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Current Proteomics, XXXX, *XX*, 1-12

RESEARCH ARTICLE

In Vitro and *In Silico* Evaluation of Caffeic and Ferulic Acids Involvement in the Translocation of Glucose Transporter 4

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Abstract: ***Background*:** Insulin is a key hormone in our systems. Upon binding of insulin to its receptors in fat and muscle tissues, tens of proteins in the insulin signaling pathway are involved in the process of GLUT4 vesicle recruitment to the Plasma Membrane (PM) and the absorption of serum glucose. Deficits in the aforementioned pathway lead to insulin resistance and eventually to Type II Diabetes Mellitus.

***Objective*:** We appreciate the contribution of phytochemicals in the treatment of diabetes. Yet, *in vitro* and *in silico* studies are needed to validate the safety and efficacy of the phytochemicals, plus their action mechanisms.

***Methods*:** Herein, we tested two phytochemicals, caffeic acid and ferulic acid *in vitro* and *in silico*. We shed light on the insulin signaling proteins as plausible therapeutic targets using *in silico* studies, *via* AutoDock and SwissADME.

***Results*:** Results obtained *in vitro* indicate that Caffeic Acid (CA) increased GLUT4 translocation at 125μM by 31% in the absence of insulin, and 24.5% in presence of insulin, when compared to the control. Ferulic Acid (FA) was less potent as an enhancer of GLUT4 translocation. Best docking results were found for the binding of the phytochemicals CA and FA to PDK1, AKT, IRS1 and PTEN proteins of the insulin signaling, with comparable results.

***Conclusion*:** These findings indicate that CA and FA possess a limited anti-diabetic potency by increasing GLUT4 trafficking to the PM in skeletal muscles. These results suggest that these compounds are candidates for further investigation in pre-clinical and clinical stages of drug discovery.

ARTICLE HISTORY

Received: June 21, 2024
Revised: August 23, 2024
Accepted: September 01, 2024

DOI: 10.2174/0115701646334678240913105521

Keywords: Type 2 diabetes mellitus, phytochemicals, *in silico*, caffeic acid, ferulic acid, GLUT4, insulin signaling, docking.

1. INTRODUCTION

Diabetes is a devastating disorder defined by elevated glucose levels in the blood. The disease is a consequence of defects in insulin production, insulin action, or the two factors altogether [1, 2]. The main types of diabetes include Type I Diabetes Mellitus (T1DM) and Type II Diabetes Mellitus (T2DM) [3-5]. T1DM is called insulin-dependent diabetes or juvenile-onset diabetes. It is a result of the autoimmunity of the body that causes the destruction of pancreatic beta-cells, which are responsible for insulin production. The body, in turn, produces little or no insulin, resulting in insulin deficiency [6]. T2DM is also called non-insulin-dependent or adult-onset diabetes. Insulin resistance, which is characterized by a decreased sensitivity of body cells to the signals of insulin, is what causes the disease to start. As the condition progresses, the ability to make insulin gradually declines over time [7, 8]. Insulin is a polypeptide pancreatic hormone that plays an essential role in regulating blood glucose. In reaction to elevated blood glucose levels, insulin is quickly released. Different organs along insulin's physiological journey are meticulously involved in the downstream response to insulin signaling [9, 10]. Insulin binding to the insulin receptor stimulates several signaling cascades [11]. The process begins when insulin binds to the IR receptor on the plasma membrane of muscle or fat tissue and induces the IR tyrosine kinase activity, causing autophosphorylation

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[12]. As a result, transmembrane domains are structurally re-ordered, which makes them close to each other. Consequently, IR tyrosine kinase is activated to phosphorylate its main substrates, IRS1 and IRS2 [13]. Consequently, class I Phosphoinositide 3-kinases (PI3K) are attracted, and the membrane domains are augmented with Phosphatidylinositol 3,4,5-trisphosphate (PIP3) [14]. An interaction of PIP3 with the PH domain of AKT follows, which makes the protein ready for phosphorylation [15]. This activation process happens indirectly *via* 3-phosphoinositide-dependent protein kinase-1 (PDK1). PDK1 has two major domains: a PH domain and a kinase domain [16]. When the PI3P activates the PH domain, the kinase domain activates the AKT protein at THR308 [17]. Activated AKT1 and AKT2 travel to the endomembrane system [18], and they phosphorylate AS160 [19]. The GAP (GTPase-activating proteins) action of the TBC domain of AS160 inhibits Rab (Ras-associated binding) family small GTPases [20]. When AS160 is phosphorylated due to insulin signaling, its GAP activity is repressed [21]. The distribution of GLUT4 is performed *via* insulin-regulated vesicular traffic in the pathway [22]. Herein, Rab GTPases control vesicle fission [23-25], destination [26], and fusion [27-29]. AS160-targeted Rabs include the Rabs 8A, 10, 13 and 14 [30-32]. In addition to AS160, PTEN (Phosphatase and TENsin homolog) is another regulatory protein found in the insulin cascade, where it functions as a phosphatase [33]. In humans, the protein is expressed by the *PTEN* gene. Two domains make up this protein: a catalytic domain and a tensin-like domain, much as other members of the protein tyrosine phosphatase family [34]. PTEN favorably dephosphorylates phosphoinositide substrates, which differentiates it from other proteins in the family [33]. It is a negative regulator for the intracellular amounts of phosphatidylinositol-3,4,5-trisphosphate. It also inactivates the IR1 and IR2, leading to the suppression of the insulin signaling pathway, Fig. (**1**) [35-37]. On the other side, the non-canonical cascade causes insulin to induce RAC1 to load up on GTP. RAC1 activation leads to GLUT4 translocation [38-40].

Two further proteins are crucial components in the drug discovery pipeline: Protein Phosphatase 2A (PP2A) and Protein Tyrosine Phosphatase 1B (PTP1B). A malfunctioning Protein Phosphatase 2A (PP2A) has been associated with the occurrence of T2DM [43]. PP2A blocks the activity of AKT when it is active [44], thus acting as a negative effector of insulin signaling. Activation of PTP1B decreases the phosphorylation of IR protein, which is associated with inhibited insulin signaling and glucose uptake, and, consequently, insulin resistance [45].

Indeed, in insulin resistance and T2DM, insulin signaling is impaired, and it thus does not promote the translocation of GLUT4 to the Plasma Membrane (PM). Several anti-diabetic synthetic and natural drugs circumvent insulin resistance by augmenting the translocation of GLUT4 *via* pathways that are insulin-independent [46, 47].

The utilization of medicinal plants in drug development aids in isolating and developing new bioactive lead compounds [48-51]. According to the World Health Organization (WHO), around 80% of the population in developing countries depends on traditional medicine in the treatment of day-to-day diseases.

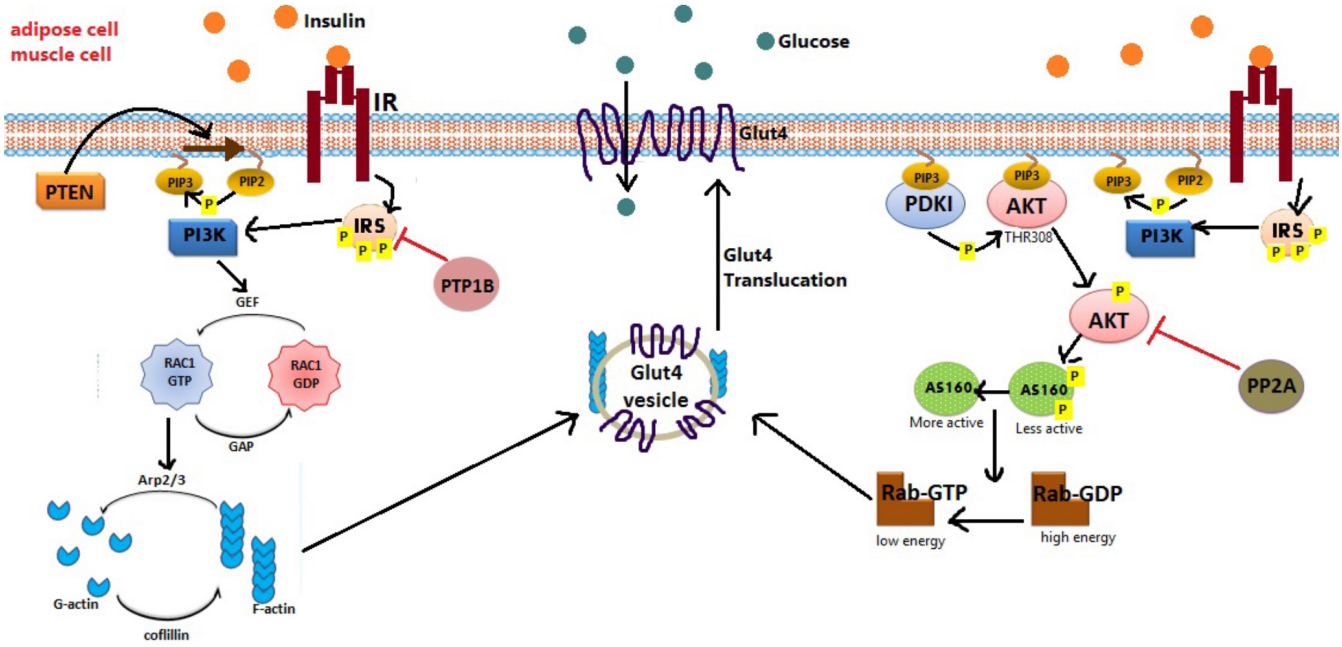

Fig. (1). GLUT4 translocation *via* insulin signaling cascade: All studied proteins in this study are shown in the figure [41, 42]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The chemical compounds that are experimentally reported to have positive effects in GLUT4 translocation include caffeic acid and ferulic acid [52-54]. The 2D structures for the two compounds are retrieved *via* Open Babel [55] and are shown in Fig. (**2a** and **b**). We appreciate that the two compounds have the same phenolic structure except for an additional methyl group in ferulic acid. Indeed, we previously detected caffeic acid and ferulic acid in several anti-diabetic extracts from medicinal plants [53, 56], especially *Gundelia tournefortii* [52, 53]. *Gundelia tournefortii* is a wild and edible Mediterranean plant scientifically tested to have a wide variety of medicinal actions, including anti-diabetic, anticancer, antibacterial, and anti-epileptic effects [52, 57, 58].

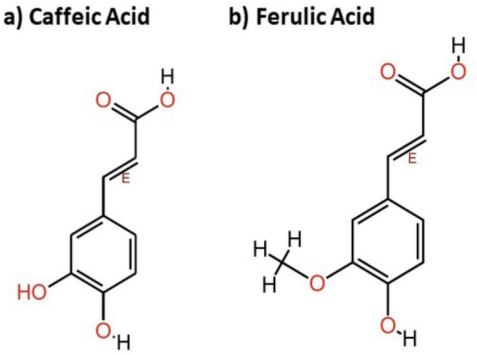

Fig. (2). Chemical structures of **a**) caffeic acid; **b**) ferulic acid. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

In vitro studies have been extensively utilized to understand the mechanisms by which plausible drugs stimulate GLUT4 translocation to the plasma membrane [53, 54, 59]. Additionally, the prediction of *in silico* ADME/Tox (Absorption, Distribution, Metabolism and Excretion/Toxicity) is a powerful tool for studying the bioactive properties of plausible drugs [60, 61]. The use of these models pre-clinically is important since late-stage failures in clinical trials result in substantial wastes of time and money, among other adverse effects.

We aim in this study to predict the drug-likeness of the selected two phytochemicals, caffeic acid, and ferulic acid, as potential drugs in fighting insulin resistance using the *in vitro* test of GLUT4 localization to the plasma membrane, as well as molecular docking. This aids in shedding light on the role of these chemical compounds as effectors on hub proteins in the insulin signaling cascade: PI3K, PDK1, AKT, RAC1, PTP1B, IRS1, 1RS1, 1IR3, PTEN, PP2A, and AS160. The protein-ligand docking was performed between each ligand and each one of the key protein targets found in the insulin cascade in muscles. *In vitro* studies that were carried out in this study validate the effect of the two phytochemicals in translocating GLTU4 to the cell membrane. We predict that these two phytochemicals can work as effectors for PDK1, AKT, IRS1, and PTEN. Accordingly, GLUT4 translocation to the plasma membrane increases.

2. MATERIALS AND METHODS

2.1. *In vitro* Evaluation of GLUT4 Translocation by Caffeic Acid and Ferulic Acid

2.1.1. Materials

Rat L6 muscle cell lines stably expressing myc-tagged GLUT4 (L6-GLUT4myc) were obtained from Kerafast (Boston, MA, USA). α-MEM (modified Eagle's medium), fetal bovine serum, and all other animal cell culture reagents used here were obtained from the biological industries (Beit Haemek, Israel). Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit antibodies were bought from Promega (Madison, WI, USA). Caffeic acid, ferulic acid, the polyclonal anti-myc (A-14), and other standard chemicals were purchased from Sigma.

2.1.2. Cell Culture

L6-GLUT4myc cells were maintained in myoblast monolayer culture. All cells were grown under an atmosphere of 5% CO2 and 95% air in α-MEM supplemented with 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin antibiotic, and 10% fetal bovine serum.

2.1.3. MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was optimized for the L6 cell lines used in the experiments and was performed to measure cell viability, as we reported earlier [56]. Cells at a density of $2A\text{-}10^2$ I1/$4L^{-1}$ per well were plated in 96-well plates and were left for 24 h, such that they attached to the plate during the twenty-four-hour period. CA and FA were introduced at cumulative concentrations (0-1 mM) for 24 h. The medium was then substituted with 200 I1/4L fresh medium/well containing 0.5 mg mL^{-1} MTT salt, and it was cultured for an additional 4h in the tissue incubator. The supernatant was replaced with 100 I1/4L of 1mM HCl in 100% isopropanol. The absorbance was latermeasured with a microplate reader (Anthos) at 570 nm. The effect of CA and FA on cell viability was calculated according to the below formula:

Percent viability=(A_{570nm} of plant extract treated sample/$_{A570nm}$ of none treated sample) A-100

2.1.4. Determination of Surface GLUT4myc

Surface myc-tagged GLUT4 was measured in intact cells, as previously described [62]. To this aim, an anti-myc antibody and a secondary antibody conjugated to horseradish peroxidase were used. Cells were then grown at a density of $4X10^{10}$ mL^{-1} per well and subsequently plated in 24-well plates and were left to be fastened to the plate for 24 h. CA and FA were added for 24 h, and the cells were serum-starved at the last 3 h and treated in the absence and presence of 1 μM insulin for 20 min. The cells were washed with PBS at a temperature of 4 °C, fixed for 15 min with 3% paraformaldehyde (on ice), blocked with 3% (v/v) goat serum for 10 min, reacted with anti-*myc* antibody (1:200)

for 1 h, and kept on ice. Cells were then washed extensively with PBS, reacted with the secondary antibody (1:1000) for 1 h, and washed with PBS. After that, 0.5 mL of *o*-phenylenediamine dihydrochloride solution was introduced to all wells. Afterward, we waited for the yellow color to develop for 20-30 min in the linear range at room temperature (in the dark). The reaction was stopped with 0.5 mL of 3 N HCl per well. Absorbance was measured with a microplate reader (Anthos) at 492 nm. Background (blank) absorbance was obtained in six wells in the absence of anti-*myc* antibody treatment.

2.1.5. Statistical Analysis

Data were presented as mean ± standard error. Statistical analysis was performed using SPSS version 23, followed by the t-comparison test. Statistical significance was assessed at a p-value < 0.05. All experiments were repeated in triplicates.

2.2. *In silico* Experiments for Target Druggability and Drug-likeness

2.2.1. Docking Experiments Investigating Ferulic Acid and Caffeic Acid in their Binding to the Protein Targets of the Insulin Signaling Pathway

Using the PubChem database, we derived the 2D structures and IUPAC names for CA and FA [63]. Using the systematic IUPAC structures, the SMILES structures of the two compounds were determined [64]. The Open Babel server was used to produce the PDB structures for the two chemicals used as inputs in the AutoDock tools, version 1.5.7 [55, 65, 66]. The compound ID of the 3D structures of ligands were: CID: 689043 for caffeic acid and CID: 445858 for ferulic acid.

We used the RCSB database [67] to extract the structures of the PI3K, PDK1, AKT, RAC1, PTP1b, IRS1, PP2A, IR, AS160, and PTEN proteins in the *holo*- form, where the protein is bonded to a positive control (PI3K, PDB ID: 6OAC [68]), (PDK1, PDB ID: 3HRF [69]), (AKT, PDB ID: 2UVM [70]), (RAC1, PDB ID: 2FJU [41]), (PTP1b, PDB ID: 7KLX [71]), (IRS1, PDB ID: 5U1M [72]), (PP2A, PDB ID: 2IE4 [73]), (IR, PDB ID: 1IR3 [74]), (AS160, PDB ID: 3QYB [75]), and (PTEN, PDB ID: 1D5R [76]), respectively. Afterwards, the ligand was removed, and the protein structure was converted to the *apo*- form.

These files were then prepared as input files for the docking protocol using the 4.2 version of the AutoDock software [66]. As described previously by us [77], and for all docking experiments, the target protein was retained rigid. The center of mass of the protein constituted the geometric center of the grid. Thus, the geometric center was selected to make it possible to scan the whole surface of the protein. The genetic algorithm was applied to prepare for a docking protocol with a rigid protein and a flexible ligand [78]. During preparation, some modifications were performed, such as adding polar hydrogen atoms and removing water and hetero-atoms from the protein crystal structure. This helped in preventing undesirable interactions during docking [79]. Afterward, a grid box was created, which defines the surface region to be scanned by the ligand at the protein surface. Any region outside the box is not explored during docking. The dimensions of the parallel rectangular lattice boxes were prepared. The dimensions for the grid boxes covering PI3K, PDK1, AKT, RAC1, PTEN, AS160, PTP1b, PP2A, IRS1, and IR total surfaces were (126 Å x 126 Å x 126 Å), (126 Å x 126 Å x 126 Å), (126 Å x 86 Å x 108 Å), (126 Å x 100 Å x 100 Å), (46 Å x 76 Å x 54 Å), (52 Å x, 62 Å x 54 Å), (62 Å x 54 Å x 52 Å), (70 Å x 116 Å x 116 Å), (44 Å x 42 Å x 48 Å), and (126 Å x 126 Å x 126 Å). This process was repeated twenty independent times for each of the two phytochemicals. After running the docking protocol, the resulting PDB files were extracted. Subsequently, results with the best scoring were selected, and the PDB files for the phytochemicals that showed the lowest free energies with protein were analyzed. Files with the lowest free energies, RMSD values, and inhibition constants were ranked first, and the results they contained were analyzed. PyMol 2.3 software was then utilized for molecular 3D visualization and analysis [80, 81]. The 'best fit' of the proteins and ligands and plausible non-covalent interactions were analyzed. Various types of interactions occur at the binding interface between the protein and ligand, such as electrostatic, hydrophobic, and Van der Waals force interactions [79, 82].

2.2.2. ADMEtox Properties

The study was run to assess the drug-likeness potential of the two compounds (CA and FA) using the SwissADME tool [60]. SwissADME is accessed at http://www.swissadme.chin, which is a free web browser that shows the SwissADME submission page. Input parameters include drug-likeness, medicinal chemistry pharmacokinetics, and physicochemistry friendliness properties. ADME is used to predict the (Absorption, Distribution, Metabolism, and Excretion) properties of the chemical structure of the drugs. On the SwissADME website, files were converted into molecular sketchers based on ChemAxon's Marvin JS. Afterward, ADME calculations with default parameters were carried out. Prepared SDF files for ligands were screened based on Lipinski's rule of five (RO5) in order to find the drug-likeness based on physiochemical criteria [83]. The bioavailability of drugs is given a low score if molecules violate more than one of the aforementioned rules.

3. RESULTS

3.1. *In Vitro* Results

3.1.1. In Vitro Results for the Toxicity of Caffeic Acid and Ferulic Acid

The MTT assay was applied to appraise the nontoxic concentrations of CA and FA on L6-GLUT4myc cells. Cells were seeded in 96 well plates and were subjected to increasing concentrations of the two compounds (0-1 mM) for 24 hours. Extract concentrations that led to less than 5% cell death were considered safe. CA was reasonably safe at least up to 1mM, and FA was safe up to 0.5mM (Fig. **3**). The effect of these compounds on GLUT4 translocation to the PM was tested at a concentration lower relative to the safe concentrations.

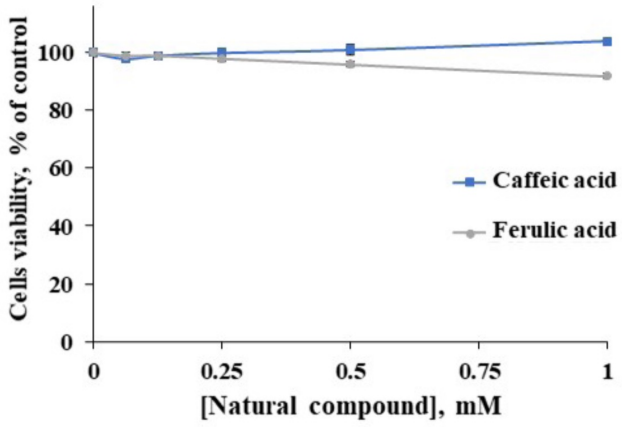

Fig. (3). Effect of CA and FA on cell viability by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were exposed to CA and FA for 24 h. Values represent means ± SEM (% of untreated control cells) of three independent experiments. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.1.2. Effects of Caffeic Acid and Ferulic Acid on GLUT4 Translocation

In muscle, the primary tissue responsible for dietary glucose uptake, insulin aids in the translocation of intracellular GLUT4 vesicles towards the PM to cause a quick rise in glucose uptake [85, 86]. However, in diabetes type II and insulin resistance, insulin signaling is impaired and thus does not promote GLUT4 translocation to the PM. The routes by which several anti-diabetic synthetic and natural drugs act help overcome insulin resistance. This can be achieved by augmenting GLUT4 translocation in insulin-dependent or independent pathways [41].

Herein, we tested the potential effect of two occurring natural compounds, *i.e*., CA and FA, on GLUT4 translocation to the PM in rat muscle cell line, namely L6-GLUT4myc. The compounds were added to the cells in the absence or presence of insulin, and the translocation of GLUT4myc to the plasma membrane was measured as described in the methods. The results we obtained indicate that insulin enhanced GLUT4 translocation from 100% (basal) to about 150% (Fig. **4**), as reported elsewhere in earlier studies [86]. CA and FA enhanced the exocytic trafficking of the GLUT4 intracellular storage vesicles to the PM in L6-GLUT4myc cells, both in the presence and absence of insulin.

We observed that CA enhanced GLUT4 translocation at 125 µM, from 100% to 131 ± 16% at basal conditions. It also increased GLUT4 translocation from 195 ± 27% to 243 ± 17% in cells treated with insulin (Fig. **4a**). FA unveiled a less effective result on GLUT4 translocation. GLUT4 translocation reached 119 ± 8% in the presence of insulin and 244 ± 30% in the absence of insulin (compared to 207 ± 15% in non-FA treated cells) in cells exposed to 125 µM FA (Fig. **4b**).

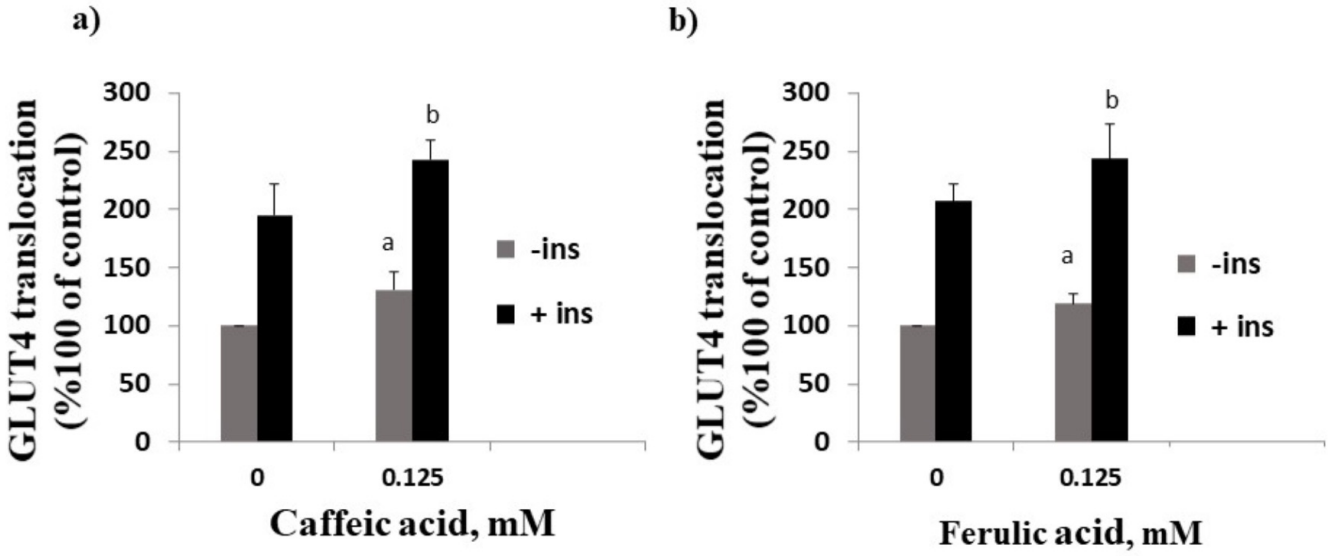

Fig. (4). Effect of CA **a**), and FA **b**), on GLUT4 translocation to the PM. 0.125µM CA and FA were introduced to L6-GLUT4myc cells (40,000 cells/well) for 24 h in the absence (-) or presence (+) of 1µM insulin for 20 min at 37°C. Surface myc-tagged GLUT4 density was measured *via* the antibody-coupled colorimetric assay. Values shown characterize means ± SEM (relative to untreated control cells) of three independent experiments. Results were considered to be statistically significant when $P < 0.05$; (**a**) compared with (-) in the control group, and (**b**) compared with (+) in the control group. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Binding free energies, inhibition constants, and structure deviation from the reference structure for the docking of the phytochemicals to the different proteins in the insulin signaling pathway.

S. No.	Protein	Caffeic-acid			Ferulic-acid		
		Autodock Binding Free Energy (joule/mol A-10^4)	Autodock Binding Constant (Ki) µM	RMSD (Å)	Autodock Binding Free Energy (joule/mol A-10^4)	Autodock Binding Constant (Ki) µM	RMSD (Å)
1	PI3K	-1.883	500.30	18.782	-1.883	500.30	18.782
2	PDK1	-2.435	54.16	18.723	-2.669	21.00	54.273
3	AKT	-2.431	55.39	26.786	-2.364	72.17	26.270
4	RAC1	-1.845	580.53	123.249	-1.787	746.11	123.058
5	PTP1B	-1.443	2.94 A-10^3	36.273	-1.406	3.46 A-10^3	37.002
6	IRS1	-2.929	7.38	10.430	-2.912	7.97	11.462
7	1IR3	-1.791	732.29	45.985	-1.791	733.66	47.225
8	PP2A	-1.435	3.05 A-10^3	67.091	-1.218	7.33 A-10^3	-
9	PTEN	-3.577	536.40 A-10^{-3}	98.465	-1.954	379.41	95.089
10	AS160	-1.736	912.34	97.421	-1.845	590.05	88.522

Note: * Ki: is the dissociation constant of a complex and molecules bound to it. RMSD is root-mean-square deviation, is used in superimposed proteins to measure the average distance between similar or identical atoms. I"G: standard Gibbs free energy for binding. Results that are shaded in red indicate positive bindings. Shaded cells in blue identify the results for best-fitting protein-ligand interfaces.

Table 2. ADMEtox properties for caffeic acid and ferulic acid.

Phytochemical	Physicochemical Properties (Lipinski's Rule of Five)				
	Molecular Weight (g mol^{-1})	H-bond acceptor	H-bond Donor	Log P	Bioavailability score
Caffeic-acid	180.165	4	3	0.97	0.56
Ferulic-acid	194.18	4	2	1.62	0.85

Phytochemical	Bioavailability score	Solubility			Pharmacokinetics	
		Log S (ESOL)	Log S (Ali)	Log S (SILICOS-IT)	GI absorption	CYP enzymes inhibitors
Caffeic-acid	0.56	Very soluble	soluble	soluble	high	No
Ferulic-acid	0.85	soluble	soluble	soluble	high	No

3.2. *In Silico* Analysis

3.2.1. Docking Analysis of Ligands Against Protein Targets: PI3K, PDK1, AKT, RAC1, PTP1B, IRS1, 1IR3, PP2A, PTEN and AS160

Based on the RMSD values, the free energy of binding, and the equilibrium constants for binding (Table **1**), AKT, PDK1, IRS1, and PTEN had the lowest values of the calculated equilibrium constants for binding in their docking to both FA and CA in several hundred nanomolar to few micromolar concentrations; what shows the most stable binding interfaces among the studied ligand-protein complexes. Comparable results for binding free energies were found for the binding of the aforementioned proteins to the two phytochemicals, suggesting a good fit at the protein-ligand interface. However, other proteins in the insulin signaling pathway modeled equilibrium constants for binding on the range of hundreds of micromolar, except PTP1B and PP2A, which showed the least promising results in terms of binding to both phytochemicals (at the millimolar range of equilibrium constants of binding).

Results were scored based on the equilibrium constants and the free energy of binding to find the best docking interfaces for protein-ligand binding. We shed light on the four proteins that scored the best according to Table **1**. AKT had polar binding interfaces that induced several polar bonds with the bonded CA and FA. Residues at the interfaces included ARG, THR, GLU, and LYS. On the other hand, PDK1 showed some polar bonds (*via* GLN, THR, LYS, and ARG). Yet, it had a π-π interaction at the docking interfaces with both CA and FA, induced *via* a PHE residue in both interfaces. IRS1 interacted with the target CA and FA mainly *via* polar bonds (with ARG, ASN, and ALA amino acids). PTEN had contributions of both polar interactions with the two phytochemicals (*via* ARG and LYS residues) and of π-π interactions *via* a TYR residue, owing to its phenol ring (Fig. **5a-d**).

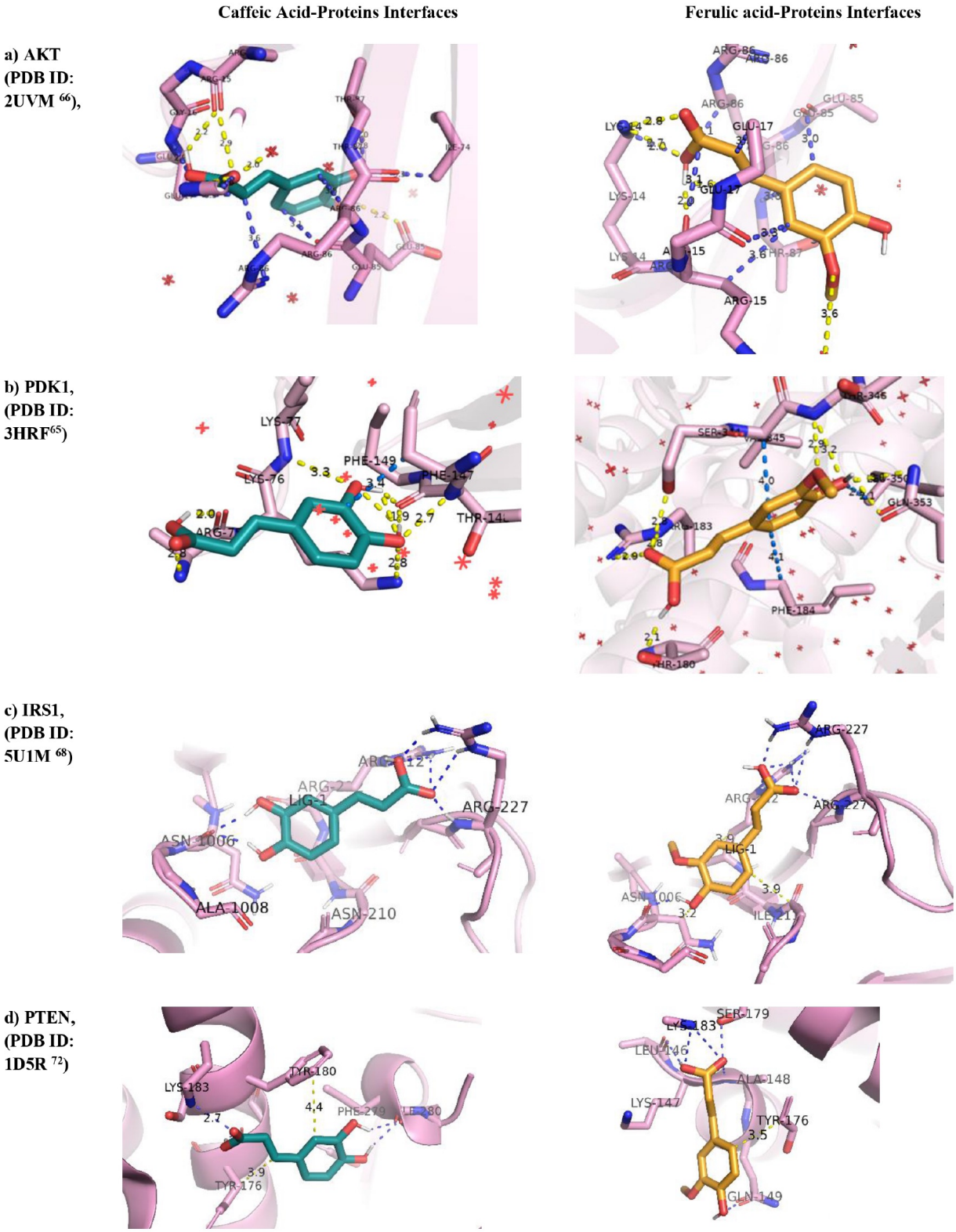

Fig. (5). The most robust binding interfaces of caffeic acid and ferulic acid to four proteins in the insulin signaling pathway: **a**) AKT, **b**) PDK1, **c**) IRS1, **d**) PTEN. Polar contacts were colored yellow, and nonpolar contacts were colored blue. Amino acids in the range of 5 Å from the ligands are shown. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2.2. Drug-likeness Analysis of CA and FA

Drug-likeness was analyzed to infer whether CA and FA have advantageous ADME properties (Table **2**). A good drug is expected to follow Lipinski's rule of five, to have good solubility, to have no excretion problems, to be a non-inhibitor of CYP- enzymes, and to be specific in nature. A molecule is deemed to be not orally active if it violates two or more of Lipinski's Five Rules (RO5): Molecular Weight (MW) of less than or equal to 500 g mol^{-1}, no more than 10 hydrogen bond acceptors, hydrogen bond donors not exceeding 5, and the number of rotatable bonds to be at most 10. Our calculations show that each ligand of the two compounds follows Lipinski's rule of five using the SwissADME online web tool (Table **2**). Thus, both compounds are orally active as drugs for humans, as they follow Lipinski's RO5 (Neither of them has a violation of two or more rules).

4. DISCUSSION

The GLUT4 cycles between intracellular vesicles and the plasma membrane. Insulin, in normal conditions, induces GLUT4 translocation to the PM, whereas glucagon activates GLUT4 translocation to the intracellular compartments [84, 85].

CA and FA were previously detected by our group in distinct antidiabetic plant extracts [53, 56]. Others reported they might exert anti-diabetic activity [87]. CA was reported to lessen insulin resistance and control glucose uptake in hepatocyte cell lines [87]. CA was also reported previously by our group to inhibit alpha-amylase and alpha-glucosidase, suggesting their plausible contribution to the treatment of diabetes [41, 77]. Interestingly, CA has also been shown to improve glucose uptake in cells, which can help in lowering blood glucose levels [88]. FA was reported to enhance enzyme activity involved in glucose breakdown and utilization. It was also shown to reduce blood glucose levels, improve endogenous antioxidant enzyme activities, and stop lipid peroxidation in pancreatic tissues in diabetic rats [89, 90]. CA and FA are known for their strong antioxidant properties. They help in reducing oxidative stress, which is linked to insulin resistance and diabetes [91, 92]. Indeed, some studies suggest that CA and FA may enhance insulin secretion from pancreatic beta cells [93]. This could be due to their ability to modulate cellular signaling pathways that are involved in insulin release. Moreover, CA and FA were reported to inhibit the interaction with the PI3K/Akt pathways in cancer cells [94, 95].

Remarkably, CA was found in *Gundelia tournefortii* L. (GT) methanol extract and an active subfraction of the same extract. CA accounted for 12% of the active subfraction that augmented GLUT4 translocation to the PM by 50% in cells exposed to 250µg/ml of this GT extract subfraction [53]. FA was found in *Abelmoschus esculenbis* (not published) and Orthosphon sraminenus extracts. These extracts augmented GLUT4 translocation to the PM [56]. Yet, to the best of our knowledge, none has reported the *direct* effect of either CA or FA *pure* extracts on GLUT4 activity or translocation to the plasma membrane of myocyte. However, the findings of the previous study are consistent with the current results under a microscope in this study, as pure CA and FA significantly improved GLUT4 translocation to the PM in L6 muscle cells.

Furthermore, the potential protein targets of CA and FA in the insulin signaling pathway were investigated in this manuscript. Indeed, insulin-independent mechanisms in the GLUT4 translocation need further investigation [46]. CA is linked to the phosphorylation of AKT [96], an upstream enhancer of GLUT4 translocation. This is in line with our docking results, which show a clear interaction between CA and AKT.

In this work on ten proteins in the insulin signaling pathway, we predicted the interactions between medical compounds and proteins and ranked them *via* scoring schemes (RMSD, free energy, and inhibition constant). According to the aforementioned criteria, AKT, PDK1, IRS1, and PTEN showed the most robust results in terms of equilibrium constant for binding when compared to other selected proteins in the insulin signaling pathway. PTP1B and PP2A showed the least promising results in terms of binding to both phytochemicals. Comparable results for binding free energies were found for the four selected proteins in their binding to the two phytochemicals, suggesting a good fit at the protein-ligand interface.

Taking together the above-reported data, one can appreciate that CA and FA possess anti-diabetic activity. Our data reported here suggest their direct interaction with key proteins in the insulin signaling pathway, which ultimately leads to significant enhancement in GLUT4 translocation to the muscle PM. Thereby, CA and FA can dramatically reduce insulin resistance, especially as they have augmented GLUT4 translocation in the presence and absence of insulin in our study.

CONCLUSION

In this work, we combined *in vitro* and *in silico* research to predict how druggable ferulic acid and caffeic acid phytochemicals are. We aimed to test their contribution to GLUT4 translocation and to predict their mechanism of action in the insulin signaling pathway. The two compounds showed auspicious results, as they demonstrated good binding to the majority of the tested proteins in the insulin signaling cascades. Our work provides the basis for ongoing pre--clinical and clinical phases in the drug discovery pipeline in suggesting the two compounds as plausible drugs to be launched.

AUTHORS' CONTRIBUTIONS

The authors confirm their contribution to the paper as follows: Najlaa Bassalat carried out the cellular experiments. Shahd Abu Naim performed the computation dockings and contributed to writing the paper. Waseim Barriah contributed to the development of chemical detection and design. Jörg Labahn conceived the presented idea and supervised the project anddiscussed the results. Siba Shanak developed

the computational docking tools, supervised the work of Shahd Abu Naim and wrote the manuscript..) Hilal Zaid conceived the presented idea, supervised the project, and contributed to manuscript writing. All authors reviewed the results and approved the final version of the manuscript.

LIST OF ABBREVIATIONS

ADME/Tox	=	Absorption, Distribution, Metabolism and Excretion/Toxicity
CA	=	Caffeic Acid
FA	=	Ferulic Acid
GAP	=	GTPase-activating Proteins
PM	=	Plasma Membrane
PP2A	=	Protein Phosphatase 2A
PTEN	=	Phosphatase and TENsin Homolog
PTP1B	=	Protein Tyrosine Phosphatase 1B
Rab	=	Ras-associated Binding
T1DM	=	Type I Diabetes Mellitus
T2DM	=	Type II Diabetes Mellitus
WHO	=	World Health Organization

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

FUNDING

We would like to acknowledge the Federal Ministry of Education and Research- Germany; Almaqdisi- France; AAUP; and Al-Qasemi Research Foundations for providing their financial support.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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