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Research Article

Anti-Diabetic Effects of Cucurbitacins from *Ibervillea lindheimeri* on Induced Mouse Diabetes

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Mexico has a great tradition of using medicinal plants against diabetes. For example, species from the genus *Ibervillea* traditionally known as "wereque" in Mexican popular medicine have a long ethnobotanical history as anti-diabetic agents. Previous studies by our group indicated that ethyl acetate extract from *Ibervillea lindheimeri* (*I. lindheimeri*) roots reduced glucose in mice with chemically induced diabetes. In this work, the primary metabolites of the ethyl acetate extract of *I. lindheimeri*; 23,24-dihydrocucurbitacin D (1); 2-O-β glucopyranosyl-23,24-dihydrocucurbitacin D (2), and acetylated compounds (3) and (4) obtained from 1 and 2, respectively, were evaluated as anti-hyperglycemic agents in a murine model of chemically induced diabetes. Our results showed that cucurbitacins 1, 2, and 4 reduced glycemia in diabetic CD1 mice compared to the control diabetic group. In addition, the results suggest that compounds 1, 2, and 4 promote glucose transporter type 4 (Glut4) translocation to the plasma membrane (PM) mainly in epididymal adipose tissue (EAT), AMP-activated protein kinase (AMPK) activation in soleus muscle (SM) or dual activation of AMPK, and protein kinase B (AKT) in EAT in an insulin-independent manner when compared to controls. All results together indicate that the isolated cucurbitacins are responsible for the anti-diabetic properties of *I. lindheimeri* acting predominantly on adipose tissue and call attention to this species as a new source of anti-diabetic compounds.

1. Introduction

Diabetes mellitus type 2 is the most common metabolic illness in the world. It is characterized by hyperglycemia, dyslipidemia, and other metabolic disorders. Although there are several treatments for the symptoms and complications of this disease, such as insulin secretagogues, insulin sensitizers, and α-glycosidase inhibitors [1], none of these treatments guarantees complete glycemic control. Moreover, all of them have uncomfortable secondary effects for patients [2]. Therefore, it is necessary to discover or design new compounds that regulate glucose homeostasis without undesirable secondary effects. Plants are a vital source of unique pharmacologically active compounds; nature has fashioned an unlimited array of molecular entities with the potential for bioactive agents, drug development, and bases for developing new, more effective drugs [3]. Moreover, 50% of the approved drugs during the past 30 years are directly or indirectly from natural products [4]. Nevertheless, there is a lack of data about their biological activity, efficacy, and action mechanisms because of their enormous variety and complexity.

In Mexico, the species *Ibervillea lindheimeri* from the family Cucurbitaceae, which is locally known as "wereque," grows [5]. This plant is a thin, creeping perennial vine with tendrils growing from a large thick caudex [6]. An earlier

chemical analysis of this species led to isolating 23,24-dihydrocucurbitacin F,23,24-dihydrocucurbitacin D and its 2-O-β glucopyranosyl derivative; these compounds were isolated from the ethyl acetate extract [5]. Cucurbitacins are a group of triterpenoids isolated as bitter compounds from the Cucurbitaceae family species and are characterized by a tetracyclic cucurbitane nucleus skeleton 19-(10⟶9b)-abeo-10α- lanost-5-ene [7]. Anti-inflammatory, anti-tumor, anti-atherosclerotic, and anti-diabetic properties, among others, have been documented for this kind of triterpene [8]. From the wide range of biological activities of cucurbitacins, we are interested in their anti-diabetic activity, such as that previously indicated for *Ibervillea sonorae* or *Momordica charantia*, which have been shown to have hypoglycemic activity and enhance glucose clearance [9].

The primary cellular mechanism that diminishes glycemia after carbohydrates are ingested is glucose transport into its target organs, primarily skeletal muscle and adipose tissue; glucose transporter 4 (Glut4) is the major glucose transporter protein that carries this uptake. Significantly, in diabetes mellitus type 2, Glut4 and glucose metabolism are decreased in muscle and adipose tissue [10]. This transporter mediates glucose uptake in adipocytes and muscle by rapid translocation from intracellular storage sites to the plasma membrane (PM). Glut4 translocation may be induced via the canonical insulin pathway, where protein kinase B (AKT) is a central mediator, or through the critical regulator of energy homeostasis AMP-activated protein kinase (AMPK) activation [10]. After this point, redundant signaling mechanisms, starting with the phosphorylation of AS160 by these kinases, leading to Glut4 translocation to the cell surface, are induced [11].

The importance of Glut4 in pathophysiological states lies in the fact that the decrease in its intracellular concentrations, or its translocation to the PM, results in an insulin-resistant (IR) state and hyperglycemia. The present study evaluated the glycemic effects of 23,24-dihydrocucurbitacin D (1) and 2-Oβ-glucopyranosyl-23,24-dihydrocucurbitacin D (2) *in vivo*. Additionally, to gain some insight into the structure/function relationship, we synthesized and evaluated the acetylated derivatives 2,16-diacetyl 23,24-dihydrocucurbitacin D (3) obtained from 1 and 2-O-β-(2′,3′,4′,6′ tetraacetyl) glucopyranosyl,16 acetyl 23,24-dihydrocucurbitacin D (4) obtained from 2. The effects of 1, 2, and 4 on Glut4 translocation and the activation of AMPK and AKT enzymes were evaluated; compound 3 was not further evaluated due to its relatively weak activity to low glycemia. Additionally, the interaction between cucurbitacins 1, 2, 3, and 4 and the kinases AMPK and AKT was assessed *in silico*.

2. Materials and Methods

2.1. Experimental Animals. Six- to eight-week-old male CD1 mice (25–30 g weight) were purchased from Instituto de Ciencias de la Salud, Universidad del Estado de Hidalgo and Facultad de Estudios Superiores (FES) Iztacala, Universidad Nacional Autónoma de México (UNAM). Mice were kept in an environment free of pathogens at the bioterium of FES-Iztacala. All animal experimentation trials were designed to minimize the number of participants and suffering. These assays were carried out in strict compliance with approved ethical standards and guidelines for the Care and Use of Laboratory Animals adopted by the US National Institutes of Health and the Mexican Regulation of Animal Care and Maintenance (NOM-062ZOO-1999, 2001). The FES-Iztacala Ethics Committee and IFC approved and revised the experimental procedures (protocol number 1027/27/06/2014), UNAM. This study followed the same procedures and guidelines to ensure the well-being of the experimental subjects of Rodriguez et al., and the description of the methods partly reproduces their wording [12].

2.2. Plant Material. Roots from *I. lindheimeri* were collected on three separate occasions between 2015 winter and 2016 summer near Peña Blanca station in Peña Miller municipality, Queretaro, Mexico, by Figueroa-Hernández. Dr. Rafael Lira from Instituto de Biología, UNAM, identified the plant. A voucher specimen was deposited at Instituto de Biología, UNAM (MEXU, 111000).

2.3. Extraction and Isolation. The roots were cleaned, dried, milled (8,482 g dry weight), and successively extracted separately at room temperature with hexane (Hx), ethyl acetate (EtOAc), and methanol (MeOH) for 24 h each. The solvents were evaporated at low pressure and 60°C, yielding 55.75 g (Hx), 84.37 g (EtOAc), and 576 g (MeOH) extracts. Cucurbitacins 1 and 2 were isolated from the EtOAc extract (50 g) using an open chromatography column (OCC) with silica gel as a stationary phase (10:1 SiO_2:extract) and eluted with Hx/EtOAc and EtOAc/MeOH mixtures of increasing polarity. An amorphous solid was isolated from the fractions eluted with 1:1 Hx/EtOAc, which was submitted to an OCC and eluted with a 1:1 Hx/EtOAc mixture using aluminum oxide (Al_2O_3) as the stationary phase. This procedure resulted in the isolation of 23,24-dihydrocucurbitacin D (1). An impure solid was isolated from the fractions eluted with 9:1 EtOAc/MeOH. Purification of this solid by crystallization procedures afforded 2-O-β glucopyranosyl 23,24-dihydrocucurbitacin D (2). In addition, 1 (800 mg) and 2 (800 mg) under acetylation conditions (pyridine 1 ml/acetic anhydride 1 ml) afforded acetylated products 3 and 4, respectively. The identification of cucurbitacins 1, 2, 3, and 4 was achieved by comparing their spectral data with those previously published (all reagents were purchased from Sigma-Aldrich, St. Louis, USA).

2.4. Nuclear Magnetic Resonance Analysis. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were acquired with Varian GeminiXL-200 and Varian VXR-300 spectrometers. Spectra of 1 and 3 were acquired using deuterated chloroform ($CDCl_3$) as the solvent. Dimethyl sulfoxide (DMSO-D6) was used to acquire spectra of 2 and 4 (both deuterated solvents were from Sigma-Aldrich).

2.5. Chemical Diabetes Induction. Diabetes was induced in randomly selected mice. The induction of noninsulin-dependent diabetes mellitus in mice was achieved by a single

intraperitoneal (ip) administration of 120 mg/kg streptozotocin (STZ; Sigma-Aldrich). This diabetes model gives rise to characteristics like diabetes mellitus type 2; it is characterized by hyperglycemia with poor insulin response while not presenting significant variations in the serum insulin levels, making it suitable for studying the pharmacological effects of glycemia [13, 14]. STZ was freshly prepared in citrate buffer (HOC(COONa) $(CH_2COONa)_2$ $2H_2O$; $C_6H_8O_7$ 0.1 M, pH 4.5) before administration (Sigma-Aldrich).

2.6. Measurement of Blood Glucose and Glucose Tolerance Test. The glycemic level was observed for 10 days after the administration of STZ. Mice with glycemia >200 mg/dL were considered diabetic. Glycemia was determined using an Accutrend GC (Roche) glucometer and reactive strips were obtained from Accu-Chek.

The activity of cucurbitacins 1, 2, 3, and 4 toward glycemia was determined in male CD1 mice fasted for 8 h. Nondiabetic mice (NDM) and diabetic mice (DM) were divided into groups of three individuals. Mice of the control group were administered orally with a vehicle solution (saline and Tween 20 (0.1%)) only. The dose of 1 used was 48 mg/kg for NDM mice, while 83 mg/kg was used for DM mice. The doses of 2, 3, 4, and metformin were 500 mg/kg for DM mice. Metformin was selected due to its anti-diabetic effectiveness and the fact that it directly interacts and activates AMPK independently from insulin, two desirable characteristics since (1) the diabetic model used in this study is characterized by high glucose with low insulin response and (2) cucurbitacins have been suggested to exert their effect via AMPK pathway. Cucurbitacins 1, 2, 3, and 4 and metformin were administered orally using saline and Tween 20 (0.1%) as the vehicle. The dose of 500 mg/kg metformin was selected because the optimal average dose of oral metformin in humans is 2 g/kg/day, equivalent to 20 mg/kg/day for humans with a weight of 100 kg. [15]. The dose of human metformin is converted to a dose equivalent to 250 mg/kg/day of mouse metformin. [16]. In our experiment, we used a mouse model of young nonobese mice, which would be equivalent to approximately 50 kg of young humans, which translates to a dose of 500mg/kg/day for mice. Cucurbitacins 1, 2, 3, 4, vehicle, and metformin, were approximately 30 minutes before a 1.5 g/kg glucose solution was orally administered to the mice. This time was selected since previous data from our group and the literature [9, 17, 18] indicate that around 30 minutes are needed for the compounds and metformin to reach their highest concentration in plasma [19]and exert their anti-diabetic effect when administered orally, most likely due to bioavailability [20]. The glycemic level was measured at minute 0 (before cucurbitacin administration) and 15, 30, 60, and 120 minutes after glucose administration. Blood samples were obtained from the tips of the tails of the mice. Blood glucose was determined using an Accutrend GC glucometer.

2.7. Evaluation of Glut4 Translocation to the Plasma Membrane. Random CD1 male mice were selected for the induction of diabetes as described in Section 2.5. The treatments were supplied 30 minutes before oral glucose administration (1.5 g/kg). Considering the glucose reduction observed in the glucose tolerance curve and literature reports, the mice were euthanized 30 minutes after glucose administration [21].

Soleus muscle (SM) and epididymal adipose tissue (EAT) were removed, washed with PBS buffer (150 mM NaCl; 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4), fixed in 15% formalin for 12 h, and embedded in paraffin. The paraffin-embedded SM and EAT were subjected to an immunohistochemistry assay with antibodies for Glut4 (SLC2A4/GLUT-4 antibody (aa333-509) LS-C343974, LS Bio 1/200 and 1/500), whose epitope includes two extramembrane regions of this transporter [22], and developed with diaminobenzidine (DAB, D8001). (All reagents were purchased from Sigma-Aldrich.) The amount of Glut4 (diaminobenzidine-HRP (horse radish peroxidase) reaction) was determined semiquantitatively using the "FIJI" version of ImageJ software with the method reported by Fuhrich, 2013. In brief, the global scale of the image analysis was 3.91 pixels = 1 μm with a pixel ratio of 1. The white balance macro written by Vytas Bindokas was used (October 2006, University of Chicago modified by Patrice Mascalchi), and the image was adjusted using the command "subtract background." The areas of interest (ROIs) corresponding to the tissues were selected using the "Brush" tool. The digitized area was subjected to the "color deconvolution" plug-in using the HDAB36 vector. The final intensity of DAB was calculated with the formula $f = 255—i$, where f = final intensity of DAB and i = mean intensity of DAB obtained from the software [23].

2.8. Measurement of Second Messengers Involved in Glut4 Translocation. Mice were euthanized as described in Section 2.6, and SM and EAT were removed, cryopreserved in nitrogen, and stored at −70°C until protein extraction [21]. Protein solutions were preserved at −70°C until use. Protein (7.5 μg/ml) was resolved in a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS–PAGE) and transferred to a nitrocellulose membrane. Anti-AKT2 [EP1676] (AB131168) and anti-AMPK alpha 2 (AB31959) antibodies were purchased from Abcam. Phospho-AMPKα (Thr172) and phospho-AKT (Ser473) antibodies were purchased from Cell Signaling Technologies. The expression of GAPDH was employed as a loading control using a donkey GAPDH antibody.

2.9. Molecular Docking. Data on the structure of mouse AMPK and human AKT2 proteins were obtained from the Protein Data Bank (accession numbers AMPK (5UFU) and AKT2 (3E8D)). The three-dimensional model of cucurbitacin 1 was obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/), while structural models of cucurbitacins 2 and 4 were generated with the software Maestro, Ver. 11.9 (Schrodinger, LLC, New York, NY, USA). For docking analysis, the inhibitors N-[2-(5-methyl-4H-1,2,4-triazol-3-yl) phenyl]-7H-pyrrolo [2,3-d] pyrimidin-4-amine and 4-[2-(4-amino-2,5-dihydro-1,2,5-oxadiazol-3-

yl)-6-{[(1S)-3-amino-1-phenylpropyl] oxy}-1-ethyl-1H-imidazo [4,5-c] pyridin-4-yl]-2-methylbut-3-yn-2-ol of AKT2 and the agonist AMP of AMPK were removed from the binding site using UCFS Chimera Software Ver. 1.13.1 (developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311) [24]. The protein and ligand structures were prepared for docking using ADT 1.5.2 software [25]. Docking analysis was conducted using AutoDock 4.2.5.1 software (available at https://autodock.scripps.edu/) [26]. After docking, 100 conformations of each compound were obtained and then clustered for analysis using ADT 1.5.2 software. The conformations selected were within the most represented cluster and corresponded to those showing the lowest binding energy values and the inhibition constant (Ki). Model analyses and figure drawings were carried out with PyMOL 2.1.0 (The PyMOL Molecular Graphics System, Version 2.1.0 Schrödinger, LLC; https://sourceforge.net/p/pymol).

2.10. Statistical Analysis. Statistical calculations were performed with the GraphPad Prism 6 for Windows software package. The results are expressed as the mean ± standard error of the mean (SEM) of $n = 3$ for CD1 mice. Data were analyzed using one-way ANOVA, and the value of $p < 0.005$ was considered significant.

3. Results and Discussion

3.1. Extraction and Isolation of Secondary Metabolites from I. lindheimeri. Three different pools of the roots of *I. lindheimeri* were collected as described in Section 2.2. These pools rendered a total of 84.37 g of EtOAc extract, corresponding to 1.14 ± 0.48% of the dry weight of the root (Table 1). In a previous study, a similar yield was reported [5].

The EtOAc extract was subjected to a chromatographic process by OCC. From the fractions eluted with 1:1 (Hx: EtOAc), a solid was isolated, which was purified by a crystallization process, yielding 23,24-dihydro cucurbitacin D (1) and representing 4.07 ± 0.74% of the EtOAc extract. From the fractions eluted with 9:1 (EtOAc:MeOH), a second compound was isolated, which, after purification by OCC, resulted in 2-(O-β-D-glucopyranosyl)-23,24-dihydrocucurbitacin D (2), corresponding to 18.33 ± 6.94% of the EtOAc extract (Figure 1).

Interestingly, although the yield of EtOAc extract was almost the same in the three studied collections, the isolated amount of 2 varied in each collection. In contrast, 1 did not suffer significant variation (Table 1). This behavior could be due to the different stress conditions of the plant over several months of collection [27, 28].

Note. HW = humid weight of the root, DW = dried weight of the root, Hx ext. = hexane extract, EtOAc ext. = ethyl acetate extract, MeOH ext. = methanol extract, 1 = 23,24-dihydrocucurbitacin D, 2 = 2-(O-b-D-glucopyranosyl)-23,24-dihydrocucurbitacin D, % EtOAc ext. = % of weight of EtOAc ext. of the DW, % 1 = % of 1 within EtOAc ext., and % 2 = % of 2 within EtOAc ext.

To gain insight into the structure/activity relationships, we synthesized 2,16-diacetyl-23,24-dihydrocucurbitacin D (3) and 2-O-β (2,3,4,6-tetracetyl) glucopyranosyl, 16-acetyl-23,24-dihydrocucurbitacin D (4) from 1 and 2, respectively (Figure 1). The presence of an acetyl residue at position C2 and the acetylation of glucose have been related to cucurbitacin biological activity [7, 29–31].

The identification of 1, 2, 3, and 4 was achieved by analyzing their ^{1}H, ^{13}C, and 2D nuclear magnetic resonance (NMR) spectra and comparing their physical and spectral data with published data [5, 32, 33].

3.2. Effects of 1, 2, 3, and 4 Cucurbitacins on Glycemia. Noninsulin-dependent diabetes *mellitus* was induced in male CD1 mice, and they were divided into groups as described in Section 2.1. Considering the low oral bioavailability (approximately 10% of the total administrated dose) reported for cucurbitacins [19] and that the dose of 500 mg/kg of AcOEt extract previously evaluated by our group (data not shown) had an anti-hyperglycemic effect and did not cause hypoglycemia or any evident toxic effect in diabetic and nondiabetic mice, along with previous studies where high doses of pure cucurbitacins lowered glycemia without provoking adverse effects [34, 35], we decided to use a dose of 500 mg/kg in the activity assays of the compounds. However, cucurbitacin 1 at a 500 mg/kg dose induced lethal hypoglycemia in mice; therefore, a dose-response curve was generated to evaluate its hypoglycemic effect in NDM (Figure 2). The results indicated a 48 mg/kg effective dose of 50 (ED_{50}). Since this dose did not lower blood glucose in the DM group, 83 mg/kg was chosen, which had an effect of 82.9% on glycemia but had a nonlethal hypoglycemic effect in mice (Figure 2).

The results showed that 1 lowered glycemia in DM at a dose of 83 mg/kg. The blood glucose level was significantly different from untreated DM ($p \leq 0.001$) and not significantly different from NDM. The other three compounds did not lower glycemia in NDM (data not shown). In DM, 1 (83 mg/kg), 2 (500 mg/kg), and 4 (500 mg/kg) showed significant differences compared with DM ($p \leq 0.001$, 0.05, and 0.001, respectively), and they did not show significant differences compared with NDM. Although treatment with 3 lowered glycemia levels, they were not significantly different from those in the DM group (Figure 3).

The results indicate that all tested cucurbitacins had quite different effects on glycemia. Cucurbitacin 1 at 83 mg/kg doses was the most effective compound, lowering glycemia to a similar extent to metformin. Unfortunately, it caused severe hypoglycemia at higher doses (data not shown) in both the DM and NDM groups. In contrast, although glycoside 2 and its acetyl derivative 4 at 500 mg/kg lowered glycemia in the DM group, they did not cause hypoglycemia or low blood glucose in NDM (anti-hyperglycemic effect), which are desirable characteristics for antidiabetic compounds. This effect has been reported for metformin, cucurbitacin derivatives, and 5-amino-1-β-D-

Table 1: Yield of 23,24-dihydrocucurbitacin D (1) and 2-(O-β-D-glucopyranosyl)-23,24-dihydrocucurbitacin D (2) after ethyl acetate extraction from the root of *Ibervillea lindheimeri*.

HW (g)	DW (g)	Ext Hx (g)	Ext EtOAc (g)	Ext MeOH (g)	% of Ext EtOAc	1 (g)	2 (g)	% 1	% 2
4,990	789	8.04	12.87	64	1.14 ± 0.48	0.47	2.35	4.07 ± 0.74	18.33 ± 6.94
14,359.3	3,360	26.39	22.5	184		1.11	2.57		
25,680	4,333	21.32	49.0	328		1.78	12.40		
45,029.3	8,482	55.75	84.37	576		3.36	17.32		

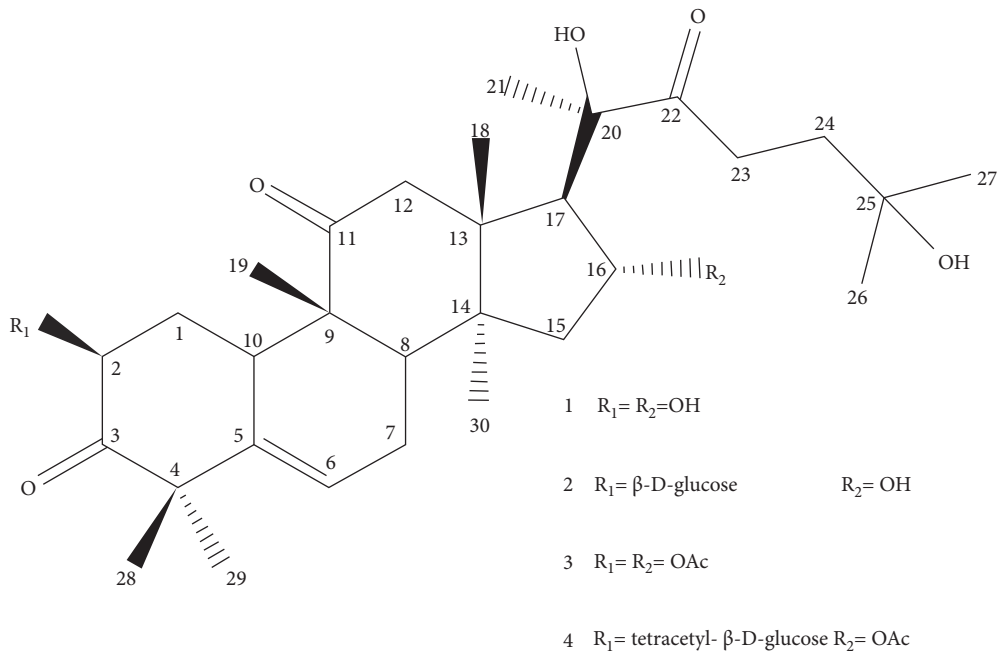


Figure 1: Chemical structures of 1-4 cucurbitane triterpenoids. 23,24-dihydrocyclyrbitacin D (1), 2-O-β glucopyranosyl 23,24-dihydrocucurbitacin D (2), 2,16-diacetyl-23,24-dihydrocucurbitacin D (3), and 2-O-β (2′,3′,4′,6′-tetraacetyl) glucopyranosyl-16-acetyl-23,24-dihydrocucurbitacin D (4).

ribofuranosyl-imidazole-4-carboxamide (AICAR), which is an activator of AMPK. This kinase is involved in glucose clearance by promoting Glut4 translocation via a mechanism of action independent of insulin [9, 36, 37]. The anti-hyperglycemic activity has also been associated with inhibiting hepatic glucose liberation [16]. Additionally, treatment with 1, 2, 3, or 4 did not cause undesired effects, such as acute diarrhea, vomiting, gastrointestinal bleeding, hypotension, or irritation of mucous membranes, as previously described for so-called cucurbitacin poisoning [38, 39], even when 2 and 4 were tested at doses as high as 500 mg/kg [7, 37].

Comparing the anti-diabetic effects of compounds 1 and 3, we observed that 1 was the most active, while its diacetylated compound 3 was the least active, even though both cucurbitacins presented a similar lowest-energy conformation (Figure 4). This result indicates that the hydroxyl groups at C1 and C16 may be essential for anti-diabetic activity. However, unexpectedly, acetylated glycoside 4, which has an acetyl group at C16, showed more significant activity than nonacetylated glycoside 2. The activity of 4 is likely due to its conformational change compared to cucurbitacins 1–3. This result suggests that 4 possibly interacts with AMPK and AKT, which are enzymes involved in anti-diabetic activity, but at different binding sites than 1 and 2 (Figure 4).

3.3. Translocation of Glut4 to the Plasma Membrane Induced by 1, 2, and 4 in Soleus Muscle and Epididymal Adipose Tissue. Once the hypoglycemic effect of 1 and the anti-hyperglycemic effects of 2 and 4 were established, their mechanism of action was investigated. We evaluated the effects of 1, 2, and 4 on Glut4 translocation from storage vesicles to the PM in SM and EAT; diacetate 3 was not evaluated since it did not show a significant effect on glycemia. We used immunohistochemistry as described in Section 2.7 as a direct visualization histological method to assess the effect of cucurbitacins on the translocation of Glut4.

Our results showed that 1, 2, and 4 increased the presence of Glut4 in the PM of SM, as indicated by a higher intensity of DAB measured with FIJI software, compared to the DM control group (Figure 5(a)). However, only the administration of cucurbitacin 1 promoted Glut4 translocation in the DM group, in such a way that the presence of this transporter in PM was similar to that of the NDM control group, and both were significantly different from the DM group treated with the vehicle ($p \leq 0.001$; Figure 5(b)). Notably, the administration of 2 induced a comparable effect to that induced by metformin dispensation. Nevertheless, neither of the treatments was significantly different from the DM + Vehicle group. It has been reported that metformin

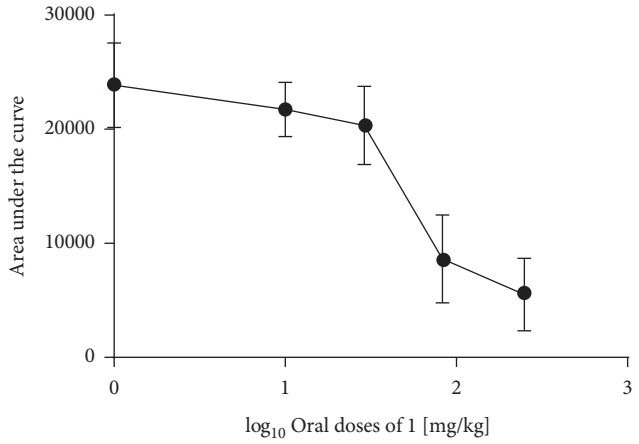


Figure 2: Dose-response curve of 23,24-dihydrocucurbitacina D (1). The area under the glucose tolerance curve as an integrated expression of glycemia. The error bars represent the standard error mean of measurements for $n = 3$.

administration stimulates Glut4 in human skeletal muscle cells [40, 41]. In our case, the stimulation induced by metformin was less than that induced by 1. Since 1 is the only compound able to induce Glut4 translocation in skeletal muscle, which accounts for the uptake of 70% of postprandial carbohydrates, it would explain the hypoglycemic effect presented by this molecule. The effect of the compounds on EAT was evaluated in material obtained from the same mice from which MS samples were removed. The overall Glut4 levels detected in the PM of NDM EAT were lower than those observed in SM NDM (Figure 5(b)). In NDM individuals treated with cucurbitacins and metformin, the translocation of Glut4 was similar to that observed in the NDM group without treatment (data not shown). In contrast, in the DM groups treated with cucurbitacins 1, 2, and 4, Glut4 translocation increased in a meaningful fashion. This effect was significantly different from that in the DM + Vehicle group (Figure 5(c)). It has been reported that fasting could affect the level of Glut4 present in adipose tissue, unlike skeletal muscle where the levels of Glut4 remain stable in both fasting and not fasting conditions that would explain the difference observed in the amount of Glut4 presented by both NDM controls in SM and AE. The analysis of the reduction of Glut4 amounts showed that in adipose tissue, there is a decrease in mRNA to a larger extent than Glut4 protein [42]; if we consider that our compounds enhance Glut4 translocation, it seems logical that more Glut4 is present in PM in treated groups even though there is a reduction of this transporter at the translational level. More experiments are needed to determine whether our compounds have an effect on Glut4-mRNA in the short or long term in addition to promoting Glut4 translocation.

Our results showed that 1 promoted the translocation of Glut4 to the PM in both MS and EAT tissues (Figures 5(b) and 5(c)). These activities could account for the hypoglycemic effect shown by 1. On the other hand, in the MS tissue, the translocation of Glut4 to the PM induced by 2 and 4 was not significantly different from that in the DM control (Figure 5(b)); nevertheless, the administration of 2 and 4 to diabetic mice significantly decreased glycemia compared to the DM control group (Figure 3(b)). There was a significant Glut4 translocation in EAT tissue induced by 2 and 4 (Figure 5(c)), which suggests that an increase in the presence of this transporter in the PM of adipose tissue is sufficient to contend with the hyperglycemia presented by DM. Interestingly, it has been reported that knockout (KO) organisms for Glut4 in adipose cells induced insulin resistance to a similar degree as in KO muscle [43], even though the muscle captures approximately 70% of postprandial glucose, versus 10% for adipose tissue, highlighting the importance of adipose tissue in glucose disposal [44]. Moreover, glucose uptake in skeletal muscle is also impaired *in vivo* despite preserved Glut4 expression in muscle [45]. It has been reported that overexpression of Glut4 in the adipose tissue of muscle-specific Glut4-deficient mice overcomes glucose intolerance and diabetes [46]. It has also been proposed that adipocytes detect the absence of glucose uptake and respond by secreting adipokines such as RBP4 to restrict glucose uptake in skeletal muscle and increase glucose output by the liver, thereby increasing the blood glucose level [47]. These data, along with our results indicate that EAT is particularly crucial for glucose homeostasis; however, the conditions that give rise to such different behaviors remain to be elucidated. In our case, EAT was the primary effector tissue involved in the cucurbitacin-lowering effect on glycemia and played a significant role in glucose homeostasis *in vivo*. Although *in vitro* studies of the activity of cucurbitacins have reported an increase in Glut4 translocation activity against the diabetic negative control, also observed in the present study, our dual *in vivo* approach allowed us to appreciate the different effects exerted by the same compound in different tissues of a single individual, which permitted us to highlight the main target tissue effector of the anti-diabetic activity. Compounds that promote glucose disposal by adipose tissue are ideal for the treatment of diabetes *mellitus* by safely lowering blood glucose in diabetic individuals without causing hypoglycemia.

Histology also revealed no cell damage or abnormalities in the tissue architecture of either EAT or SM.

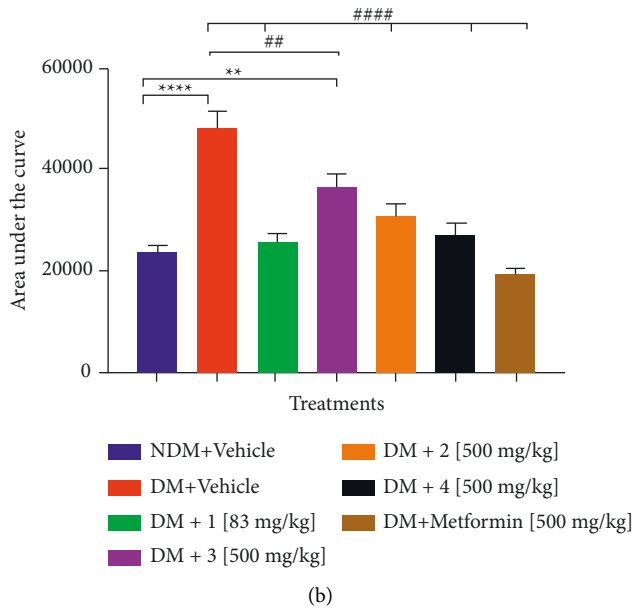
3.4. Effects of 1, 2, and 4 on AMPK and AKT Activation Involved in the Translocation of Glut4 in MS and EAT. It has been experimentally determined that the AMPK and AKT pathways are the primary controllers of Glut4 translocation in muscle and adipose tissue [10]. Western blotting was conducted as described in Section 2.8 to assess whether our compounds affect the activation of these two kinases.

According to our results in the DM control group, the expression of activated enzymes, both AMPK and mainly AKT, in the SM was almost absent. AKT activation was also diminished in EAT, validating our diabetic model since the canonical insulin signaling pathway has AKT signaling as a critical factor for inducing the translocation of this transporter, therefore presenting reduced translocation of Glut4 to the PM and, consequently, diminished uptake of glucose by both tissues [13].

Additionally, higher expression of proteins (second messengers) was observed in EAT than in SM (Figure 6(c) vs.

Time [min]	NDM + Vehicle	DM + Vehicle	DM + 1 [83mg/kg]	DM + 2 [500mg/kg]	DM + 3 [500mg/kg]	DM + 4 [500mg/kg]	DM + Metformin [500mg/kg]
0	75.5 ± 2.4	210.0 ± 1.9	211.0 ± 2.4	209.6 ± 2.1	209.7 ± 2.4	213.9 ± 2.3	208.8 ± 2.7
15	217.1 ± 13.5	363.8 ± 20.3	256.4 ± 7.3	320.0 ± 20.9	286.9 ± 6.9	261.5 ± 21.4	230.3 ± 15.3
30	200.5 ± 10.1	360.3 ± 23.4	232.1 ± 6.9	312.1 ± 25.1	269.4 ± 20.1	232.4 ± 20.4	184.9 ± 17.4
60	148.1 ± 10.1	307.8 ± 23.4	152.9 ± 14.6	242.1 ± 19.2	204.1 ± 20.9	166.8 ± 19.4	117.1 ± 6.9
120	98.3 ± 6.0	230.0 ± 21.4	98.6 ± 14.9	151.6 ± 12.7	120.0 ± 16.0	105.5 ± 15.8	63.0 ± 4.2
180	91.9 ± 9.8	179.5 ± 15.4	71.1 ± 12.0	107.3 ± 10.8	77.9 ± 12.1	75.3 ± 16.5	40.1 ± 4.4

(a)



(b)

Figure 3: Effect of 1-4 cucurbitacins on glycemia. (a) Glycemia presented by treated groups during the glucose tolerance test curve of the evaluated groups; ± standard error mean of measurements for $n = 3$. (b) Area under the curve of the glucose tolerance test as the integrated expression of the glycemia concentration; the error bars represent the standard error mean of measurements for $n = 3$. NDM = non-diabetic mice, DM = diabetic mice, 1 = 23,24-dihydrocucurbitacin D, 3 = 2,16-diacetyl-23,24-dihydrocucurbitacin D, 2 = 2-*O*-β glucopyranosyl-23,24-dihydrocucurbitacin D, and 4 = 2-O-β (2,3,4,6-tetracetyl) glucopyranosyl-16-acetyl-23,24-dihydrocucurbitacin D. The bars show the SEM. ** and **** show significance at $p \leq 0.01$ and $p \leq 0.0001$, respectively, versus NDM + vehicle; $^{\#\#}$ and $^{\#\#\#\#}$ show significance at $p \leq 0.01$ and $p \leq 0.0001$, respectively, versus DM + vehicle.

Figure 6(h) and Figure 6(e) vs. Figure 6(j)), and under the conditions of this study, both AMPK and AKT were active in the NDM group (Figure 6(f). This behavior has been documented for adipocytes, where it has been shown that AMPK can upregulate AKT activation in adipocytes by the PI3K/AKT pathway with an increase in the levels of phosphatidylinositol (3,4,5)-trisphosphate even in the absence of insulin, thus suggesting insulin-independent AMPK-dependent AKT activation [48].

In EAT, activation of the AKT enzyme was observed in the groups treated with 1, 2, and metformin, similar to the NDM group, yet the complete expression of this enzyme was different in every treated group. These results indicate that Glut4 translocation depends on the amount of activated enzyme rather than the total enzyme available (Figure 6(i)). On the other hand, all three cucurbitacins and metformin could induce AMPK activation compared to the DM control group. Compounds 1 and 4 and metformin showed activated AMPK expression similar to that in the NDM control group (Figure 6(g)). However, the full expression of this enzyme was different in every treated group. These results support the proposal that Glut4 translocation depends on the amount of activated (phosphorylated) enzyme, independent of the total expressed protein, and suggest that the doses used can only activate a certain amount of enzyme. Cucurbitacin 4 did not cause an increase in AKT phosphorylation (Figure 6(i)); nevertheless, it promoted AMPK activation, Glut4 membrane translocation, and

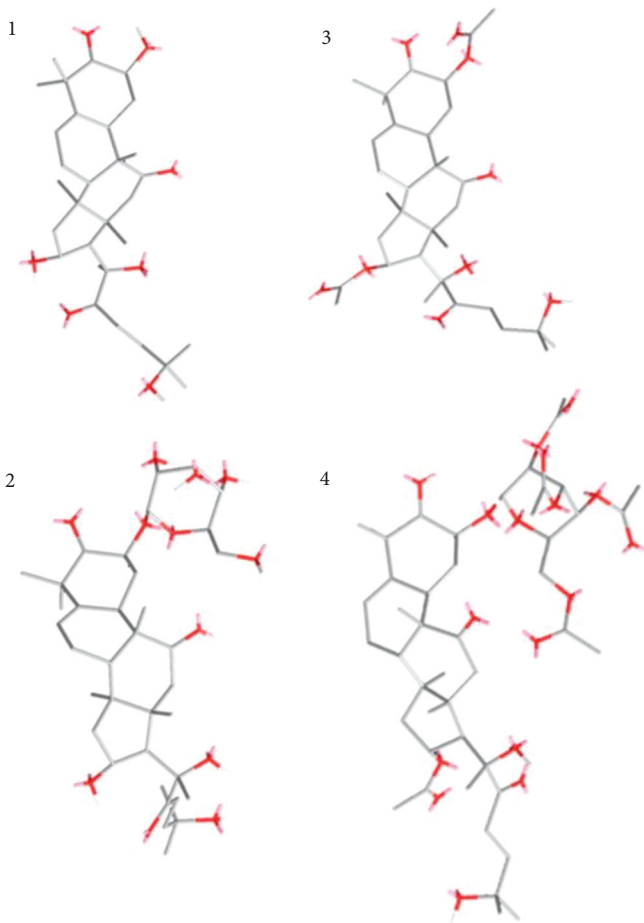


Figure 4: The lowest-energy conformations of 1, 2, 3, and 4 were determined with chembio 3 ultra 13.0. Only the polar hydrogens are shown (energy 1 = 79.305, energy 2 = 103.2813, energy 3 = 85.2036, and energy 4 = 127.29 Kcal/mol).

hyperglycemia reduction, which suggests that the activation of this kinase pathway by cucurbitacin 4 plays a vital role in the anti-diabetic activity of this compound independent of AKT activation. Treatment with cucurbitacin 2 induced the least AMPK phosphorylation compared with those of the 1, 4, and metformin groups, with phosphorylation amounts between those of the NDM and DM control groups (Figure 6(g)). Nonetheless, treatment with this compound can induce Glut4 translocation at the same level as the other cucurbitacins and metformin (Figure 5(c)). This result could indicate that the achieved *p*AMPK levels are sufficient to promote Glut4 translocation effectively. On the other hand, 2 also increased *p*AKT, similar to the NDM control (Figure 6(i)), so dual activation of these pathways could be responsible for the observed Glut4 translocation induced by 2 (Figure 5). Another explanation would be that the activated AMPK levels were enough to induce AKT activation via PI3K [48], and the dual activation of both translocation signaling enzymes, AMPK and AKT, influences the presence of Glut4 in the PM in EAT. Further experiments are needed to determine whether the cucurbitacins increase insulin sensitivity in EAT, as the activation of AKT suggests in the treatments with 1 and 2 in addition to AMPK activation being both pathways necessary for Glut4 translocation and the subsequent reduction in glycemia, or if AMPK is the key protein responsible for directly activating the translocation of this transporter and potentiating this effect by also activating AKT. However, the behavior observed in the group treated with 4, where the sole activation of AMPK is enough to induce Glut4 membrane translocation, and hyperglycemia reduction suggests that in EAT, the activation of AMPK and not AKT is primarily responsible for the increase in Glut4 translocation to the PM in diabetic individuals treated with cucurbitacins 1, 2, and 4. Furthermore, aqueous extracts from another member of the *Ibervillea* genus have been shown to induce glucose uptake by both murine and human adipocyte cell lines in the absence or presence of insulin signaling pathway inhibitors [49]. Additionally, extracts from *Citrullus colocynthis*, rich in cucurbitacins, have been described to induce Glut4 translocation in 3T3 adipocytes even without insulin and increase AKT phosphorylation/activation without influencing the phosphorylation of the insulin receptor, suggesting that cucurbitacins can positively influence the activation of AKT in adipocytes in an insulin-independent manner [50].

In SM, the full expression of AMPK was diminished in the DM groups treated with cucurbitacins or metformin compared to NDM (Figure 6(c)). However, the groups treated with 1, 4, and metformin had higher phosphorylation of AMPK than the NDM group (Figure 6(b)). Moreover, administration of 1 increased pAMPK to a greater extent than in the metformin group. This activity could account for the Glut4 translocation induced by 1.

This translocation was similar to that in the NDM group and higher than that in the metformin group (Figure 5(b)). Cucurbitacin 2 induced AMPK activation comparable to that in the NDM control group, while 4 showed higher activation than 2. However, both were less active than metformin (Figure 6(b)). It is possible that the induced increments of pAMPK by 2 and 4 were not enough to promote Glut4 translocation in a significant manner (Figure 5(b)). None of the cucurbitacin treatments had a positive effect on AKT phosphorylation in SM, since all groups maintained low levels of *p*AKT (Figures 6(d) and 6(e)). Taking into account that the model of diabetes induction utilized in this study interrupts the insulin signaling pathway (insulin-resistant model) [13], which mediates the translocation of Glut4 via the activation of AKT, these results indicate that the cucurbitacins used in this study do not activate the AKT pathway but do activate AMPK in the absence of insulin in muscle. These outcomes are consistent with a study on *M. charantia* cucurbitacins, where anti-diabetic activities have also proven to be effective even when an inhibitor of insulin has been used *in vitro* [9]. In addition, AMPK activation has been reported to inhibit AKT activation in muscle tissue [51, 52]. Furthermore, 1 was able to increase AKT expression in SM, although the mechanism of how cucurbitacins exert this effect remains to be elucidated.

Considering our results and the available evidence on the importance of adipose tissue on energy homeostasis, we propose that cucurbitacins from *I. lindheimeri* help contend with hyperglycemia and enhance glucose clearance by

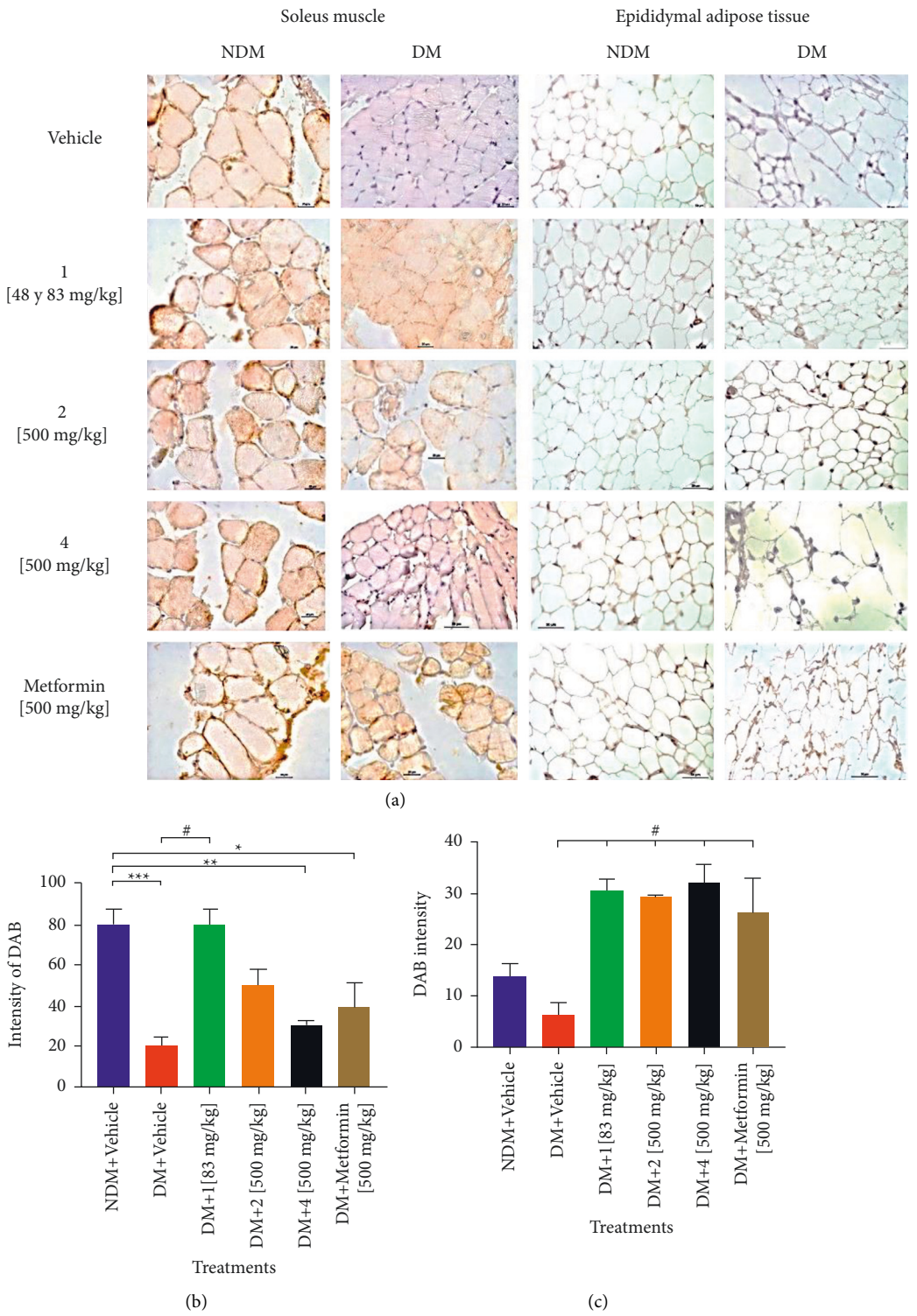


Figure 5: Immunohistochemistry of Glut4 in soleus muscle and epididymal adipose tissue. (a) Immunohistochemistry of SM and EAT of nondiabetic (NDM) and diabetic (DM) CD1 mice treated with the vehicle = saline 0.9% with Tween 20 0.1%, metformin, 1, 2, and 4; scale 40x; and bar = 20 μm. (b) Diaminobenzidine (DAB) intensity as an expression of the presence of Glut4 in PM of SM. (c) DAB intensity as an expression of the presence of Glut4 in PM of EAT. The DAB intensity was calculated with the "Fiji" version of ImageJ software. $*$, $**$, and $***$ show significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively, versus NDM + vehicle; $^{\#}$ shows significance at $p \leq 0.05$ versus DM + vehicle. The error bars represent the standard error mean of measurements for $n = 3$.

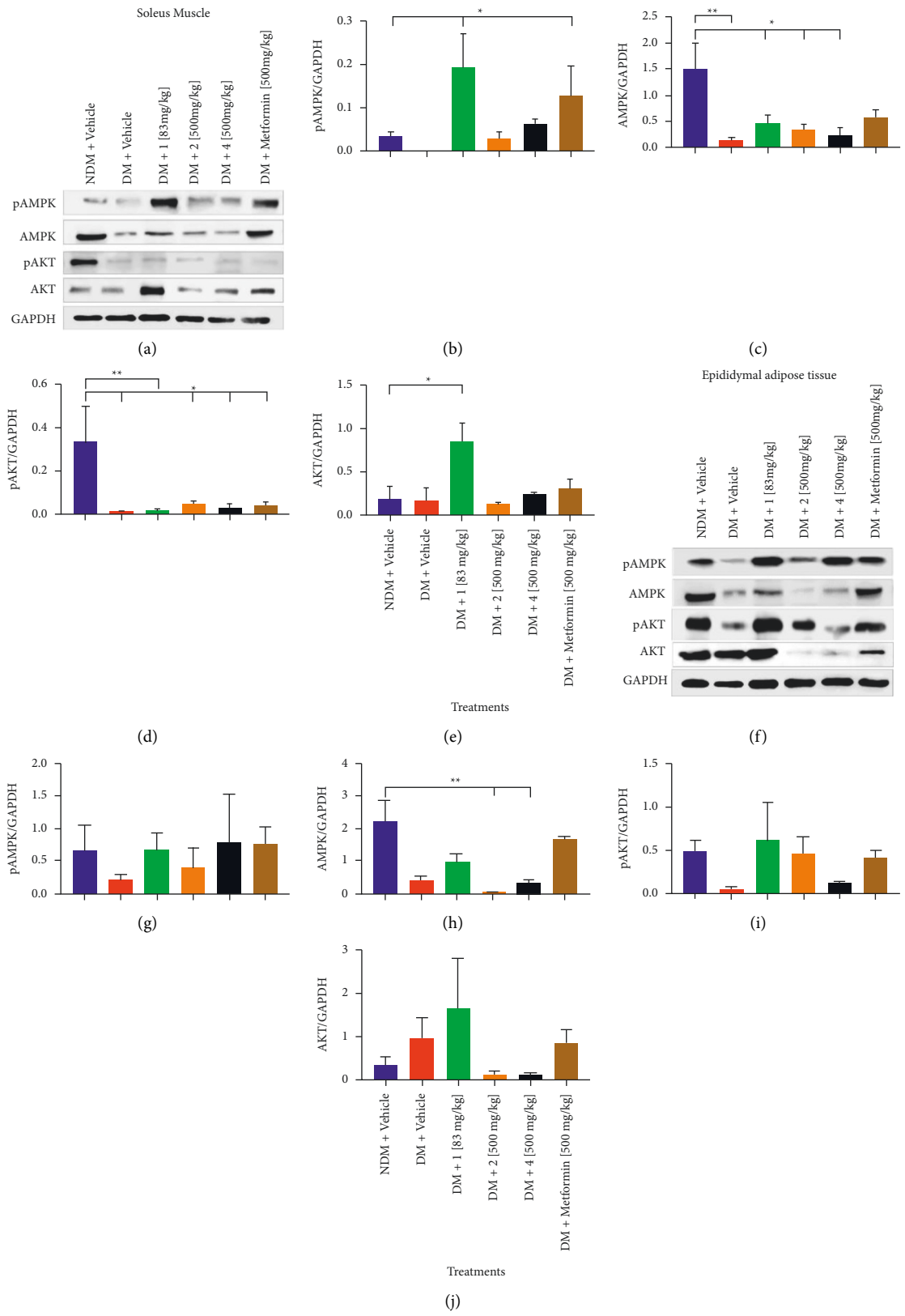


Figure 6: Measurement of second messengers involved in Glut4 translocation in soleus muscle and epididymal adipose tissue. (a) Representative western blot of pAMPK, AMPK, pAKT, and AKT expression in the soleus muscle of the treated groups. (b) Densitometry analysis of the pAMPK/GAPDH ratios in SM. (c) Densitometry analysis of the AMPK/GAPDH ratios in SM. (d) Densitometry analysis of the pAKT/GAPDH ratios in SM. (e) Densitometry analysis of the AKT/GAPDH ratios in SM. (f) Representative western blot of pAMPK, AMPK, pAKT, and AKT expression in epididymal adipose tissue from the treated groups. (g) Densitometry analysis of the pAMPK/GAPDH ratios in EAT. (h) Densitometry analysis of the AMPK/GAPDH ratios in EAT. (i) Densitometry analysis of the pAKT/GAPDH ratios in EAT. (j) Densitometry analysis of the AKT/GAPDH ratios in EAT. The error bars represent the standard error mean of measurements for $n = 3$.

Table 2: Results of docking analysis of cucurbitacins 1, 2, and 4 with (A) the active site of AKT and (B) cystathionine β-synthetase (CBS) sites of AMPK.

Site of interaction	Cucurbitacin 1		Cucurbitacin 2		Cucurbitacin 4	
	B.E. (kcal/mol)	K_d (μM)	B.E. (kcal/mol)	K_d (μM)	B.E. (kcal/mol)	K_d (mM)
(A) AKT						
ATP binding site	−7.97	1.43	−6.05	37.05	−3.05	5.83
(B) AMPK						
Cbs1	−6.46	18.4	−9.08	0.22	−3.23	4.26
Cbs3	−6.31	23.57	−7.32	4.11	−3.3	N.B.
Cbs4	−6.36	21.83	−3.46	2,890	−1.73	54.39

Note. B.E. = binding energy and K_d = estimated dissociation constant. N.B. = no binding found.

stimulating the uptake of this carbohydrate mainly in adipose tissue by promoting Glut4 translocation to the PM via AMPK activation in an insulin-independent manner. This mechanism of action highlights cucurbitacin 1, 2, and 4 as potential treatments for the treatment of both diabetes mellitus types where the absence of insulin signaling either due to the lack of this hormone in diabetes mellitus type 1 (DMT1) or resistance to it in diabetes mellitus type 2 (DMT2) prevents the correct transport and use of glucose, leading to metabolic syndrome, lack of glycemic control, and loss of glucose homeostasis.

3.5. Molecular Docking. According to our results, 1, 2, and 4 increase glucose uptake by promoting Glut4 translocation via AMPK or both AMPK/AKT activation. To evaluate whether cucurbitacins 1, 2, and 4 could bind to AKT and AMPK structures, a docking analysis was conducted as described in Section 2.10. AMPK is a heteromeric protein kinase composed of one catalytic (α) and two regulatory (β and γ) subunits that senses the ratio of adenine nucleotides by competitive binding of AMP, ADP, and ATP to four cystathionine β-synthetase (CBS) motifs, which function as adenine nucleotide-binding sites [53, 54].

The docking results indicated that cucurbitacin 1 may bind to CBS1, CBS3, and CBS4 with high affinity. Interestingly, the analysis indicated that the affinity of compound 2 with CBS sites 1 and 3 was approximately 80- and 6-fold higher than that of compound 1, respectively, but the affinity with CBS4 was low (Table 2).

In addition, 1 was stabilized within 4 Å of the same amino acids that interact with the adenine nucleotides at motifs CBS1, CBS3, and CBS4. This compound showed H-bonds between R2-OH and the oxygen in the peptide bond of Arg152; the –OH from C20, also with the O in the peptide bond of Arg152; and the -OH from C25 with the –O in the Thr89 side chain in CBS1. Thr89 is known to form an H-bond with the phosphate of AMP under physiological conditions. In CBS3, 1 showed possible H-bonds between the oxygen at C1 and the hydrogen of N3 of the side chain of His298; the –OH of C20 with the –O in the peptide bond of Gly275; and the –OH of C25 with the –O of the peptide bond of Arg299. Both His298 and Arg299 make H-bonds with AMP with phosphate and adenine, respectively. Additionally, although R1OH did not show interactions in this analysis, the hydrogen of the –OH was only within 2.6 Å of the N3 of His298 and could also form an H-bond with this atom. In site CBS4, 1 showed H-bonds between the –O of C1 and the –H of N of the peptide bond of Ala205. This amino acid forms an H-bond with the adenine of AMP, and there is also an H-bond between the –OH of R1 and the –O of the peptide bond of Ile204 (Figure 7(b)) [55, 56].

Cucurbitacin 2 was located within 4 Å of almost the same amino acids interacting with the adenine nucleotides at motifs CBS1, CBS3, and CBS4. It is more voluminous than 1, so 2 could not interact with the same amino acids that 1 did. It was more than 4 Å from Arg152 in site CBS1; from Gly275, Asp245, Ser242, and Arg70 in site CBS3; and from Ser316, Ser226, Ala227, Ile312 (although it was close to Ile311), and Ser314 in site CBS4. This cucurbitacin showed possible H-bonds between R1-GlucC2-OH and the oxygen of Val83 and R1GlucC6-OH with the oxygen of the peptide bond of Met85 in motif CBS1. Met85 is known to form an H-bond with the ribose of AMP under physiological conditions. Motif CBS3, showed potential H-bonds among the –OH of R1GlucC4 with the –O of the peptide bond of Glu296 and the –O of R2C16 with the –H of N in the peptide bond of Leu277. This amino acid forms an H-bond with the adenine of AMP [55], and the –OH of C20 forms a bond with the –O of the peptide bond of Arg299. Finally, in site CBS4, cucurbitacin 2 had potential H-bonds between the –O at R1GlucC2 and the –H of the N of the peptide bond of Ala205 (Figure 7(b)).

Of the amino acids located within 4 Å, Val276 and Leu277 in motifs CBS3 and Ile312 in CBS4 have been shown to be essential for the proper functioning and activation of AMPK [55]. His151 binds the phosphate groups, and His298 forms hydrogen bonds with the phosphates of adenine nucleotides at both CBS3 and CBS4 [56]. These docking results suggest that cucurbitacins 1 and 2 may not only be in the proximity of important amino acids for the activation of AMPK but may also interact in the CBS motifs in a similar way to AMP activating this kinase. The docking analysis also indicated that C1, C2, and their substituents are likely to interact with CBS3 and CBS4, which are the most important for the sensing of adenine nucleotides and AMPK activation; this is congruent with the results previously reported for this domain and important for other biological effects of cucurbitacins [57, 58].

Remarkably, when comparing the affinity results with the activation results of AMPK (Figure 6) for cucurbitacin 2, we observed that even when it has a high affinity for these

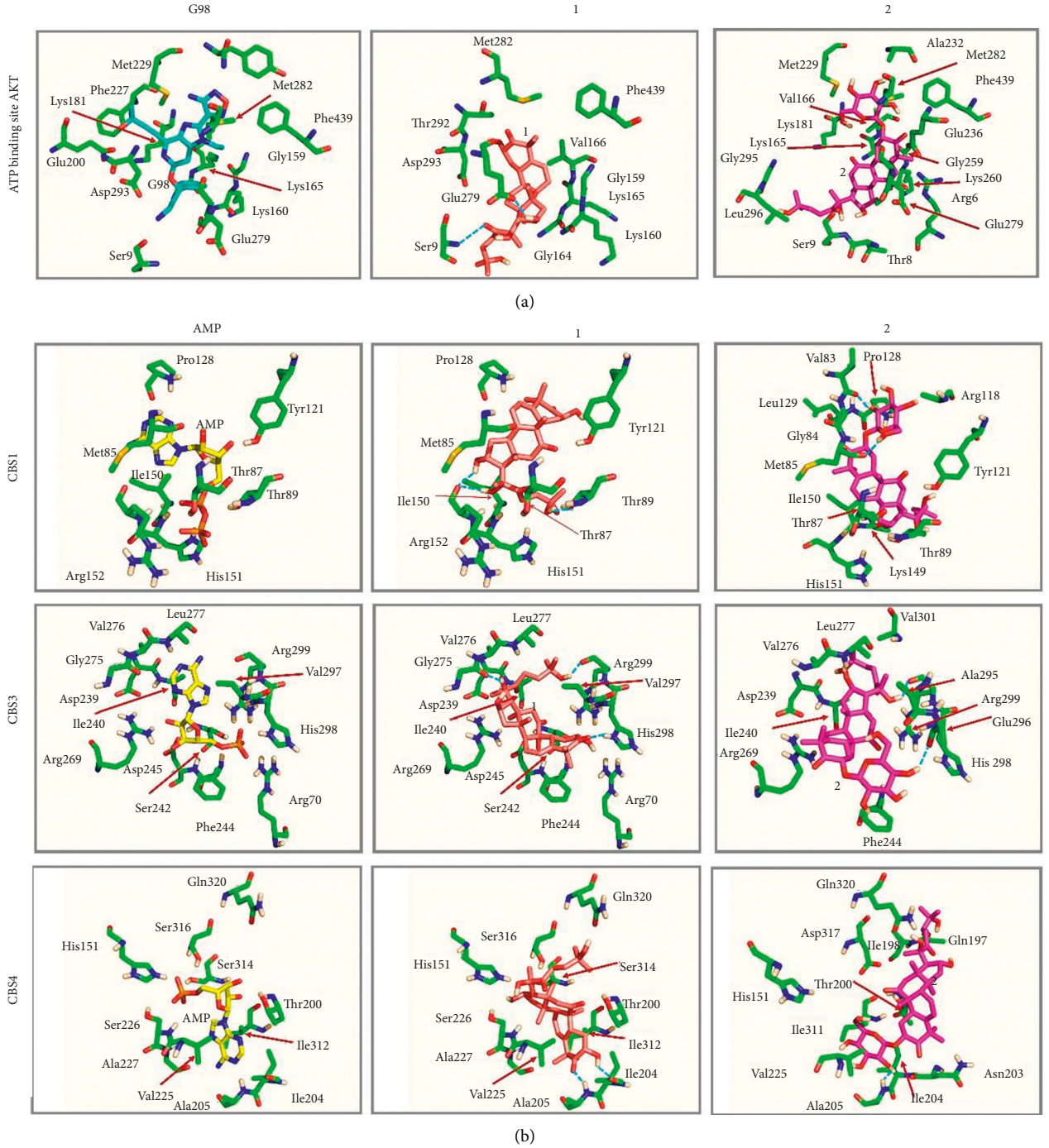


Figure 7: Binding of compounds 1 and 2 to AKT and AMPK. (a) Binding of compounds 1 and 2 to the ATP binding site of AKT showing inhibitor G98, and compounds 1 and 2 bound. (b) Binding of compounds 1 and 2 to the cystathionine β-synthetase (CBS) sites CBS1, CBS3, and CBS4 of AMPK. Panels show the residues of the active site of the enzymes located within 4 Å of compounds 1 and 2. Amino acids (colored in green) and compounds 1 (colored in pink) and 2 (colored in magenta) are shown as sticks. For AKT, the inhibitor G98 is shown in cyan, while for AMPK, ADP bound to CBS1 and AMP bound to CBS3 and CBS4 are shown in yellow. H-bonds of the compounds with residues of the binding site of the enzymes are indicated by orange dashed lines. The figure was drawn with PyMOL version 2.1.0 (Schrödinger, LLC; https://sourceforge.net/p/pymol).

two sites, particularly with CBS3, which is considered the sensor site of adenine nucleotides, the activation of this kinase is almost half of that observed in DM treated with 1 in EAT and far lower in SM. Some studies have demonstrated that precisely, this binding of AMP to site CBS4 stabilizes the molecule in a conformation that allows the preference of binding for AMP to site CBS3, maintaining the relation of a high affinity for AMP/low affinity for ATP. When CBS4 does not bind AMP, this affinity relation is reversed, increasing the affinity for ATP and preventing the sensing of

adenine nucleotides and therefore the activation of AMPK [55]. Our docking results indicate that these compounds are likely to interact with AMPK in a similar way to AMP and ADP in the motifs CBS1, CBS3, and CBS4, so it seems logical that by not having a high affinity for site CBS4, cucurbitacin 2 has a lesser effect on the activation of this enzyme.

Cucurbitacin 4 was bound to a different site of the structure near the other compounds but with very low affinity. Although docking analysis indicated that 4 (a more voluminous compound) does not bind adequately to either of the structures, these results do not rule out the interaction of this compound with the kinases. Since protein structures are quite dynamic, it may be possible that in another conformation, this protein may be able to bind compound 4 with higher affinity.

AKT is a serine-threonine protein kinase activated by the phosphorylation of two critical residues, Thr308 and Ser473, which induces a substantial conformational change, increasing its kinase activity [59]. Docking analysis of our compounds against this kinase indicated that 1 and 2 may bind with high affinity to a site close to the binding site of the competitive inhibitor of AKT G98, with theoretical dissociation constant (K_d) values of 1.43 and 37.05 μM, respectively (Figure 7(a) and Table 2). It was also observed that unlike the G68 inhibitor, compounds 1 and 2 do not block the access of ATP to the binding site. With respect to 4, the results indicated that it may bind with low affinity to a different site near the entrance to the ATP binding site. Relating this result with Figure 6(i) indicates that although these interactions are close to an inhibition site, unexpectedly, they promote the activation of AKT in EAT. It has been reported that ATP-competitive inhibitors cause hyperphosphorylation of AKT by stabilizing a conformation in which both phosphorylated sites are inaccessible to phosphatases [59, 60]. As our compounds show high affinity for the binding site of the competitive inhibitor but do not block access to ATP, it is possible that they help stabilize the conformation of AKT to maintain phosphorylation by AMPK, allowing the binding of ATP and creating a positive feedback loop for the activation of AKT. Similarly, with 4 being the most voluminous compound, its steric impediment would prevent the formation of the stable conformation of AKT, which would explain why we did not observe an increase in *p*AKT in the groups treated with this cucurbitacin. Nevertheless, further studies are needed to corroborate this hypothesis.

Although, to the best of our knowledge, there is no study of the structure activity of the activation of AMPK by cucurbitacins; it appears that the activation of AMPK by cucurbitacin-type triterpenes is particularly structure-dependent. For example, in our case, glucoside 2 induced lower AMPK phosphorylation than its aglycone 1, being in the most active compound SM. Furthermore, cucurbitacin B, whose only structural dissimilarity from 1 is the presence of a 23–24 double bond, improved insulin tolerance through translocation of Glut4 by activation of the PI3K/AKT signaling pathway [61]. Another case is 3b, 7b, 25-trihydroxy-Cucurbita-5,23 (E)-dien-19al, which activates AMPK not allosterically but via CaMKKb in L6 myotubes [62]. Nevertheless, the malonic ester of this cucurbitacin enhances glucose uptake via insulin receptor substrate 1 (IRS-1) rather than AMPK in C2C12 myotubes and *in vivo* in ICR mice; interestingly, this study also describes a tissue-dependent behavior, such as the cucurbitacins from *I. lindheimeri*, between skeletal muscle and adipose tissue [63].

These results indicate that cucurbitacins may indeed bind and modulate the activity of AKT and AMPK kinases, promoting Glut4 translocation to the PM in a tissue-dependent manner.

4. Conclusion

The results obtained show that cucurbitacins 1 and 2 and acetylated derivative 4 reduce glycemia *in vivo* in a model of chemically induced diabetes. Treatment with these cucurbitacins seems to low glycemia, stimulating glucose uptake by promoting Glut4 translocation to the PM of their target tissues, mainly in adipose tissue. Cucurbitacins 1, 2, and 4 also induced AMPK activation in MS and the dual activation of AMPK and AKT in EAT in an insulin-independent manner. Docking analysis suggests that 1 and 2 can bind to CBS3 activation and CBS4 stabilization sites of AMPK. Additionally, they can bind with high affinity to a site close to the competitive inhibitor AKT G98 binding site possibly helping to stabilize its structure, promoting its activation in adipose tissue.

Our results indicate that adipose tissue plays a key role in the development of diabetes highlighting it as a therapeutic target for the treatment of diabetes with compounds able to increase glucose uptake in this tissue. Furthermore, since the activity of these cucurbitacins does not depend on the insulin signaling pathway, they are useful for both the treatment of DMT1 and DMT2, where the absence or resistance to insulin prevents glucose uptake through its canonical route. Treatment with 1, 2, and 4 could help better control glycemia, and DMT1 likely decreases the insulin dose requirement.

Finally, these results indicate that *I. lindheimeri* constitutes a potential new source for obtaining new compounds for the treatment of diabetes. Therefore, more *in vivo* and *in vitro* studies are necessary to fully characterize the pharmacokinetics and the threshold of activated proteins required to exert their biological activities in a tissue-dependent manner, as well as to explore the effect of these cucurbitacins as chronic treatments.

Data Availability

The NMR and docking data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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References

[1] American Diabetes Association, "8. Pharmacologic approaches to glycemic treatment: standards of medical Care in diabetes-2018," *Diabetes Care*, vol. 41, 2018.

[2] K. Imam, *Management and Treatment of Diabetes Mellitus*, Springer, New York, NY, USA, 2013.

[3] C. Veeresham, "Natural products derived from plants as a source of drugs," *Journal of Advanced Pharmaceutical Technology & Research*, vol. 3, no. 4, pp. 200-201, 2012.

[4] D. J. Newman and G. M. Cragg, "Natural products as sources of new drugs over the 30 years from 1981 to 2010," *Journal of Natural Products*, vol. 75, no. 3, pp. 311–335, 2012.

[5] J. L. Figueroa-Hernández and M. Martínez-Vázquez, "Chemical constituents from ibervillea lindheimeri (A. gray) greene," *Biochemical Systematics and Ecology*, vol. 54, pp. 237–239, 2014.

[6] "Text available under a CC-BY-SA creative commons attribution license, ibervillea lindheimeri," 2005, https://www.llifle.com/Encyclopedia/SUCCULENTS/Family/Cucurbitaceae/31787/Ibervillea_lindheimeri.

[7] J. C. Chen, M. H. Chiu, R. L. Nie, G. A. Cordell, and S. X. Qiu, "Cucurbitacins and cucurbitane glycosides: structures and biological activities," *Natural Product Reports*, vol. 22, no. 3, pp. 386–399, 2005.

[8] U. Kaushik, V. Aeri, and S. R. Mir, "Cucurbitacins—an insight into medicinal leads from nature," *Pharmacognosy Reviews*, vol. 9, pp. 12–18, 2015.

[9] M.-J. Tan, J.-M. Ye, N. Turner et al., "Antidiabetic activities of triterpenoids isolated from bitter melon associated with activation of the AMPK pathway," *Chemistry & Biology*, vol. 15, no. 3, pp. 263–273, 2008.

[10] S. Huang and M. P. Czech, "The GLUT4 glucose transporter," *Cell Metabolism*, vol. 5, no. 4, pp. 237–252, 2007.

[11] D. Leto and A. R. Saltiel, "Regulation of glucose transport by insulin: traffic control of GLUT4," *Nature Reviews Molecular Cell Biology*, vol. 13, no. 6, pp. 383–396, 2012.

[12] T. Rodriguez, T. Pacheco-Fernández, A. Vázquez-Mendoza et al., "MGL1 receptor plays a key role in the control of T. cruzi infection by increasing macrophage activation through modulation of ERK1/2, c-jun, NF-κB and NLRP3 pathways," *Cells*, vol. 9, p. 108, 2020.

[13] K. Hayashi, R. Kojima, and M. Ito, "Strain differences in the diabetogenic activity of streptozotocin in mice," *Biological and Pharmaceutical Bulletin*, vol. 29, no. 6, pp. 1110–1119, 2006.

[14] M. Ito, Y. Kondo, A. Nakatani, and A. Naruse, "New model of progressive non-insulin-dependent diabetes mellitus in mice induced by streptozotocin," *Biological and Pharmaceutical Bulletin*, vol. 22, no. 9, pp. 988-989, 1999.

[15] G. Tucker, C. Casey, P. Phillips, H. Connor, J. Ward, and H. Woods, "Metformin kinetics in healthy subjects and in patients with diabetes mellitus," *British Journal of Clinical Pharmacology*, vol. 12, no. 2, pp. 235–246, 1981.

[16] M. Foretz, B. Guigas, L. Bertrand, M. Pollak, and B. Viollet, "Metformin: from mechanisms of action to therapies," *Cell Metabolism*, vol. 20, no. 6, pp. 953–966, 2014.

[17] J. Guerrero-Analco, O. Medina-Campos, F. Brindis et al., "Antidiabetic properties of selected mexican copalchis of the rubiaceae family," *Phytochemistry*, vol. 68, no. 15, pp. 2087–2095, 2007.

[18] K. H. Kim, I. S. Lee, J. Y. Park, Y. Kim, E. J. An, and H. J. Jang, "Cucurbitacin B induces hypoglycemic effect in diabetic mice by regulation of AMP-activated protein kinase alpha and glucagon-like peptide-1 via bitter taste receptor signaling," *Frontiers in Pharmacology*, vol. 9, pp. 1–13, 2018.

[19] N. Hunsakunachai, N. Nuengchamnong, W. Jiratchariyakul, T. Kummalue, and P. Khemawoot, "Pharmacokinetics of cucurbitacin B from *Trichosanthes cucumerina* L. in rats," *BMC Complementary and Alternative Medicine*, vol. 19, pp. 157–212, 2019.

[20] Y. Hashimoto, M. Tanaka, H. Okada et al., "Postprandial hyperglycemia was ameliorated by taking metformin 30 min before a meal than taking metformin with a meal; a randomized, open-label, crossover pilot study," *Endocrine*, vol. 52, no. 2, pp. 271–276, 2016.

[21] N. Yamamoto, Y. Yamashita, Y. Yoshioka, S. Nishiumi, and H. Ashida, "Rapid preparation of a plasma membrane fraction: western blot detection of translocated glucose transporter 4 from plasma membrane of muscle and adipose cells and tissues," *Current Protocols in Protein Science*, vol. 29, 2016.

[22] M. Mueckler, C. Caruso, S. A. Baldwin et al., "Sequence and structure of a human glucose transporter," *Science*, vol. 229, no. 4717, pp. 941–945, 1985.

[23] M. Daniele, G. Fuhrich, B. A. Lessey et al., "Comparison of HSCORE assessment of endometrial β3 integrin subunit expression with digital HSCORE using computerized image analysis (ImageJ)," *Anal Quant Cytopathol Histpathol*, vol. 35, pp. 210–216, 2013.

[24] E. F. Pettersen, T. D. Goddard, C. C. Huang et al., "UCSF chimera? a visualization system for exploratory research and analysis," *Journal of Computational Chemistry*, vol. 25, no. 13, pp. 1605–1612, 2004.

[25] G. M. Morris, R. Huey, W. Lindstrom et al., "AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility," *Journal of Computational Chemistry*, vol. 30, no. 16, pp. 2785–2791, 2009.

[26] R. Huey, G. M. Morris, A. J. Olson, and D. S. Goodsell, "A semiempirical free energy force field with charge-based desolvation," *Journal of Computational Chemistry*, vol. 28, no. 6, pp. 1145–1152, 2007.

[27] R. Croteau, T. M. Kutchan, and N. G. Lewis, "Secondary metabolites," *Biochemistry and Molecular Biology of Plants*, vol. 7, pp. 1250–1318, 2000.

[28] A. A. Alghasham, "Cucurbitacins: a promising target for cancer therapy," *International Journal of Health Sciences*, vol. 7, no. 1, pp. 77–89, 2013.

[29] A. Rojas, L. Hernandez, R. Pereda-Miranda, and R. Mata, "Screening for antimicrobial activity of crude drug extracts and pure natural products from mexican medicinal plants," *Journal of Ethnopharmacology*, vol. 35, no. 3, pp. 275–283, 1992.

[30] G. v. Dang, B. M. Rode, and H. Stuppner, "Quantitative electronic structure-activity relationship (QESAR) of natural cytotoxic compounds: maytansinoids, quassinoids and cucurbitacins," *European Journal of Pharmaceutical Sciences*, vol. 2, pp. 331–350, 1994.

[31] Y. Yamada, K. Hagiwara, K. Iguchi, S. Suzuki, and H. Hsu, "Isolation and structures of arvenins from anagallis arvensis L. (Primulaceae). New cucurbitacin glucosides," *Chemical and Pharmaceutical Bulletin*, vol. 26, no. 10, pp. 3107–3112, 1978.

[32] S. Fujita, R. Kasai, K. Ohtani et al., "Dammarane glycosides from aerial parts of neoalsomitra integrifoliola," *Phytochemistry*, vol. 38, no. 2, pp. 465–472, 1995.

[33] Y. Yamada, K. Hagiwara, K. Iguchi, Y. Takahasi, and H.-Y. Hsu, "Cucurbitacins from Anagallis arvensis," *Phytochemistry*, vol. 17, no. 10, p. 1798, 1978.

[34] L. Harinantenaina, M. Tanaka, S. Takaoka et al., "Momordica charantia constituents and antidiabetic screening of the isolated major compounds," *Chemical and Pharmaceutical Bulletin*, vol. 54, no. 7, pp. 1017–1021, 2006.

[35] F. Rivera-Ramírez, G. N. Escalona-Cardoso, L. Garduño-Siciliano, C. Galaviz-Hernández, and N. Paniagua-Castro, "Antiobesity and hypoglycaemic effects of aqueous extract of ibervillea sonorae in mice fed a high-fat diet with fructose," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 968984, 6 pages, 2011.

[36] K. Yagasaki, "Anti-diabetic phytochemicals that promote GLUT4 translocation via AMPK signaling in muscle cells," *Nutrition and Aging*, vol. 2, no. 1, pp. 35–44, 2014.

[37] J. T. Siqueira, E. Batistela, M. P. Pereira et al., "Combretum lanceolatum flowers ethanol extract inhibits hepatic gluconeogenesis: an in vivo mechanism study," *Pharmaceutical Biology*, vol. 54, no. 9, pp. 1671–1679, 2016.

[38] C. H. Ho, M. G. Ho, S.-P. Ho, and H. H. Ho, "Bitter bottle gourd (*Lagenaria siceraria*) toxicity," *Journal of Emergency Medicine*, vol. 46, no. 6, pp. 772–775, 2014.

[39] M. Saade, J. Magdalou, N. Ouaini, and H. Greige-Gerges, "Stability of cucurbitacin E in human plasma: chemical hydrolysis and role of plasma esterases," *Biopharmaceutics & Drug Disposition*, vol. 30, no. 7, pp. 389–397, 2009.

[40] R. Lage, C. Diéguez, A. Vidal-Puig, and M. López, "AMPK: a metabolic gauge regulating whole-body energy homeostasis," *Trends in Molecular Medicine*, vol. 14, no. 12, pp. 539–549, 2008.

[41] V. Sarabia, L. Lam, E. Burdett, L. A. Leiter, and A. Klip, "Glucose transport in human skeletal muscle cells in culture. Stimulation by insulin and metformin," *Journal of Clinical Investigation*, vol. 90, no. 4, pp. 1386–1395, 1992.

[42] M. Camps, A. Castelló, P. Muñoz et al., "Effect of diabetes and fasting on GLUT-4 (muscle/fat) glucose-transporter expression in insulin-sensitive tissues. Heterogeneous response in heart, red and white muscle," *Biochemical Journal*, vol. 282, no. 3, pp. 765–772, 1992.

[43] D. E. James, K. M. Burleigh, and E. W. Kraegen, "Time dependence of insulin action in muscle and adipose tissue in the rat in vivo: an increasing response in adipose tissue with time," *Diabetes*, vol. 34, no. 10, pp. 1049–1054, 1985.

[44] E. D. Abel, O. Peroni, J. K. Kim et al., "Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver," *Nature*, vol. 409, no. 6821, pp. 729–733, 2001.

[45] Y. Minokoshi, C. R. Kahn, and B. B. Kahn, "Tissue-specific ablation of the GLUT4 glucose transporter or the insulin receptor challenges assumptions about insulin action and glucose homeostasis," *Journal of Biological Chemistry*, vol. 278, no. 36, pp. 33609–33612, 2003.

[46] E. Carvalho, K. Kotani, O. D. Peroni, and B. B. Kahn, "Adipose-specific overexpression of GLUT4 reverses insulin resistance and diabetes in mice lacking GLUT4 selectively in muscle," *American Journal of Physiology—Endocrinology And Metabolism*, vol. 289, pp. E551–E561, 2005.

[47] Y. Tamori, H. Sakaue, and M. Kasuga, "RBP4, an unexpected adipokine," *Nature Medicine*, vol. 12, no. 1, pp. 30-31, 2006.

[48] R. Tao, J. Gong, X. Luo et al., "AMPK exerts dual regulatory effects on the PI3K pathway," *Journal of Molecular Signaling*, vol. 5, pp. 1–9, 2010.

[49] R. Zapata-Bustos, Á. J. Alonso-Castro, M. Gómez-Sánchez, and L. A. Salazar-Olivo, "Ibervillea sonorae (cucurbitaceae) induces the glucose uptake in human adipocytes by activating a PI3K-independent pathway," *Journal of Ethnopharmacology*, vol. 152, no. 3, pp. 546–552, 2014.

[50] F. Drissi, F. Lahfa, T. Gonzalez et al., "A *Citrullus colocynthis* fruit extract acutely enhances insulin-induced GLUT4 translocation and glucose uptake in adipocytes by increasing PKB phosphorylation," *Journal of Ethnopharmacology*, vol. 270, Article ID 113772, 2021.

[51] Y. Zhao, X. Hu, Y. Liu et al., "ROS signaling under metabolic stress: cross-talk between AMPK and AKT pathway," *Molecular Cancer*, vol. 16, pp. 79–12, 2017.

[52] J. Ning, G. Xi, and D. R. Clemmons, "Suppression of AMPK activation via S485 phosphorylation by IGF-I during hyperglycemia is mediated by AKT activation in vascular smooth muscle cells," *Endocrinology*, vol. 152, pp. 3143–3154, 2011.

[53] S.-C. Lin and D. G. Hardie, "AMPK: sensing glucose as well as cellular energy status," *Cell Metabolism*, vol. 27, no. 2, pp. 299–313, 2018.

[54] F.-J. Xin, J. Wang, R.-Q. Zhao, Z.-X. Wang, and J.-W. Wu, "Coordinated regulation of AMPK activity by multiple elements in the α-subunit," *Cell Research*, vol. 23, no. 10, pp. 1237–1240, 2013.

[55] X. Gu, Y. Yan, S. J. Novick et al., "Deconvoluting AMP-activated protein kinase (AMPK) adenine nucleotide binding and sensing," *Journal of Biological Chemistry*, vol. 292, no. 30, pp. 12653–12666, 2017.

[56] Y. Yan, X. E. Zhou, H. E. Xu, and K. Melcher, "Structure and physiological regulation of AMPK," *International Journal of Molecular Sciences*, vol. 19, 2018.

[57] J. L. Ríos, I. Andújar, J. M. Escandell, R. M. Giner, and M. C. Recio, "Cucurbitacins as inducers of cell death and a rich source of potential anticancer compounds," *Current Pharmaceutical Design*, vol. 18, pp. 1663–1676, 2012.

[58] S. O. Chung, Y. J. Kim, and S. U. Park, "An updated review of cucurbitacins and their biological and pharmacological activities," *EXCLI journal*, vol. 14, pp. 562–566, 2015.

[59] T. Okuzumi, D. Fiedler, C. Zhang et al., "Inhibitor hijacking of Akt activation," *Nature Chemical Biology*, vol. 5, no. 7, pp. 484–493, 2009.

[60] K. Lin, J. Lin, W.-I. Wu et al., "An ATP-Site On-Off Switch that Restricts Phosphatase Accessibility of Akt," *Science Signaling*, vol. 5, 2012.

[61] F. S. A. Saadeldeen, Y. Niu, H. Wang et al., "Natural products: regulating glucose metabolism and improving insulin resistance," *Food Science and Human Wellness*, vol. 9, no. 3, pp. 214–228, 2020.

[62] T. J. Iseli, N. Turner, X. Y. Zeng et al., "Activation of AMPK by bitter melon triterpenoids involves CaMKKβ," *PLoS One*, vol. 8, Article ID e62309, 2013.

[63] J. H. Han, N. Q. Tuan, M. H. Park et al., "Cucurbitane triterpenoids from the fruits of momordica charantia improve insulin sensitivity and glucose homeostasis in streptozotocin-induced diabetic mice," *Molecular Nutrition & Food Research*, vol. 62, Article ID e1700769, 2018.