylation of ERK1 and ERK2.

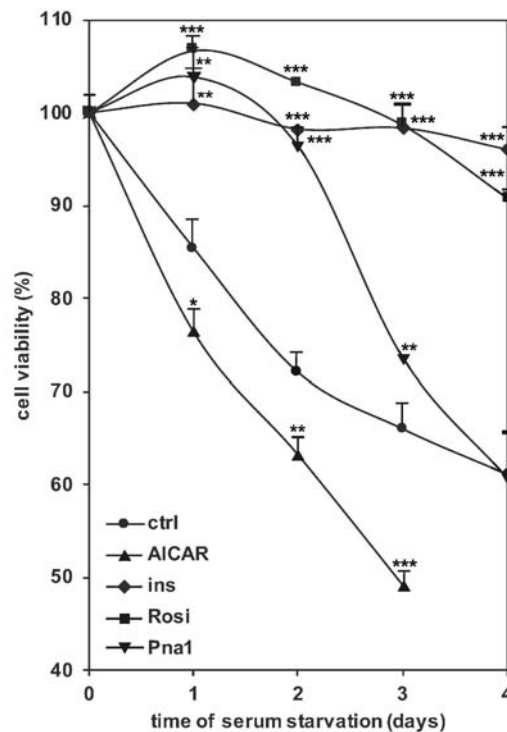


< **Figure 6.** Extract Pna1 enhances insulin action by increasing PKB activity. (A) Adipocytes were incubated for 5 or 15 min in the absence or presence of 1 or 100 nM insulin and 100 $\mu\text{g}/\text{ml}$ Pna1. Vehicle-treated cells were incubated with 0.5% DMSO in the absence of Pna1. As a control for AMPK activation, adipocytes were incubated for 60

min with 2 mM AICAR or 2 mM phenformin. Lysates were subjected to SDS-PAGE and immunoblotting using the indicated antibodies. In the ERK blots, the upper band is ERK1 and the lower band is ERK2 (**B**) The results of four independent experiments were quantified and expressed as percentage of the amount of signal of cells that had been incubated for 15 min with 100 nM insulin in the absence of Pna1. *, $P < 0.05$ and **, $P < 0.01$, vs identical incubations without Pna1. >

3.6. Extract Pna1 mimics insulin in enhancing cell survival

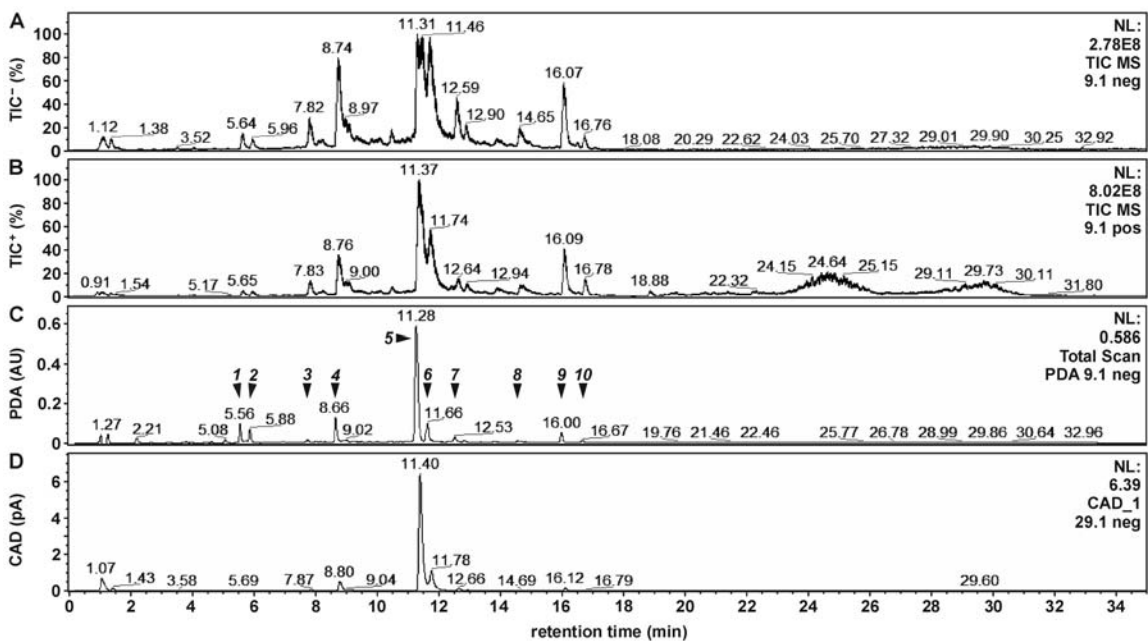
Given that extract Pna1 enhanced insulin action towards GLUT4 and PKB activity, and that both insulin and PKB are known potentiating factors in cell survival, Pna1 was tested for its effect on serum deprivation-induced cell death (Fig. 7). Mature adipocytes were incubated for up to 4 days without serum in the absence or presence of AMPK activator AICAR, insulin, PPAR γ agonist rosiglitazone, or extract Pna1. Subsequently, cell viability was measured using Alamar Blue reagent resazurin. This demonstrated that both insulin and rosiglitazone efficiently protected the cells from serum starvation-induced reductions in cell viability, in contrast to AICAR which significantly deteriorated cell viability. Remarkably, Pna1 fully protected the cells from the 15% and 28% reduction in cell viability during the first and second day of starvation, respectively. After four days of starvation, viability of Pna1-treated cells was no longer different from control cells.



< **Figure 7.** Extract Pna1 enhances cell viability during serum starvation. Adipocytes were deprived from fetal bovine serum (FBS) for 1 to 4 days in the absence or presence of 2 mM AICAR, 1 nM insulin, 1 μ M rosiglitazone, or 100 μ g/ml Pna1. Viability was measured using resazurin and expressed as percentage of the viability of non-deprived cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, vs control ('ctrl') cells. >

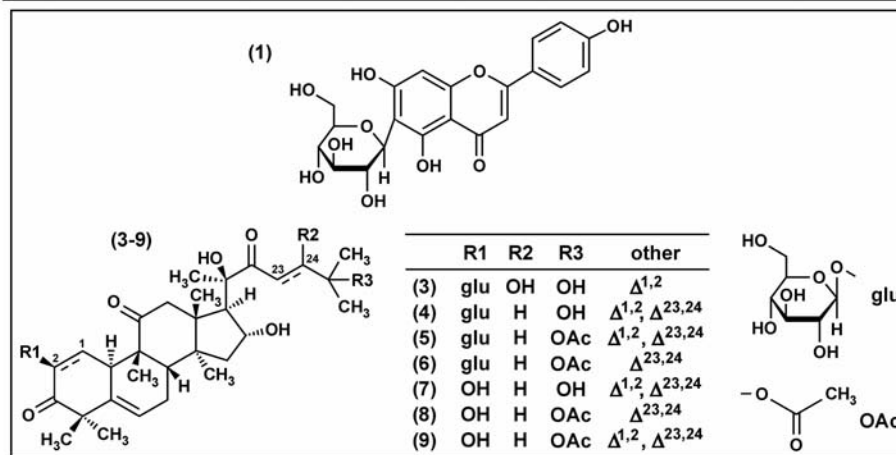
3.7. Identification of plant metabolites in extract Pna1

To have an idea of which molecules within extract Pna1 could be involved in its insulin-enhancing effect, the secondary metabolites present in this extract were identified by means of LC–PDA-CAD-MS. This revealed that ten compounds were present in Pna1 to a considerable extent (Fig. 8). Almost all compounds were detectable by positive and negative ion ESI MS and UV (Fig. 8A to 8D, respectively). The CAD trace is the less sensitive of the used detectors but is independent of the structure of the analyte (universal detector) and thus proportional to the amount of detected compound. Therefore, CAD data were used to quantify the relative concentrations of the compounds (Table 1). The CAD response shows that all compounds of Pna1 were detectable by MS and UV. Most of the compounds were identified by comparing the obtained spectra with those from literature (Chawech et al., 2015; Dhakad et al., 2017; Gurudeeban et al., 2010; Hussain et al., 2014). Two flavonoids were detected, isovitexin (compound 1) and a second flavonoid of which we were not able to firmly determine its identity, but which we believe is a methylated form of either vitexin or isovitexin (compound 2), with molecular weights of 432 and 462 g/mol, respectively (Table 1). Seven known cucurbitacins were identified (compounds 3-9), with Tr values reaching from 7.75 to 16 min: the 2-O- β -D-glucopyranosyl forms of cucurbitacins J, I, E, and B, and cucurbitacins I, B, and E. Glucopyranosyl-cucurbitacin E (compound 5), also known as colocynthin and elaterinide, accounted for more than 60% of the extract. A final peak was retrieved at Tr 16.67 min (compound 10). The corresponding metabolite had the molecular configuration of a cucurbitacin but had not been described yet in literature. We speculate that one of these 10 compounds, or possibly a combination of several, displays the insulin-enhancing activity that was discovered in Pna1. Of note, the first two peaks visible in all spectra (at Tr 1.07 and 1.43 in the CAD spectrum) corresponded to a total of 4.5% surface area and represented compounds that were not retained on the column. Analyses of these two peaks demonstrated that pectin (galacturonic acid polymers) and one or several hexoses were the main compounds of the first peak while the second first peak corresponded to citric acid. The LC–PDA-CAD-MS analysis of extract Sna1 (data not shown) demonstrated that these two initial peaks made up 25% of the total CAD surface area of Sna1, with the first larger peak corresponding to hexoses and malic acid and the second smaller peak to citric acid. The molecular composition of the rest of extract Sna1 largely resembled that of Pna1 in that all compounds of Pna1 (except compound 10) were also present in Sna1. Compounds 1 to 4 were respectively 8-, 11-, 1.8-, and 1.8-fold more present in Sna1 than in Pna1. In contrast, compounds 5 to 9 were respectively 15, 60, 40, 90, and 60% less present in Sna1 than in Pna1.



< **Figure 8.** Liquid Chromatography–PhotoDiode Array–Charged Aerosol Detector–ESI Mass Spectrometry (LC–PDA–CAD–MS) analyses of Pna1. **(A)** Negative total ion current chromatogram ((-)TIC). **(B)** (+)TIC. **(C)** PDA. **(D)** CAD. Peaks indicated in C are listed and further described in Table 1. >

N°	Tr (min)	% area	UV λ max (nm)	negative ion m/z	positive ion m/z	MW (g/mol)	molecular formula	compounds	ref
1	5.56	0.17	218, 270, 332	431.0973	433.1129	432	C ₂₁ H ₂₀ O ₁₀	isovitexin (apigenin-6-C-glucoside)	Yoshikawa et al., 2007
2	5.88	0.14	218, 270, 348	461.1078	463.1233	462	C ₂₂ H ₂₂ O ₁₁	methoxy-(iso)vitexin***	-
3	7.75	0.97	224, 255	739.3521*	712.3903**	694	C ₃₆ H ₅₄ O ₁₃	2-O- β -D-glucopyranosyl-cucurbitacin J	Yoshikawa et al., 2007
4	8.66	4.87	236	721.3421*	694.3798**	676	C ₃₆ H ₅₂ O ₁₂	2-O- β -D-glucopyranosyl-cucurbitacin I	Yoshikawa et al., 2007
5	11.28	62.68	234	763.3530*	731.3907**	718	C ₃₈ H ₅₄ O ₁₃	2-O- β -D-glucopyranosyl-cucurbitacin E (colocynthin, elaterinide)	Yoshikawa et al., 2007
6	11.66	14.48	254	765.3685*	738.4065**	720	C ₃₈ H ₅₆ O ₁₃	2-O- β -D-glucopyranosyl-cucurbitacin B (arvenin I)	Nayab et al., 2006
7	12.53	1.67	230	559.2896*	-	514	C ₃₀ H ₄₂ O ₇	cucurbitacin I (elatericin B, JSI-124)	Hussain et al., 2014
8	14.75	0.30	223, 285	603.3154*	576.3529**	558	C ₃₂ H ₄₆ O ₈	cucurbitacine B (amarin)	Hussain et al., 2014
9	16.00	1.53	230, 270	601.3002*	574.3375**	556	C ₃₂ H ₄₄ O ₈	cucurbitacin E (α -elaterin)	Hussain et al., 2014
10	16.67	0.27	225, 306	631.3104*	604.3481**	586	C ₃₃ H ₄₆ O ₉	unknown	-



< **Table 1.** LC-PDA-CAD-ESI/High Resolution MS data of *C. colocynthis* extract Pna1. Most abundant compounds are listed, together with their MS data and their relative presence in Pna1 (normalized to 100%). Corresponding peaks are indicated in Fig. 8C. Lower panel displays the molecular structures of the identified compounds. Δ indicates the presence of a double bond. * M+HCOO⁻ adduct; ** M+NH₄⁺ adduct; *** tentatively annotated according to HR MS and MS/MS analysis. >

4. Discussion

So far, two of our studies (Azzi et al., 2015; Lahfa et al., 2017), as well as two studies from other laboratories (Abdel-Hassan et al., 2000; Agarwal et al., 2012), have demonstrated acute blood glucose-lowering effects of colocynth extracts in rodents and rabbits. Since in these studies plasma insulin levels have not been determined, it is unclear whether the blood glucose-lowering effect of colocynth extracts was due to a change in either pancreatic insulin secretion or glucose uptake by muscle and adipose tissue. Here, for the first time, the acute effects of colocynth extracts on glucose uptake in adipocytes *in vitro* have been investigated. Two of the extracts, Sna1 and Pna1, enhanced the insulin-induced translocation of GLUT4 from intracellular storage sites towards the plasma membrane and accordingly increased insulin-induced glucose uptake. Several of our findings suggested that pulp extract Pna1, which increased glucose uptake more than its seed homologue Sna1, increased GLUT4 translocation and glucose uptake by acting on the same intracellular signaling cascade as the one employed by insulin. First, Pna1 increased cell surface GLUT4 levels mostly at intermediate insulin concentrations (~ 1 nM), without augmenting its surface levels at high insulin concentrations (≥ 100 nM). Second, Pna1 increased GLUT4 translocation without changing translocation kinetics, similarly to the unaltered translocation kinetics when cells are stimulated with different concentrations of insulin (Berenguer et al., 2010). Third, while Pna1 did not enhance signaling via the Shc/GRB2/SOS/ERK and AMPK pathways (Fig. 6) (Taniguchi et al., 2006), it rapidly increased insulin-induced phosphorylation of PKB on both threonine residue 308 and serine residue 473. PKB phosphorylation is known to lead to its activation and to the translocation of GLUT4 to the plasma membrane (Cong et al., 1997; Hill et al., 1999; Kohn et al., 1996). Therefore, we assume that Pna1 enhanced GLUT4 translocation through its effect on PKB. Remarkably, at 100 nM insulin, Pna1 further increased phosphorylation of PKB on both residues (Fig. 6) without enhancing insulin-induced GLUT4 translocation (Fig. 5), suggesting that at 100 nM insulin, in the absence of Pna1, a sufficient number of PKB molecules was activated to stimulate GLUT4 translocation to a full extent. This is in accordance with other studies that have shown that, while being a crucial node in insulin signaling, only a limited number of PKB molecules need to be phosphorylated for maximal PKB-relayed downstream effects (Ng et al., 2010; Tan et al., 2012). Interestingly, insulin is known to induce phosphorylation of PKB on Thr308 and Ser473 via two distinct kinases. Phosphorylation on Thr308 within PKB's activation loop by PDK1 leads to partial PKB activation, while phosphorylation on Ser473 within PKB's carboxy-terminal hydrophobic motif primarily by mammalian target of rapamycin complex 2 (mTORC2), but also by the related kinase DNA-PK, is required for full activation of PKB (Hemmings and Restuccia, 2012; Manning and Toker, 2017). Fully active PKB is known to play an important role in cellular growth, proliferation, and survival (Hemmings and Restuccia, 2012; Manning and Toker, 2017). In accordance, the effect of Pna1 on PKB is likely to be implicated in the Pna1-induced enhancement of cell viability. When present during 24 hours in medium containing 2% fetal bovine serum (FBS) instead of the regular 10%, the Pna1-induced increase in cell viability (affected by the 2% FBS) might be mediated via an effect of Pna1 on PKB-dependent signal transduction initiated by growth factors present in FBS (Fig. 4C). Pna1 also enhanced cell viability when cells were cultured for several days in the complete absence of serum (Fig.

7). While Pna1 fully protected the cells from a 28% reduction in cell viability during the first 48 hours of deprivation, Pna1 no longer displayed any beneficial effect on viability after 96 hours of deprivation, when cell viability was reduced to 60%. This transient effect of Pna1 on cell viability may have been caused by an enhancement of the PKB signaling induced by growth factors from serum that was ongoing when the serum was removed. Alternatively, it cannot be excluded that Pna1 by itself may induce phosphorylation of PKB to a very minor (non-measurable) extent, which may possibly also result in a temporary increase in cell viability. In any case, no major toxicity issues were revealed for the two ethyl acetate fractions of aqueous extracts of seed and pulp, Sna1 and Pna1. This is in accordance with previous reports that have demonstrated that, at least *in vivo*, aqueous colocynth extracts are not toxic (Dhakad et al., 2017; Gurudeeban et al., 2010), in contrast to alcoholic extracts that at elevated doses lead to diarrhea, which is most likely related to the laxative effect of the colocynth fruit (Shi et al., 2014).

At present, the mechanism by which Pna1 enhances PKB phosphorylation has not been identified. Pna1 does not appear to act on protein tyrosine phosphatase PTP1B, implicated in the dephosphorylation of the insulin receptor, since PTP1B inhibitors are known to increase insulin receptor tyrosine phosphorylation, in contrast to Pna1 (Erbe et al., 2009; Xie et al., 2003; Zhang et al., 2006). Therefore, Pna1 must have acted (directly or indirectly) on the insulin signaling cascade, anywhere between IRS1 and PKB, on either IRS1, PI 3-kinase, plasma membrane PIP3 levels, PDK1, or PKB (Hemmings and Restuccia, 2012; Taniguchi et al., 2006). Several hypotheses explain how Pna1 may increase PKB phosphorylation. First, Pna1 may increase PKB phosphorylation by inhibiting one of the two phosphatases that dephosphorylate PKB: protein phosphatase 2A (PP2A), dephosphorylating Thr308, or PH-domain leucine-rich-repeat-containing protein phosphatase PHLPP, which dephosphorylates Ser473 (Hemmings and Restuccia, 2012; Manning and Toker, 2017). These phosphatases assure the feedback inhibition of PKB activation, which limits PKB phosphorylation in magnitude and in time. Second, Pna1 might inhibit phosphatase and tensin homolog PTEN, responsible for the dephosphorylation of PIP3 into PIP2, which is a negative feedback mechanism for insulin-activated PI 3-kinase (Chen, C.Y. et al., 2018). This would lead to increased PIP3 concentrations within the plasma membrane and in an enhanced downstream signaling, starting with increases in the amount of PDK1 and PKB at the plasma membrane (Manning and Toker, 2017). Accordingly, a reduction in PTEN activity leads to increased PKB phosphorylation, especially in insulin-stimulated cells (Ma et al., 2018). Third, Pna1 may inhibit the insulin-activated inositol hexakisphosphate (IP6) kinase IP6K1 and thus the formation of IP7 out of IP6. IP7 competes with PIP3 for binding to the pleckstrin homology (PH) domain of PKB (Mackenzie and Elliott, 2014; Zhang et al., 2019). Therefore, if Pna1 inhibits IP6K1, this would lead to a reduction in the IP7-dependent limitation of PKB activation, allowing more PKB molecules to dock onto PIP3 at the plasma membrane and to be phosphorylated by PDK1. Interestingly, IP6K1 ablation enhances hormone-induced PKB phosphorylation on Thr308 and Ser473 without augmenting basal PKB phosphorylation (Chakraborty et al., 2010). Finally, the Pna1-induced enhancement of PKB phosphorylation may involve the nutrient-sensing kinase mTORC1 or mTORC1-regulated S6 kinase 1 (S6K1) (Dann et al., 2007; Fenton and

Gout, 2011; Yoon, 2017). Both kinases phosphorylate IRS1 on serine residues, especially under conditions of insulin resistance, which disrupts its interaction with either PI 3-kinase or the insulin receptor, dependent on the serine residue that is phosphorylated, leading to submaximal PKB activation upon insulin stimulation (Boura-Halfon and Zick, 2009; Cops and White, 2012; Fenton and Gout, 2011; Yoon, 2017; Zhang et al., 2019). Hence, both mTORC1 and S6K1 may be targets of Pna1. Interestingly, as in Pna1-treated cells, inhibition of S6K1 increases PKB phosphorylation in the presence but not in the absence of insulin (Chen, J. et al., 2018). Of note, S6K1 also phosphorylates mTORC2, which leads to its inhibition, resulting in a decrease in the mTORC2-mediated phosphorylation of PKB on Ser473 (Cops and White, 2012; Manning and Toker, 2017) and probably also in a reduction in Thr308 phosphorylation, since Ser473 phosphorylation stabilizes phosphorylation at Thr308 (Manning and Toker, 2017). Thus, if Pna1 inhibits S6K1, this would not only result in a reduction in IRS1 serine phosphorylation but also in an independent increase in mTORC2 activity, both contributing to increases in insulin-induced PKB phosphorylation and glucose uptake (Shum et al., 2016; Um et al., 2004). In summary, several direct and indirect feedback mechanisms exist that limit the phosphorylation and activation of PKB and of which one may be the target of Pna1. These include phosphatases PP2A, PHLPP, and PTEN, and kinases IP6K1, mTORC1, and S6K1. Whether and which of these enzymes are implicated in the Pna1-induced increase in PKB phosphorylation and GLUT4 translocation remains to be determined.

While colocynth extracts have been widely studied for their anti-diabetic effects *in vivo*, the active components responsible for these actions have not been identified so far (Hussain et al., 2014). The metabolites that were identified in extract Pna1 are cucurbitacins B (corresponding concentration in 100 µg/ml of extract: 0.54 µM), E (2.8 µM), and I (3.2 µM), their 2-O-β-D-glucopyranosyl forms (20.1 µM, 87.3 µM, and 7.2 µM, respectively), 2-O-β-D-glucopyranosyl-cucurbitacin J (1.4 µM), the flavone isovitexin (0.39 µM), and a methylated form of either vitexin or isovitexin (0.30 µM). Together, these nine molecules accounted for 87% of the composition of Pna1 and were also detected in extract Sna1. At present it is not known which of these molecules are responsible for the insulin-enhancing effects of Pna1. Remarkably, Sna1 increased insulin-induced GLUT4 translocation at a concentration of 20 µg/ml, but not at 4 µg/ml nor at 100 µg/ml. In addition, while 4 µg/ml of Pna1 increased cell surface GLUT4 levels in non-stimulated cells but not in insulin-treated cells, 100 µg/ml of Pna1 resulted in the opposite effects. We hypothesize that this is due to the presence of stimulating as well as inhibitory molecules in these extracts (concerning both insulin-mimicking and insulin-enhancing activity), each of which displays maximum effects at a distinct concentration. The nine molecules that were identified in extracts Pna1 and Sna1 have been reported to display a wide range of biological activities. Flavonoid glycoside isovitexin is known for its antioxidant and anti-inflammatory effects, via activation and inhibition of the Nrf2 and NFκB pathways, respectively (He et al., 2016; Lv et al., 2016). It also displays anti-cancer (Ganesan and Xu, 2017; He et al., 2016), insulinotropic (Folador et al., 2010) and α-glucosidase-inhibiting activity (Choo et al., 2012). Cucurbitacins B, E, I, at prolonged treatment, are all cytotoxic, inhibit STAT3 (Chan et al., 2010; Liu et al., 2020; Oi et al., 2016; Seo et al., 2014), disrupt cytoskeleton organization (Knecht et al., 2010; Sari-

Hassoun et al., 2016; Yin et al., 2008), inhibit PKB phosphorylation (Si et al., 2019; Zhou et al., 2017; Zhu et al., 2018), and are particularly studied for their anti-microbial (Hussain et al., 2014) and anti-cancer effects (Garg et al., 2018; Li et al., 2018; Si et al., 2019). Cucurbitacin B was also found to display insulinotropic activity (Kim et al., 2018). Anti- but also pro-oxidant effects, likely dependent on experimental conditions, have been reported for cucurbitacins B and I (Lin et al., 2019; Lopez-Haber and Kazanietz, 2013; Ren et al., 2015; Yang and Kim, 2018). Cucurbitacin E was reported to increase AMPK activity (Wu et al., 2016; Zha et al., 2015) and to display anti-inflammatory (Qiao et al., 2013; Shang et al.) and anti-obesity effects (Murtaza et al., 2017). Much less is known about the biological activity of the 2-O- β -D-glucopyranosyl forms of cucurbitacins B, E, and I, nor of 2-O- β -D-glucopyranosyl-cucurbitacin J. Cytotoxicity has been reported for glucopyranosyl-cucurbitacin B (Minh et al., 2015). Antioxidant and proliferation-inhibitory activity was found for the glucopyranosyl forms of cucurbitacins B and E (Tannin-Spitz et al., 2007a; Tannin-Spitz et al., 2007b) and anti-allergic activity has been demonstrated for glucopyranosyl-cucurbitacin E (Yoshikawa et al., 2007). Since the glucopyranosyl forms of cucurbitacins B, E, and I were the three major constituents of extracts Pna1 and Sna1, it seems likely that one or several of these three molecules display insulin-enhancing activity.

Various plant metabolites have been shown to display beneficial effects on GLUT4 or on cellular glucose uptake, either in adipocytes or in myocytes. Some of these metabolites acutely increase cell surface GLUT4 levels, but, unlike Pna1, always display insulin-mimicking activity. These include the sesquiterpenic phytohormone abscisic acid (Bruzzone et al., 2012; Lanzerstorfer et al., 2014), alkaloids berberine (Lee et al., 2006; Zhou et al., 2007) and mahanine (Nooron et al., 2017), phenolic compounds such as caffeic acid (Huang et al., 2009), cinnamic acid (Huang et al., 2009), cyanidin-3-rutinoside (Choi et al., 2017), daidzein (Cheong et al., 2014), epigallocatechin gallate (Takagaki et al., 2019; Ueda et al., 2008), gallic acid (Lanzerstorfer et al., 2014; Prasad et al., 2010), isorhamnetin (Jiang et al., 2019), piceatannol (Minakawa et al., 2012), quercetin (Jiang et al., 2019), scopoletin (Jang et al., 2020), and tannic acid (Lanzerstorfer et al., 2014), amino acid 4-hydroxyisoleucine (Jaiswal et al., 2012) and the iridoid oleuropein (Fujiwara et al., 2017). The molecular mechanisms by which these phytochemicals act on GLUT4 most often remain to be determined. Exceptions are abscisic acid (Bruzzone et al., 2012), mahanine (Nooron et al., 2017), and quercetin (Jiang et al., 2019), which were shown to act on GLUT4 by inducing phosphorylation of PKB, and berberine, which appeared to affect GLUT4 through AMPK and PTP1B (Chen et al., 2010; Lee et al., 2006; Zhou et al., 2007). Our study has indicated that also the colocynth contains insulin-mimicking activity, at least regarding GLUT4, for instance in the 80% methanolic extracts of the colocynth seed (Snh1-2 and Sdh1-3), though this was not further investigated.

5. Conclusion

The current study has demonstrated that the cucurbitacine glycoside-rich ethyl acetate fraction of the colocynth non-defatted aqueous pulp extract displays insulin-enhancing but not insulin-mimicking activity towards PKB phosphorylation, cell surface GLUT4

levels, and cellular glucose uptake. To the best of our knowledge, this is the first time that such an activity is described for a plant extract or a plant compound. The insulin-enhancing activity of the colocynth fruit described here is likely to contribute to its anti-diabetic effects in traditional medicine. Since this extract appeared not to be toxic to cells, not even during prolonged treatments, it likely contains a compound that may have potential for the development of a novel therapeutic strategy for the treatment of T2D. It is important to note that Pna1 by itself did not activate PKB, since, at least in mice, a life-long enhancement of hormone-induced PKB activation has been demonstrated not to lead to an increase in tumor incidence (Chakraborty et al., 2010), in contrast to unconditional increases in PKB activity, that are detrimental with respect to tumor development (Manning and Toker, 2017).

Declaration of competing interests

The authors declare no conflict of interests.

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Author contributions

RG, FD, NF, and FL conceived the study. RG, NF, and FD coordinated the study. TG, FD, and RG performed the experiments. TG, NF, RG, FP, JFT, FD, and MH analyzed the data. RG and FD wrote the manuscript, with contributions from NF, JFT, FP, FL, TG, MH. The final version of the manuscript was reviewed and approved by all authors.

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