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PHF5, a poly-herbal formulation with antidiabetic potential: in vitro and in silico investigation on HepG2 Cells via PKB/Akt and AMPK pathways

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Abstract

Background Amidst the rising global prevalence of diabetes, exploring novel anti-diabetic agents remains a crucial endeavor. This study investigated the biochemical mechanism of action of a poly-herbal formulation (PHF5) on HepG2 cell lines as well as molecular interactions between bioactive compounds of PHF5 and PKB/Akt, AMPK. PHF5 was formulated from leaves of *Ocimum gratissimum, Vernonia amygdalina, Gongronema latifolium, Gnetum africanum,* and *Aloe barbadensis*.

Method The study employed an experimental design encompassing both in vitro and in silico analysis. HepG2 cells were treated with PHF5 in in vitro studies that looked at parameters like cell viability, glucose uptake, and lipid accumulation. Also, glycation and fructosamine formation were studied in bovine serum albumin (BSA) that had been exposed to fructose and PHF5. In silico investigations utilized virtual screening and molecular docking simulations to elucidate the interactions of phytochemicals from PHF5 with key target enzymes involved in glucose metabolism.

Results It was found that PHF5 contained key phenolics such as quercetic, rutin etc. through HPLC profiling. In silico modeling demonstrated favorable binding of rutin and quercetin in PHF5 to PKB/Akt and AMPK, key proteins involved in glucose metabolism. The finding here suggests an antidiabetic action of PHF5, which is mediated via activation of the P13K/Akt pathway leading to trafficking of GLUT4 and simulation of insulin secretion. The findings also revealed significant enhancements in cell viability and glucose uptake, coupled with reduced lipid accumulation in HepG2 cells following treatment with PHF5. Additionally, PHF5 demonstrated a mitigating effect on glycation and fructosamine formation.

Conclusion This study sheds light on the diverse phytochemical composition of PHF5, highlighting potential interactions with crucial enzymes involved in glucose metabolism. The observed promising outcomes points at the potential of PHF5 as a valuable anti-diabetic agent.

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Keywords Poly Herbal formula 5 (PHF5), Diabetes, HepG2 cell lines, Molecular docking, Glucose metabolism

Introduction

Diabetes mellitus, a prevalent metabolic disorder characterized by elevated blood glucose levels, poses a significant global health concern, associated with a myriad of complications affecting multiple organ systems and leading to increased morbidity and mortality rates [27]. The management of diabetes primarily involves maintaining blood glucose levels within a specified range through lifestyle modifications, oral anti-diabetic agents, and insulin therapy. However, current treatment approaches may have limitations such as adverse effects, inadequate glycemic control, and high costs, underscoring the need for alternative therapeutic strategies (American Diabetes Association, 2021). Natural products, particularly plant-derived compounds, have gained attention for their potential to manage diabetes and its complications. Plants, owing to their rich phytochemical composition, offer a vast reservoir of bioactive molecules with diverse pharmacological properties [3]. Polyherbal formulations, blending various plant extracts, have shown promise in traditional medicine systems for treating diabetes and related disorders [18]. PHF5, a specific polyherbal formulation, has garnered interest due to its potential antidiabetic effects demonstrated in preclinical studies. This study aims to comprehensively evaluate PHF5, focusing on its potential as an antidiabetic agent. We employed in vitro models to assess its impact on key cellular processes related to glucose metabolism, including the effects on fructosamine formation and the glycation of bovine serum albumin (BSA). Furthermore, we quantified the total phenolic compounds and characterized the phytochemical profile of PHF5 through HPLC analysis. In silico molecular docking studies were conducted to predict the interactions between the phytochemicals in PHF5 and target enzymes involved in glucose metabolism, specifically considering phytochemicals such as caffeic acid, catechin, epigallocatechin, gallic acid, p-coumaric acid, protocatechuic acid, quercetin, rutin, and vanillic acid. These phytochemicals were chosen due to their potential relevance to diabetes management and their presence in PHF5. The study aims to elucidate the connections between these in vitro parameters and their implications in the context of diabetes.

Material and methods

Chemicals and reagents

Tris, sodium citrate, dithiobisnitrobenzoate, D-glucose, 0.2 M hydrogen peroxide, Xanthine Oxidase, Potassium dichromate (5%), Cumene Hydroperoxide, 0.3 M Disodium hydrogen phosphate, NaCl, 4.5,5-dithio-bis-2-nitrobenzoic, thiobarbituric acid, Picric acid (10 mM), Sodium borate (10 mM) were sourced from Thermo Fisher Scientific. EDTA, Sodium nitroprusside, Diluted phenol, Diluted sodium hypochlorite, Sodium hydroxide (0.4N) were obtained from Carl-Roth, Germany. Metaphosphoric acid, disodium salt of EDTA (0.1%), Metformin (Sigma-Aldrich, Steinheim, Germany), insulin ELISA assay kit, AMPKinase ELISA kit, GLUT1 ELISA kit, GLUT4 ELISA kit were purchased from Abcam, Cambridge, UK. The Randox commercial kit was also utilized.

Plant collection: Leaves of *Aloe barbadensis* (AB), *Gnetum africanum* (GA), *Gongronema latifolium* (GL), *Vernonia amygdalina* (VA), and *Ocimum gratissimum* (OG) were procured from Oriugba, a rural market in Umuahia, Abia State. Taxonomic authentication was performed by Mr. Ibe K. Ndukwe, a taxonomist at the Herbarium Unit of the Forestry and Environmental Management Department of Michael Okpara University of Agriculture, Umudike, Abia State. The leaves were meticulously picked, sorted, and air-dried at a temperature of 27 °C until a constant weight was achieved. Subsequently, they were pulverized into a powder using an ETKAL 868 electric power blender and stored in air-tight plastic containers with appropriate labeling.

HPLC analysis

Sample preparation: PHF5 plant samples (10 mg) were subjected to sonication in 1 mL of HPLC-grade methanol for 60 min. Subsequently, the samples were centrifuged at 10,000 g for 10 min, and the resulting supernatants were filtered through a 0.22 µm Millipore filter. Prior to HPLC/DAD analysis, 10 µL aliquots of the filtered supernatants were injected onto the system.

HPLC/DAD analysis: The HPLC system, equipped with a Diode Array Detector (DAD), utilized a reversed phased column (RP): Nova Pack C-18 (4.6 mm × 250 mm, 5 µm diameter particles). The column temperature was maintained at 30 oC. A mobile phase comprising water/phosphoric acid (0.1%) (Solvent A) and methanol (Solvent B) was employed. A solvent gradient of A (75–0%) and B (25–100%) for 20 min, 100% B for 4 min, and a return to initial conditions for 10 min were utilized. The flow rate was Set at 1.0 mL/min, and spectral data were recorded at 330 nm using Diode Array Detection.

Analytical reference standards including quercetin, gallic acid, and chlorogenic acid (≥ 98% purity; Sigma-Aldrich, USA) were used for compound identification and quantification, based on retention time and UV spectra. Cinnamic acid (≥ 98% purity; Sigma-Aldrich) was used as an internal standard, added at a concentration of 10 µg/mL to all samples and standards to normalize

retention time variation and improve quantification accuracy.

Phenolic compound identification: Phenolic compounds were identified by comparing the retention times and spectra of each peak with those of known standards analyzed under identical conditions.

Replicates and controls: All experiments were conducted in triplicate to ensure the reliability of the results. Known standards were included to validate the identification of phenolic compounds.

Cell culture and maintenance: Human liver carcinoma cell lines (HepG2) were sourced from the School of Pharmacy, University College London. Cells were cultured in DMEM (Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS: GIBCO 10010) and antibiotics (Penicillin 100 IU/mL, Streptomycin 100 µg/mL). The culture medium was refreshed every 2–3 days, and sub-culturing was performed every 3–4 days until cells reached 80% confluence. The cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

Cell treatment and extract preparation: Prior to experiments, HepG2 cells were adjusted to a density of 7.5 × 10^4 cells/mL. PHF5 extract, prepared in 1% v/v DMSO, was added at different concentrations ranging from 15 to 120 mg/L to the culture medium. Extract treatments occurred after 48 h of cell seeding.

Cell line authentication

To authenticate the identity and purity of the HepG2 cell line, Short Tandem Repeat (STR) profiling was performed using a commercially available STR authentication kit (e.g., Promega GenePrint® 10 System) according to the manufacturer's protocol. DNA was extracted from cultured cells at early passages, and the resulting STR profiles were compared with reference profiles available in the ATCC and DSMZ databases. Mycoplasma contamination was also routinely checked using PCR-based MycoAlert™ Mycoplasma Detection Kit.

Triglyceride accumulation estimation

To examine the role of PHF5 in triglyceride accumulation, HepG2 cells were cultured in the simultaneous presence different concentrations of PHF5 extract (25 µg/mL and 50 µg/mL) or (Rosiglitazone; 1 µM, 0.4 µg/ml) as positive control in fresh medium of DMEM (Invitrogen, UK) containing 10% fetal bovine serum (FBS: GIBCO 10010) and antibiotics {Penicillin (100 IU/mL) and streptomycin (100 µg/mL)}and cultured for 7 days. The cells were fixed by replacing growth medium with 100 µL of 10% formaldehyde in PBS. After fixing the cells for 1 h at room temperature, the formaldehyde solution was removed and the cells were washed twice with 200 µL of PBS. A volume of 100 µL crystal violet (0.1% w/v in distilled water) was added to each well for 30 min at room temperature. Excess dye was removed by washing the plates with water and later dried the plates in the oven at 37 °C. A volume of 50 µL of oil red O (0.5%w/v prepared in isopropanol) was added to each well and incubated at 37 °C for 15 min. The stained cells were washed with 100 µL acetic acid (10%v/v). After Oil Red- O staining, cells were stained with crystal violet to normalize cell density. Triglyceride accumulation was quantified by measuring the absorbance at 595 nm.

Sulphorhodamine B (SRB) assay for cytotoxicity: HepG2 cells (1 × 10^5 cells/well) were Seeded in poly-l-lysine pre-coated 96-well plates and allowed to adhere for 48 h. After treatment, cells were fixed with ice-cold trichloroacetic acid (TCA) for 1 h at 4 °C. Following fixation, plates were washed, air-dried, and stained with 0.4% w/v SRB solution. Absorbance was measured at 492 nm using a microplate reader after elution with 10 mM Tris-buffer (pH 10.5).

Glucose utilization assay: HepG2 cells were Seeded at a density of 6000 cells per well in 96-well plates and cultured for 3 days without medium change. On day three, PHF5 extract or metformin (positive control) was added to achieve a final concentration of 12.5 µg/well. After 48 h, the medium was replaced with incubation medium (8 mM glucose DMEM + 10% BSA). Glucose utilization was assessed after 180 min by measuring absorbance at 510 nm using a Multiscan MS microtiter plate reader after incubation with glucose oxidase reagent (SERA-PAK Plus, Bayer).

Calculation of pharmacokinetic parameters

To enhance the molecules, we evaluated all phytoconstituents to determine their adherence to Lipinski's rule of five and assess their binding affinity with the PKB/Akt and AMPK. The characteristics of these phytoconstituents were computed using the SwissADME online tool (http://www.swissadme.ch/index.php) [6, 11].

Molecular docking studies

We conducted molecular docking (MD) on Hp Elitebook 1030 g2 X360 using PyRx Virtual Screening Tool [10]. The structures of all the Nine phytochemicals that are present in PHF5 namely, caffeic acid (PubChem CID: 689,043), catechin (PubChem CID: 9064), epigallocatechin (PubChem CID: 72,277), gallic acid (PubChem CID: 370), p-coumaric acid (PubChem CID: 637,542), protocatechuic acid (PubChem CID: 72), quercetin (PubChem CID: 5,280,343), rutin (PubChem CID: 5,280,805) and vanillic acid (PubChem CID: 8468) and Metformin (PubChem CID: 4091), a clinical antidiabetic drug that targets protein kinases and acts as AMPK activator was used as control ligand. (.sdf File format) were downloaded from the National Center for Biotechnology Information PubChem https://pubchem.ncbi.nlm.nih.gov/. Docking

calculations were performed using AutoDock Vina. The intermolecular interactions of caffeic acid, catechin, epigallocatechin, gallic acid, p-coumaric acid, protocatechuic acid, quercetin, rutin, vanillic acid and metformin with the crystal structures of protein kinase B (PKB/Akt) (PDB ID:1O6L) and activated- AMP Kinase (AMPK) (PDB ID: 4CFF) were studied and then visualized using DSV. The enzyme structures, with the aid of Discovery Studio Visualizer 2019, were optimized, purified and prepared for MD.

The 3D crystal structures of protein kinase B (PKB/Akt) (PDB ID:1O6L) and activated- AMP Kinase (AMPK) (PDB ID: 4CFF) were downloaded from RSCB Protein Data Bank (PDB) (https://www.rcsb.org/). Using Biovia Discovery Studio version v21.1.0.20298 (Accelrys Software Inc., San Diego, CA), the enzyme was prepared by deleting water molecules and hetatoms, addition of polar hydrogen atoms and Gasteiger charges to the 3D structure. The macromolecule file was written as.pdb file format for further analysis. For the purpose of MD simulation, the three-dimensional grid box (size_x = 39.075313A°; size_y = 30.779000A°; size_z = 114.355375A°) for PKB/Akt and (size_x = 20.432776A°; size_y = 42.808776A°; size_z = 176.086514A°) for AMPK was built using AutoDock Tools (ADT) version 4.2. Ten docking runs were simulated for each ligand and the best pose was selected based on the binding energy [10].

Statistical analysis

The presented results are expressed as the mean ± standard deviation (SD) from three independent experiments (n = 5). To assess the statistical significance of the obtained data, a One-way Analysis of Variance (ANOVA) was employed. Mean comparisons were conducted using the Duncan multiple test. The statistical analysis was executed with GraphPad Prism version 8.4, and a confidence interval of $p < 0.05$ was established to determine statistical significance.

Results

HPLC quantification of phenolic acids

PHF5's complex composition was clarified by the HPLC/DAD analysis, which also revealed a rich profile of bioactive compounds (Fig. 1). Gallic acid, epigallocatechin, catechin, protocatechuic acid, and quercetin stood out among the identified compounds as major contributors, indicating a wide range of possible health benefits linked to PHF5. Rutin and p-coumeric acid were also found in PHF5, adding to the phytochemical profile's complexity and potentially advancing its therapeutic value. The result from quantification of total phenolic compounds was shown to be 122.79 ± 1.7. The HPLC–DAD retention profiles for the standard gallic acid, epigallocatechin, chlorogenic acid, cyanuric acid, luteolin, catechin, protocatechuic acid, caffeic acid, vanillic acid, catechol, ferulic acid, isoflavone, rutin, p-coumaric acid, and quercetin is shown in Table 1. The plant extract's major peaks corresponded with the gallic acid, epigallocatechin, catechin, protocatechuic acid, caffeic acid, vanillic acid, rutin, p-coumaric acid, and quercetin. Fig. 2

Effects of PHF5 on HepG2 Cell lines

Cytotoxic effect of the plant crude extracts in HepG2 cells

The in vitro cytotoxic activity of PHF5 was measured by the SRB assay against HepG2 liver cell line at various concentrations. The cytotoxicity results obtained from SRB toxicity assay is presented in Table 2. Our findings showed that PHF5 displayed low level of toxicity to HepG2 cells with 91.95% of the HepG2 cells still viable at the highest administered concentration (120 µg/ml) of PHF5. At lower concentrations of 15 µg/ml, 30 µg/ml and 60 µg/ml, PHF5 displayed no toxicity but rather proliferated the cells. This proliferation was significant compared to the control at 15 µg/ml. Doxorubicin at 5 µg/ml showed a 47.83% cell death (52.17% were still viable).

The effect of PHF5 and Metformin on glucose utilization in HepG2 cells

The result of exposure of HepG2 liver cells to PHF5 and metformin on glucose utilization is shown in Table 3 as percentage change of that of the control cells. The assessment of glucose utilization in HepG2 cells shows a concentration-dependent response to PHF5. Notably, at 50 µg/mL, PHF5 significantly ($p < 0.05$) enhanced glucose uptake (110.64%) compared to the control cells. This effect was comparable to that of the standard antidiabetic drug, metformin (147.77%).

The effect of PHF5 on lipid accumulation in HepG2 cells

The investigation into triglycerides accumulation in HepG2 cells revealed a promising anti-lipogenic potential of PHF5. The effect of PHF5 on lipid accumulation in HepG2 cells is shown in Table 4. At both concentrations (25 µg/mL and 50 µg/mL), PHF5 significantly ($p < 0.05$) reduced triglyceride accumulation, contrasting with the pro-lipogenic effect of Rosiglitazone, a known antidiabetic drug associated with increased lipid accumulation [15].

In silico predicted ADMET profiles

The physico-chemical properties of the nine compounds are shown in the Table 5.

Molecular docking scores of ligands and target proteins PKB/Akt and AMPK.

The major bioactive constituents present in PHF5 were docked against PKB/Akt (Fig. 3) and AMPK (Fig. 4),

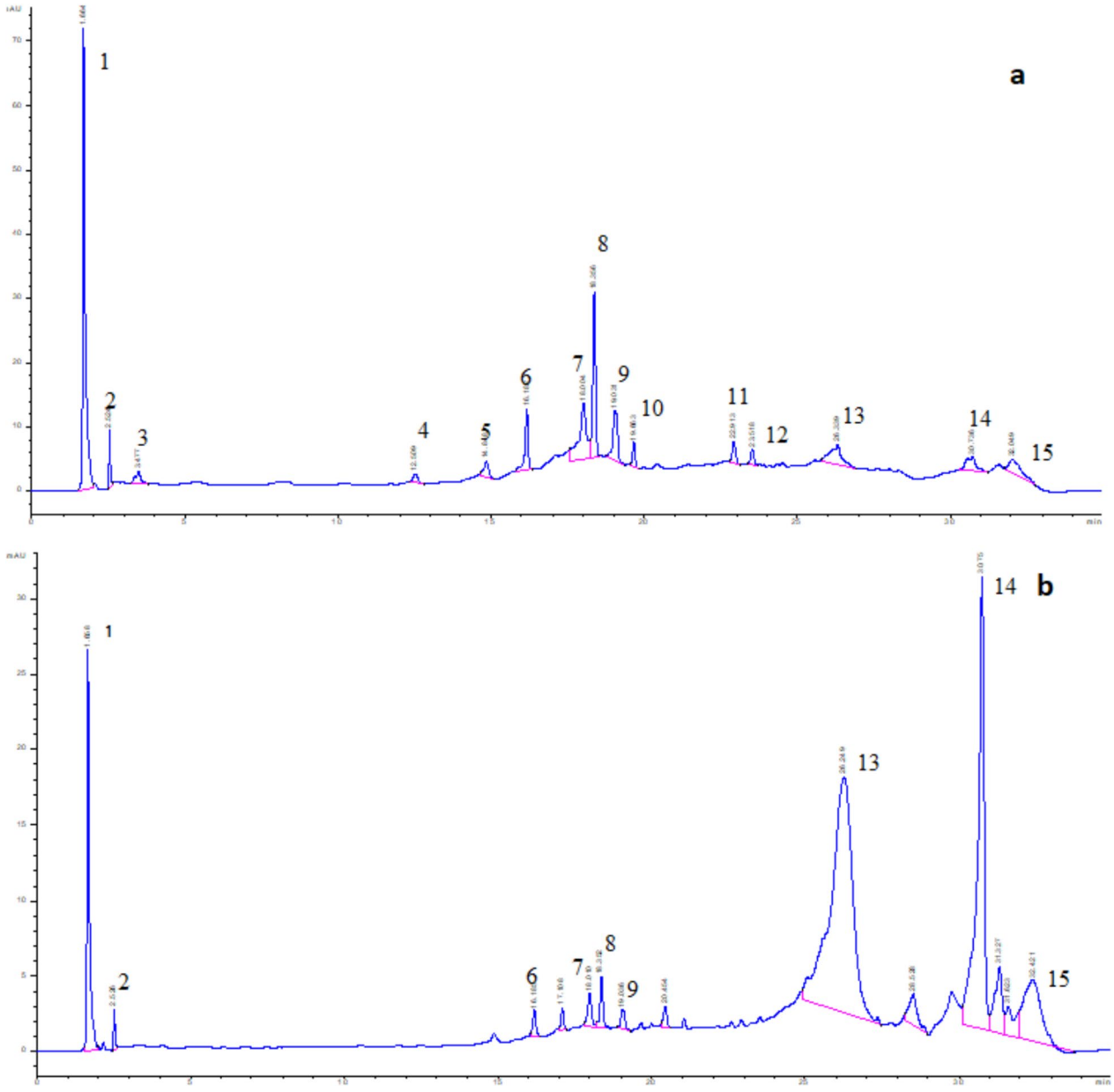

Fig. 1 HPLC–DAD chromatograms at 310 nm for (**a**) standard phenolic acids and (**b**) PHF5. The peaks in PHF5 correspond to gallic acid (1), epigallol (2), catechin (6), protocatechuic acid (7), caffeic acid (8), vanillic acid (9), rutin (13), and p-coumaric acid (14) and quercetin (15)

two major kinase enzymes involved in insulin sensitivity pathway and their docking score calculated based on the gradient optimization algorithm using AutoDock Vina. Our findings revealed that the selected compounds were able to bind appreciably to the target proteins. The docking scores (binding free energy, ΔG, kcal/mol) of the interactions and the calculated ligand efficacy (binding free energy divided by the number of heavy atoms, LE = (ΔG)/N) are presented in Table 6. Analyses of the binding interactions was based on the ΔG of the best docked pose (the more negative the binding energy, the better the docking score). We observed that the compounds showed binding energy that ranged from −6.0 kcal/mol to −10.0 kcal/mol with PKB/Akt (Fig. 5) and −6.0 kcal/mol to −11.2 kcal/mol with AMPK (Fig. 6). The standard drug, metformin showed a binding energy of −4.7 kcal/mol and −5.8 kcal/mol with PKB/Akt and AMPK respectively.

Rutin and quercetin exhibited the highest docking scores against the two targets with rutin showing docking scores of −10.0 kcal/mol and −11.2 kcal/mol against PKB/Akt and AMPK respectively while quercetin showed docking scores of −8.8 kcal/mol and −9.5 kcal/mol against PKB/Akt and AMPK respectively. Our results

Table 1 List of the identified compounds in PHF5 by HPLC/DAD

Peak number	Retention time (min)	Standards	PHF5 compounds
1	1.6644	Gallic acid	Gallic acid
2	2.524	Epigallocatechin	Epigallocatechin
3	3.477	Chlorogenic acid	N/D*
4	12.509	Cyanuric acid	N/D*
5	14.948	Luteolin	N/D*
6	16.182	Catechin	Catechin
7	18.004	Protocatechuic acid	Protocatechuic acid
8	18.356	Caffeic acid	Caffeic acid
9	19.031	Vanillic acid	Vanillic acid
10	19.663	Catechol	N/D
11	22.913	Ferulic acid	N/D
12	23.518	Isoflavone	N/D
13	26.339	Rutin	Rutin
14	30.736	p- coumeric acid	p- coumeric acid
15	32.049	Quercetin	Quercetin

*N/D –Not detected

showed that that the docking score of the standard drug, metformin was lower that the selected ligands against both target proteins.

Our results also showed that rutin formed four hydrogen bond interactions with four amino acid residues of PKB/Akt (GLY164, GLY295, LYS181, LYS160) while quercetin formed three hydrogen bond interactions with THR292, LYS181 and ALA232 residues of PKB/Akt (Fig. 7). Rutin formed hydrogen bond interactions with five amino acid residues of AMPK (ARG369, PRO367 ARG269, LEU277, ARG299) while quercetin showed hydrogen bond interactions with one amino acid residue (ARG299) and other bonds (pi-Sigma and pi-Alkyl) of AMPK (Fig. 8).

Discussion

Herbal formulations have gained great importance in the management of diseases, mostly due to their efficacy with little-known side effects as well as their easy accessibility. There have been reports that polyphenolic compounds are able to curb the onset of prolonged diabetes mellitus and its complications [5]. However, there has been no report on the effect of a formular prepared from the leaves of *Ocimum gratissimum, Vernonia amygdalina, Gongronema latifolium, Gnetum africanum,* and *Aloe barbadensis* on experimental type 2 diabetes.

Caffeic acid has shown potential for improving insulin sensitivity and reducing blood glucose levels in diabetic individuals through its antioxidant and anti-inflammatory properties [22]. Catechin, a flavonoid present in various plants, has demonstrated anti-diabetic effects by enhancing insulin secretion, improving glucose metabolism, and reducing oxidative stress [4]. Epigallocatechin, a polyphenol found in green tea, has exhibited the ability to improve glucose tolerance and insulin sensitivity, making it a potential therapeutic agent for diabetes management [24]. Gallic acid has been reported to possess anti-diabetic properties by enhancing insulin sensitivity and exerting protective effects on pancreatic beta cells [23]. p-coumaric acid has shown potential in ameliorating hyperglycemia and insulin resistance, suggesting its possible role in diabetes management [1]. Protocatechuic acid has demonstrated anti-diabetic effects by improving insulin sensitivity and reducing blood glucose levels [19]. Quercetin, a flavonoid found in many plants, exhibits anti-diabetic properties by reducing blood glucose levels, improving insulin secretion, and enhancing insulin sensitivity [7]. Rutin, a flavonol glycoside, has demonstrated potential for managing diabetes by improving insulin resistance and reducing oxidative stress [12]. Vanillic acid has shown anti-diabetic effects by enhancing insulin sensitivity and reducing blood glucose levels, making it a potential therapeutic agent for diabetes management [1].

Figure 1 provides the structures and names of selected phytoconstituents from each plant. Rutin and Epigallocatechin violate Lipinski's rule, according to Table 5. The molecules exhibit log P values ranging from 0.21 to 1.63, indicating optimal lipophilicity, a crucial factor influencing their functionality in the body [16]. Lipophilicity, assessed through Log P, measures a compound's permeability for reaching target tissues in the body [17]. All molecule weights are below 500 Da, promoting active transport through biological membranes. Further optimization involves calculating pharmacokinetics and drug-likeness properties. Compounds like gallic acid, caffeic acid, vanillic acid, rutin, protocatechuic acid, quercetin, epigallocatechin, and quercetin have a hard time getting through the blood–brain barrier, which means they can't be used as medicines for the central nervous system. However, log Kp (skin penetration, cm/s) and bioavailability values meet acceptable limits. Gastrointestinal absorption is high for all compounds, except rutin.

Cytotoxicity of PHF5

The assessment of the toxicity of herbal medicine is an important step towards the development of acceptable alternative antidiabetic therapies. This present study showed that HepG2 cells treated with the poly-herbal formulation (PHF5) displayed as much as over 90% viability even at the highest treated concentration of 120 µg/ml, suggesting a relatively low cytotoxic effect of PHF5. At lower concentrations (15 µg/ml, 30 µg/ml, and 60 µg/ml), PHF5 was even able to induce proliferation of the HepG2 cells. This low level of cytotoxicity displayed by PHF5 puts it at an advantage as a prospective, safe traditional antidiabetic medicine.

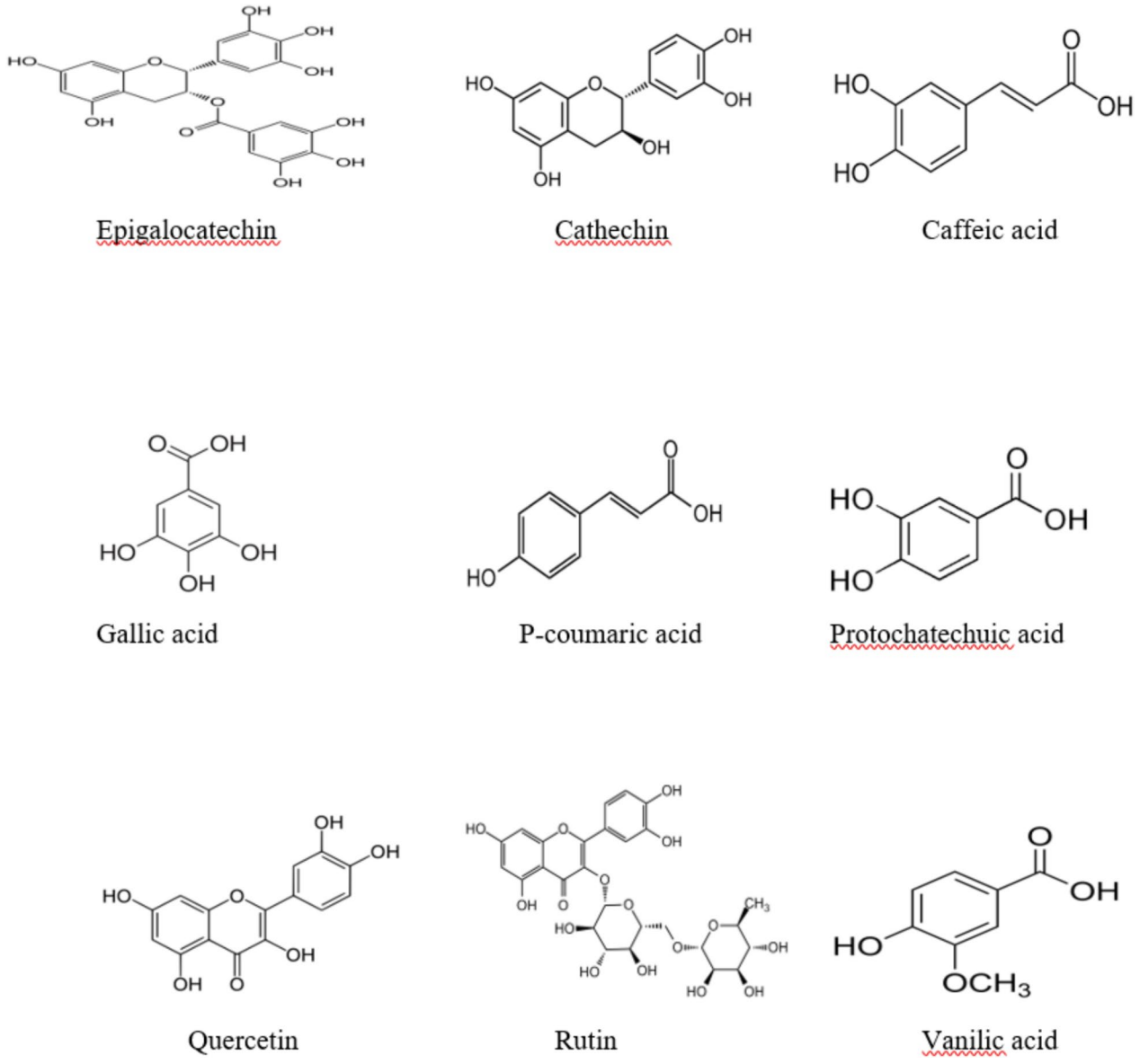

Fig. 2 Name and structure of detected phytoconstituents

Table 2 Cytotoxic effect of the plant crude extracts in HepG2 cells

Conc. (µg/ml)	SRB cell viability (% of Control)
Control	100
Doxorubicin (5 µg/ml)	52.17 ± 6.85***
15 µg/ml	112.75 ± 9.06*
30 µg/ml	106.70 ± 6.54
60 µg/ml	102.83 ± 6.72
120 µg/ml	91.95 ± 7.05

Data are expressed as % of control ± SD (n = 6).***p < 0.05 compared to the control; *p < 0.05 compared to the metformin

Table 3 The effect of PHF5 and Metformin on glucose utilization in HepG2 cells

Treatment	Glucose utilization (% of Control)
Control	100 ± 0.06
Metformin	147.77 ± 0.18***
PHF5 25 µg/mL	74.08 ± 0.1***†††
PHF5 50 µg/mL	110.64 ± 0.26***†††

Data are expressed as % of control ± SD (n = 6).***p < 0.05 compared to the control treatment; †††p < 0.05 compared to the metformin treatment

Impact of PHF5 on glucose uptake in HepG2 cells

In the bid to further understand the mechanism of possible antidiabetic activity of PHF5, a number of cell-based assays were carried out in this present study. Glucose uptake by HepG2 cells was assessed in the presence of

Table 4 The effect of PHF5 on lipid accumulation in HepG2 cells

Treatment	Lipid accumulation (% of Control)
Control	100
Rosiglitazone	188.10 ± 3.15***
PHF5 25 µg/mL	82.08 ± 4.0***
PHF5 50 µg/mL	81.21 ± 3.53***

Data are expressed as % of control ± SD (n = 4).***p < 0.05 compared to the control

PHF5, and our findings demonstrated that PHF5 significantly increased glucose uptake in the cells compared to the untreated control cells. Our results showed that treatment of the cells with metformin significantly enhanced glucose utilization in the hepatic cells as well. Metformin, a hypoglycemic drug that belongs to the class of biguanides, exerts its hypoglycemic activity via activation of AMP-activated protein kinase in the liver, leading to enhanced hepatic insulin sensitivity, improved lipid metabolism, and the utilization of glucose [26]. The presence of polyphenols in the formulation could be responsible for the enhanced glucose uptake exhibited by PHF5. Polyphenols and flavonoids have been reported in previous studies to inhibit glucose release from the liver and also improve glucose uptake in hepatic cells [13]. This improved glucose uptake demonstrated by PHF5 is probably via the activation of the insulin signaling pathway, consequently leading to the translocation of glucose transporters (GLUT4) to facilitate the cellular uptake of glucose [25].

Impact of PHF5 on lipid accumulation in HepG2 cells

In this study, PHF5 was shown to reduce fat accumulation by using a HepG2 cell model. Rosiglitazone treatment resulted in a marked triglycerides accumulation in HepG2 cells demonstrated by Oil Red O staining. However, PHF5 at the treated concentrations remarkably reduced the accumulation of triglycerides suggesting the ability of PHF5 to prevent fat deposition. These results are in congruence with other studies showing amelioration of hepatic steatosis when exposed to polyphenols [21]. The mechanisms underlying triglyceride-lowering effects of PHF5 can be Linked to the regulation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) intracellular signaling pathway.

Molecular docking simulations

Molecular docking is an important tool largely used for computational drug design and discovery. Protein–ligand docking is employed in the prediction of the position and orientation of a ligand as it is bound to a protein receptor or enzyme. In order to understand the possible mechanism by which PHF5 elicits its antidiabetic activity, as evidenced by the in vivo and in vitro studies we carried out, molecular docking and virtual screening

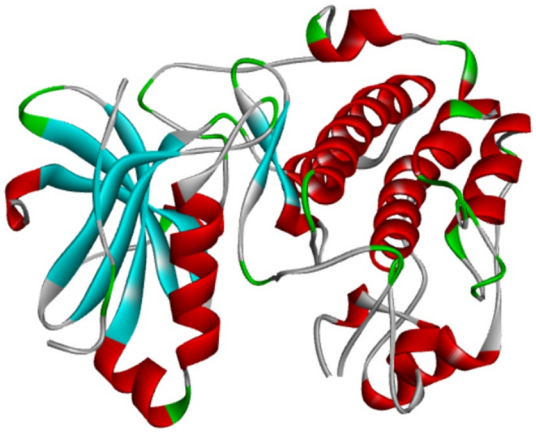

Fig. 3 The crystal structure of protein kinase B (PKB/Akt) (PDB ID:1O6L)

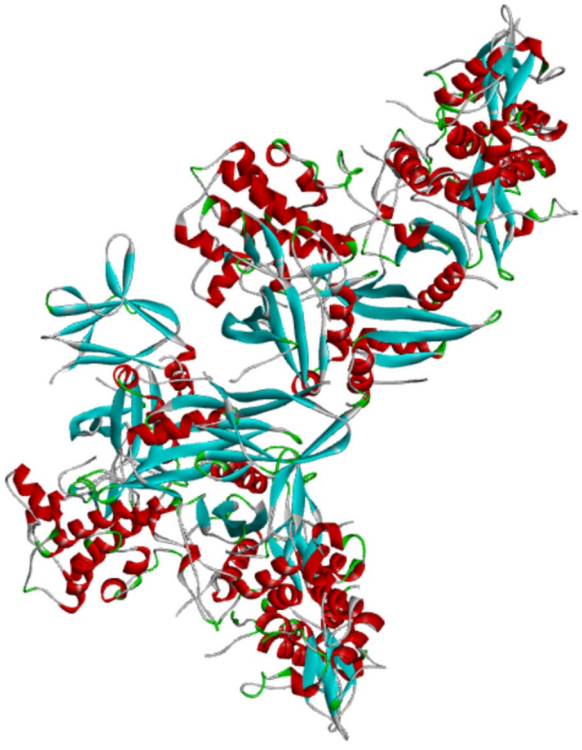

Fig. 4 The crystal structure of activated- AMP Kinase (AMPK) (PDB ID: 4CFF)

were performed using phytocompounds found in PHF5 against target enzymes AMPK and PKB/Akt, which are key in pathways involved in the metabolism of glucose. Metformin, a potent activator of AMPK, is used as the current therapy in the management of type 2 diabetes, and it was used in this study as a standard drug. The use of metformin is intended for the reversal of hyperglycemia and the improvement of glucose uptake.

The metformin-targeted kinase enzyme, AMPK, is involved in the control of fatty acid and glycogen synthesis. It is implicated as the core enzyme in type 2 diabetes and a number of metabolic syndromes and can be

Table 5 Results of predicted ADME properties of the 9 detected compounds

S/N	Compound	Canonical smiles	M.W	log Kp (cm/s)	HBA	HBD	Log p	TPSA	N ROT	WS	GI.A	BP	Pg	LV	B.S	S.A
1	Catechin	OC1CC2OC(C3CCC(C(C3)O)O)C(CC2C(C1)O)O	290.27	−7.82	6	5	1.47	110.38	1	Soluble	High	No	Yes	0	0.55	3.5
2	P-Coumaric acid	OC(= O)C = CC1CCC(CC1)O	164.16	−6.26	3	2	0.95	57.53	2	Soluble	High	Yes	No	0	0.85	1.61
3	Caffeic acid	OC(= O)C = CC1CCC(C(C1)O)O	180.16	−6.58	4	3	0.97	77.76	2	Very soluble	High	No	No	0	0.56	1.81
4	Vanillic acid	COC1CC(CCC1O)C(= O)O	168.15	−6.31	4	2	1.4	66.76	2	Soluble	High	No	No	0	0.85	1.42
5	Rutin	OC1CC(O)C2C(C1)OC(C(C2 = O)OC1OC(COC2OC(C)C(C(C2O)O)O)C(C(C1O)O)O)C1CCC(C(C1)O)O	610.52	−10.26	16	10	1.58	269.43	6	Soluble	Low	No	Yes	3	0.17	6.52
6	Protocatechuic acid	OC(= O)C1CCC(C(C1)O)O	154.12	−6.42	4	3	0.66	77.76	1	Very soluble	High	No	No	0	0.56	1.07
7	quercetin	OC1CC(O)C2C(C1)OC(C(C2 = O)O)C1CCC(C(C1)O)O	302.24	−7.05	7	5	1.63	131.36	1	Soluble	High	No	No	0	0.55	3.23
8	Epigallocatechin	OC1CC2OC(C3CC(O)C(C(C3)O)O)C(CC2C(C1)O)O	306.27	−8.17	7	6	0.98	130.61	1	Soluble	High	No	No	1	0.55	3.53
9	Gallic acid	OC(= O)C1CC(O)C(C(C1)O)O	170.12	−6.84	5	4	0.21	97.99	1	Very soluble	High	No	No	0	0.56	1.22

M.W: Molecular weight (g/mol), Log Kp:Skin permeability, HBA: Number of H-bond acceptors,; HBD: Number of H-bond donors, Log p: Coefficient log p, TPSA: Topological polar surface area (Å2), N Rot: Number of rotatable bonds,WS: Water Solubility Class, GI.A: Gastrointestinal Absorption, B.P: Blood Brain-Barrier permeant, Pg: P- glycoprotein substrate, LV: Lipinski Violation, B.S: Bioavailability Score, S.A: Synthetic accessibility

Table 6 Binding energy and ligand efficacy of selected phytochemicals and target proteins PKB/Akt and AMPK

Ligands	PKB/Akt		AMPK	
	Binding energy (kcal/mol)	Ligand efficacy	Binding energy (kcal/mol)	Ligand efficacy
Caffeic acid	−6.7	−0.52	−6.6	−0.51
Catechin	−8.7	−0.41	−8.1	−0.39
Epigallocatechin	−7.9	−0.36	−8.5	−0.39
Gallic acid	−5.9	−0.49	−6.3	−0.53
p-coumeric acid	−6.5	−0.54	−6.2	−0.52
Protocatechuic acid	−6.1	−0.55	−6.1	−0.55
Quercetin	−8.8	−0.40	−9.5	−0.43
Rutin	−10.0	−0.23	−11.2	−0.26
Vanillic acid	−6.0	−0.50	−6.0	−0.50
Metformin	−4.7	−0.52	−5.8	−0.64

induced by cellular stress and hormones. Research has shown that AMPK is essential in the maintenance of glucose balance, and its activation could alleviate metabolic complications that are consequences of type 2 diabetes [9].

PKB/Akt is capable of inducing a number of downstream molecules, including the translocation of glucose transporters to the cellular surface. The P13k-PKB/Akt pathway could be induced via insulin and act as an effector of insulin secretion. Insulin signaling begins with the binding of insulin to its receptors and subsequent activation of the PI3K/Akt pathway (Bozulic and Hemmings). This interaction leads to the phosphorylation of phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3). This in turn leads to the activation of PIP3-dependent kinases (PDK-1 and PDK-2) and eventually to the activation of the PKB/Akt kinase. Subsequently, PKB/Akt catalyzes the phosphorylation of the AS160 substrate protein, which stimulates the translocation of GLUT4 glucose transporters [20].

In this study, we determined the protein–ligand binding energies and ligand efficacy of the interactions and went ahead to characterize the binding amino residues involved in the interactions via molecular docking analyses. Our findings showed that all the compounds demonstrated high binding affinities for the target proteins, but rutin and quercetin had the highest docking scores, higher than those of the standard drug metformin. The implication is that these compounds possess the potential to act as agonists to PKB/Akt and AMPK enzymes, leading to their activation and subsequently the stimulation of insulin secretion and improved glucose uptake

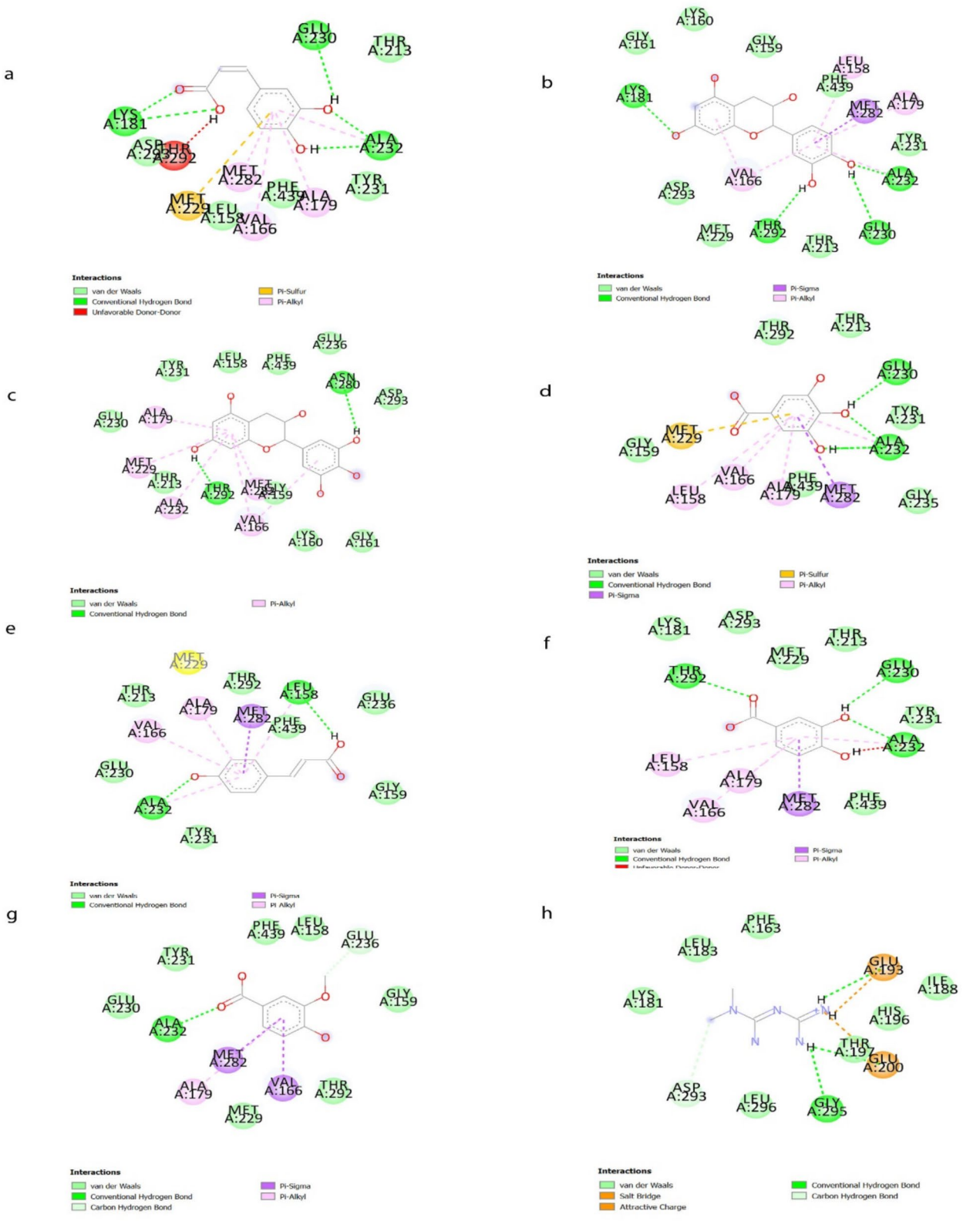

Fig. 5 2D and 3D illustration of molecular interactions between **a**) caffeic, **b**) catechin, **c**) epigallocatechin, **d**) gallic acid, **e**) p-coumeric acid, **f**) protocatechuic acid, **g**) vanillic acid and PKB/Akt

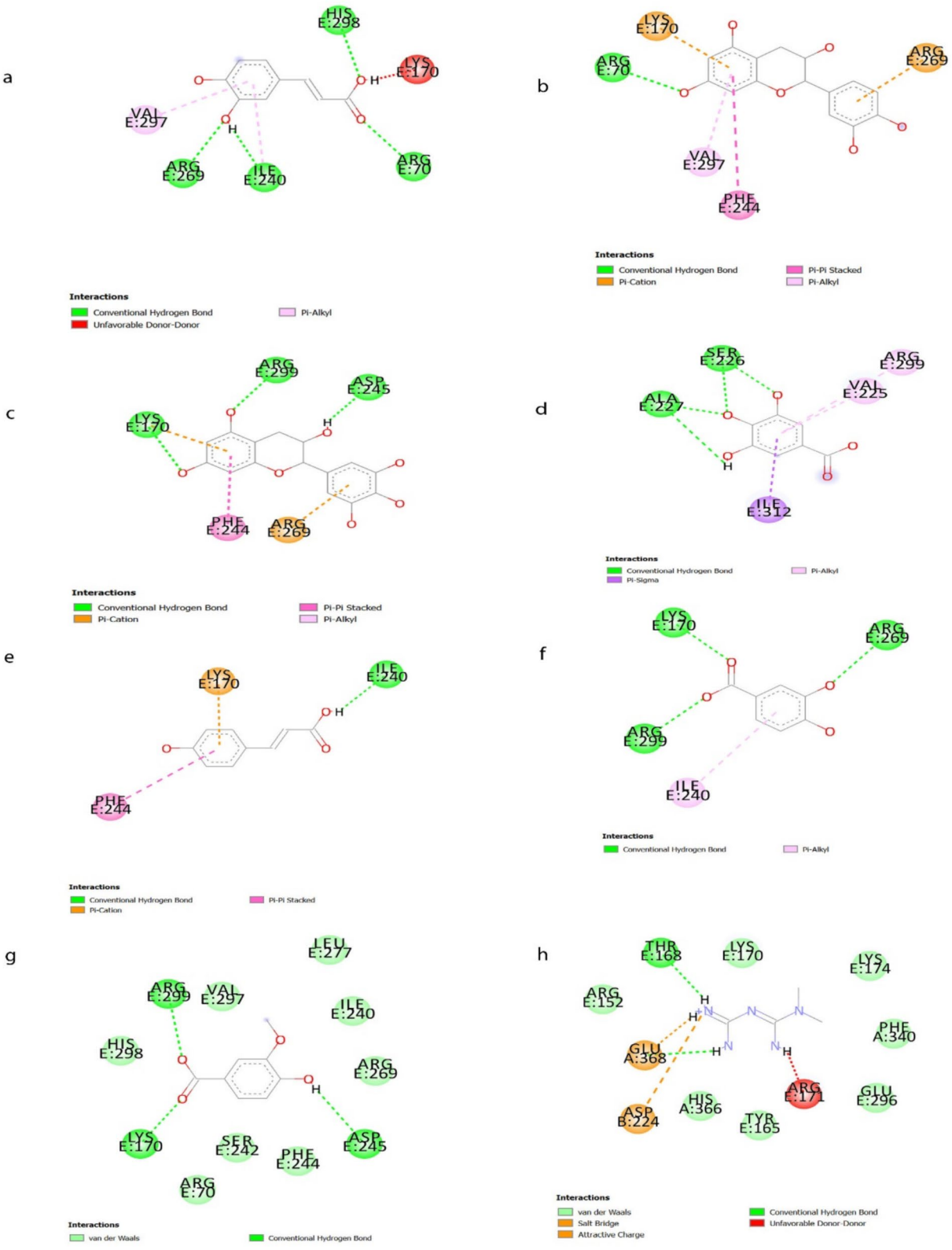

Fig. 6. 2D and 3D illustration of molecular interactions between **a**) caffeic, **b**) catechin, **c**) epigallocatechin, **d**) gallic acid, **e**) p-coumeric acid, **f**) protocatechuic acid, **g**) vanillic acid and AMPK

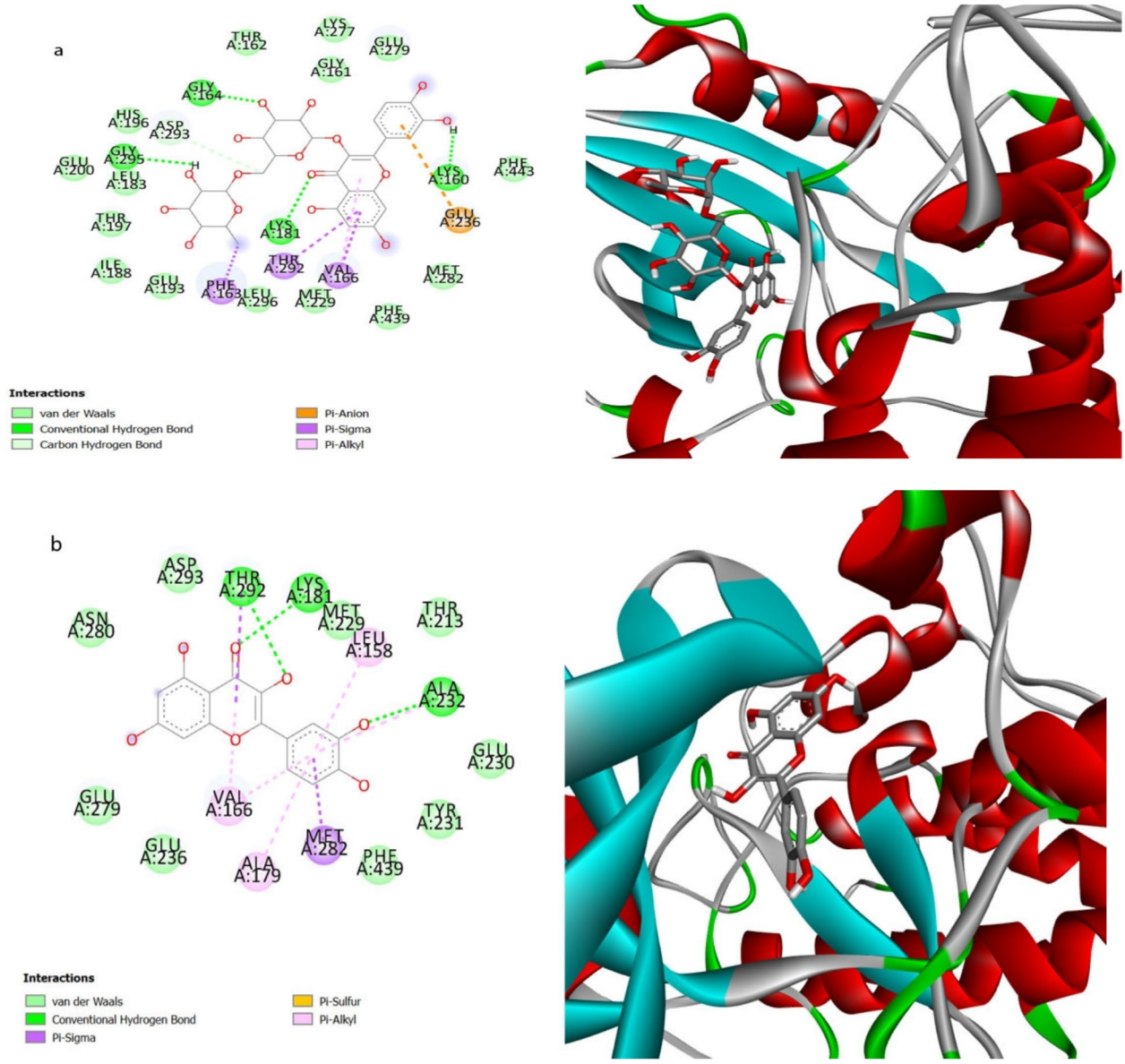

Fig. 7 2D and 3D illustration of molecular interactions between **a**) rutin and **b**) quercetin with PKB/Akt

via the PI3K/Akt pathway. A previous study by Cai and Lin on rat beta cells showed that rutin increased glucose-induced insulin secretion and preserved glucose sensing ability in high glucose conditions [8]. Rutin stimulated glucose transport into muscle by activating the synthesis and translocation of the transporter GLUT4 [14]. Evidence exists showing that quercetin activates the AMPK complex, and quercetin-induced AMPK activation down-regulates oxidative damage and augments glucose uptake in mice [2].

In vitro studies indicated that PHF5 enhanced glucose uptake in hepatic cells, lowered lipid accumulation and fat deposition, and possessed relatively low cytotoxic potential. The formulation demonstrated an ability to induce cell proliferation.

This study reported the presence of gallic acid, epigallol, catechin, protocatechuic acid, caffeic acid, vanillic acid, rutin, p-coumaric acid, and quercetin. Most of these phytochemicals have been reported to exhibit hypolipidemic, anti-oxidative, and antidiabetic effects. The computational part of this study tried to predict the molecular mechanism of the antidiabetic activity of PHF5 by determining the protein–ligand binding energies and ligand efficacy of the interactions, and this revealed that all the phytochemicals demonstrated appreciable binding affinities with the target proteins AMPK and PKB/Akt, with rutin and quercetin showing the highest binding scores. It is thus reasonable to deduce that PHF5 possessed antidiabetic activity that was demonstrated via the improvement of insulin sensitivity and stimulation of glucose

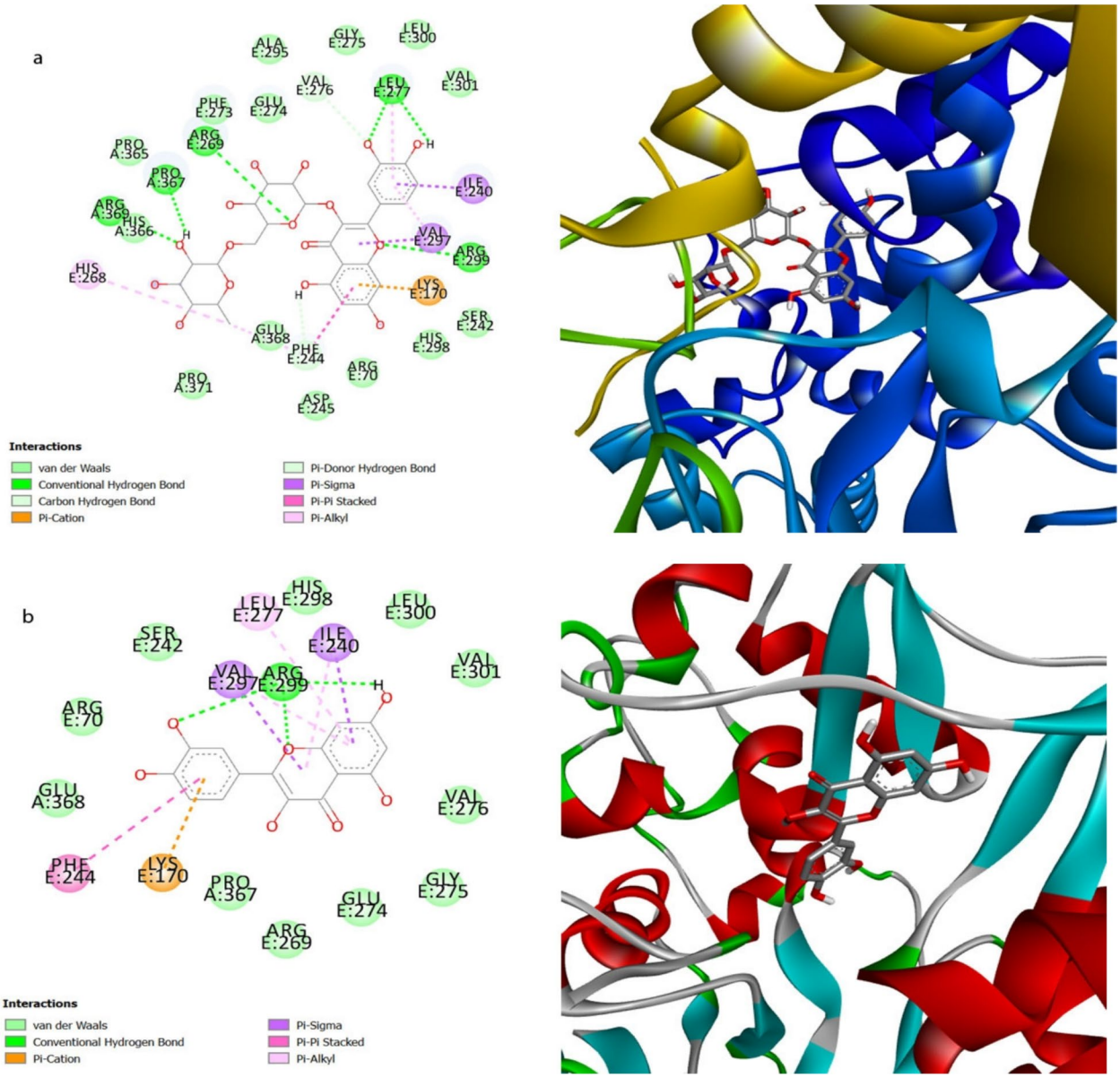

Fig. 8. 2D and 3D illustration of molecular interactions between **a**) rutin and **b**) quercetin and AMPK

transporters to enhance glucose uptake. This is achieved by the activity of possibly rutin or quercetin or the synergistic action of both against AMPK and PKB/Akt, which are key enzymes in pathways involved in the uptake and metabolism of glucose.

Data availabilty

The datasets generated during and/or analysed during the current study are included in the text.

Author contributions

ESI: Conceptualization, Investigation, Writing of manuscript. **IGC:** Data collection, Docking, Manuscript of writing. **OHC:** Lab investigation, data collection, Statistical analysis. **UEN:** Lab investigation, Review/editing. **IRC:** Analysis, Methodology, Editing. **OPN:** Conceptualization, Supervision, Review/editing. **MPE:** Project administration, Supervision, Review/editing.

Declarations

Conflict of interest

Authors declare no competing interests to the best of their knowledge.

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