Hyphaene thebaica (Doum)-derived extract alleviates hyperglycemia in diabetic rats: A comprehensive *in silico*, *in vitro* and *in vivo* study

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1. Experimental

1.1. LC-HRMS Chemical Profiling

Chemical profiling of aqueous extract was performed on an Acquity Ultra Performance Liquid Chromatography system coupled to a SynaptG2 HDMS quadrupole time-of-flight hybrid mass spectrometer (Waters, Milford, CT, USA). Chromatographic separation was performed on a BEH C18 column (2.1 × 100 mm, 1.7 µm particle size; Waters, Milford, CT, USA) with a guard column (2.1 × 5 mm, 1.7 µm particle size) and a linear solvent gradient of 0–100% eluent B at a flow rate of 0.3 $mL \cdot min^{-1}$ over 6 min, using 0.1% formic acid in water (v/v) as solvent A and acetonitrile as solvent B. The injection volume was 2 µL and the column temperature was 40°C. MS-convert software was used to convert the raw data into sliced positive and negative ionization files. Then, the obtained files were subjected to the data mining MZmine 2.10 software (Okinawa Institute of Science and Technology Graduate University, Japan) for deconvolution, peak picking, alignment, deisotoping, and formula prediction. Dictionary of Natural Products (DNP) 2018 database was used for the dereplication and identification of compounds.

1.2. Determination of Total Flavonoids Content

1 mL diluted extract, 5 mL of 80% (v/v) ethanol and 1 mL of 5% (w/w) $NaNO_2$ were mixed for 6 min, and then 1 mL of 10% $AlCl_3$ (w/w) was added and mixed, 6 min later, 10 mL of 1 mol/L NaOH was added. With 15 min standing, the absorbance of the solution was measured at 510 nm with UV-1700 spectrophotometer against the same mixture, without the sample as a blank. The calibration curve ($y = -0.0184 + 0.01251x$, where y is absorbance value of sample, x is sample concentration) ($R^2 = 0.99$).

1.3. LC-assisted Quantification of Flavonoids.

For flavonoids' quantification in the extracts, the corresponding authentic standards (compounds 1-6) were dissolved in 50% aqueous acetonitrile to prepare stock solutions (1 $mg\ mL^{-1}$).

Thereafter, they were diluted to obtain a series of working solutions that were kept at −4 °C and were then brought to room temperature just before use. Subsequently, each dried extract was dissolved in 50% aqueous acetonitrile in a 10 mL volumetric flask. After that, 1 mL was filtered through 0.22 µm membrane filter before introduction to the LC system. The same mobile phase system was used (i.e. the mobile phase used for LC-HRMS analysis) over 10 min at a flow rate of 1 mL min^{-1}. A calibration curve was then obtained for each authentic flavonoids by plotting its peak areas *vs.* its different concentrations, and then these calibration curves were used to calculate the quantity of each compound in the plant extracts. The experiments were performed in triplicate.

1.4. Molecular Docking

Docking experiments were performed by AutoDock Vina [1]. The binding site in the docked protein (PDB: 6JB1) was determined according to the co-crystalized ligand (Repaglinide). To validate the docking protocol, the co-crystalized ligand was redocked inside the protein's active site. The software according to the default parameters was able reproduce the binding pose with low root mean square deviation (RMSD = 1.1 Å). To account for these binding sites' flexibility, we used their MDS-derived conformers sampled every 10 ns for docking experiments (i.e. ensemble docking; [2]. Subsequently, the retrieved top hits were ranked according to their binding energies. The generated docking poses were visualized and analyzed using Pymol software [1].

1.5. Molecular Dynamic Simulation

Desmond v. 2.2 software was used for performing MDS experiments [3] .This software applies the OPLS force field. Protein systems were built using the System Builder option, where the protein structure was embedded in an orthorhombic box of TIP3P water together with 0.15 M Na+ and Cl− ions in 20 Å solvent buffer. Afterward, the prepared systems were energy minimized and equilibrated for 10 ns. Desmond software automatically parameterizes inputted ligands during the system building step according to the OPLS force

field. For simulations performed by NAMD [4] the parameters and topologies of the compounds were calculated either using the Charmm27 force field with the online software Ligand Reader and Modeler (http://www.charmm-gui. org/?doc=input/ligandrm, accessed on 16 April 2021) [5] or using the VMD plugin Force Field Toolkit (ffTK). Afterward, the generated parameters and topology files were loaded to VMD to readily read the protein–ligand complexes without errors and then conduct the simulation step.

1.6. Binding Free Energy Calculations

Binding free energy calculations (Δ*G*) were performed using the free energy perturbation (FEP) method (Kim et al. 2021). This method was described in detail in the recent article by Kim and coworkers 2021. Briefly, this method calculates the binding free energy ΔGbinding according to the following equation: ΔGbinding = ΔGComplex − ΔGLigand. The value of each ΔG is estimated from a separate simulation using NAMD software. Interestingly, all input files required for simulation by NAMD can be papered by using the online website Charmm-GUI (https://charmm-gui.org/?doc=input/afes.abinding, accessed on 16 April 2021). Subsequently, we can use these files in NAMD to produce the required simulations using the FEP calculation function in NAMD. The equilibration was achieved in the NPT ensemble at 300 K and 1 atm (1.01325 bar) with Langevin piston pressure (for ”Complex” and ”Ligand”) in the presence of the TIP3P water model. Then, 10 ns FEP simulations were performed for each compound, and the last 5 ns of the free energy values was measured for the final free energy values [5]. Finally, the generated trajectories were visualized and analyzed using VMD software.

1.7. MTT Assay

The RINm5F cells were seeded into 96-well microplates at a semiconfluent density (5000 cells/well). After 24 h, the medium was replaced with complete medium containing different concentrations of test compounds from 1, 10, 20, 50, 100, 200 and 500 μM. The cells were treated for 1 h. Then washed with phosphate-buffered

saline (PBS), pH 7.4, and a solution of 0.1 mg/mL MTT in PBS (pH 7.5) was added. Cells were incubated for 3 h at 37 C. Then, 200mL of 40 mM HCI (dissolved in isopropanol) was added to each well for 15 min to solubilize the formazan. The absorbance was determined at 570 nm. Data were expressed as the percentage of viable cells following treatment with the CGA compared to the control cells.

1.8. Insulin Assay

RINm5F cells were grown to 70–80% confluence in Lab-TekII eight-well glass slides and then pre-incubated for 2 h at 37 ˚C with Krebs-Ringer bicarbonate buffer (NaCl 135 mM, KCl 3.6 mM, $NaHCO_3$ 5 mM, NaH_2PO_4 0.5 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 1.5 mM), pH 7.4, and 10% bovine serum albumin, glucose 5.5. RPMI medium (300mL) was collected after of the treatments of 5μM from each test compound and glibenclamide. The insulin concentration was measured by ELISA method, following the procedure described by the provider (ALPO, USA) Insulin ELISA Kit.

Table S1. Major identified compounds in *H. thebaica* extract.

No	Name	Rt	Source	Exact mass	Ref
1	*Apignen	2.45	*H. thebaica*	270.0528	[6]
2	Luteolin*	3.86	*H. thebaica*	286.04774	[7]
3	Chrysoeriol	2.19	*H. thebaica*	300.06339	[7]
4	Myricetin*	2.14	*H. thebaica*	302.0428	[6]
5	Vitexin*	5.29	*H. thebaica*	432.383	[7]
6	Astragallin*	2.201	*H. thebaica*	448.1005	[7]
7	Quercetin 3-O- β -^{4} C_1-D-glucopyranoside	3.89	*H. thebaica*	464.37	[6]
8	Apigenin 7-glucuronide.	3.35	*H. thebaica*	446.084915	[8]
9	luteolin 7-*O*-glucuronoide	3.24	*H. thebaica*	462.07983	[8]
10	Chrysoeriol 7-O-[2″-O-β-D-galactopyranosyl]-α-Larabinofuranoside	5.56410	*H. thebaica*	594.1582	[7]
11	Nicotiflorin	4.77	*H. thebaica*	594.158	[6]
12	Isorhamnetin 3-rutinoside	2.739	*H. thebaica*	624.16904	[8]
13	Rhamnazin 3-*O*-rutinoside	5.758	*H. thebaica*	638.578	[6]
14	Chrysoeriol 7-O-[6″-O-α-L-rhamnopyranosyl]-β-D-glucopyranoside	5.04	*H. thebaica*	608.1741	[7]
15	Chlorogenic acid	5.37	*H. thebaica*	354.095085	[8]
16	*O*-caffeoyl shikimic acid	3.060	*H. thebaica*	336.085	[8]

*These compounds were identified by comparison with authentic standards.

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