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Submission date: 18-Mar-2023 02:34PM (UTC+0700)
Submission ID: 2039926224
File name: nolic_Extract_of_Red_Gedi_Leaves_Abelmoschus_manihot_L_Medic.pdf (1.74M)
Word count: 4389
Character count: 24417

Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Jan 26; 10(A):207-213.
https://doi.org/10.3889/oamjms.2022.8356
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Pharmacology*

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DPP-IV Inhibitory Activity of the Ethanolic Extract of Red Gedi Leaves *Abelmoschus manihot* L. Medic

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Edited by: Sinisa Stojanoski
Citation: Tangka J, Barung EN, Lyrawati D, Soeatmadji D, Nurdiana N. DPP-IV Inhibitory Activity of the Ethanolic Extract of Red Gedi Leaves *Abelmoschus manihot* L. Medic. Open Access Maced J Med Sci. 2022 Jan 26; 10(A):207-213. https://doi.org/10.3889/oamjms.2022.8356
Keywords: *Abelmoschus manihot* L. Medic; DPP-IV inhibitor; Molecular docking, *In vitro*
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Received: 21-Dec-2021
Revised: 11-Jan-2022
Accepted: 16-Jan-2022

Funding: DIPA for Development of Health Human Resources, Ministry of Health, Republic of Indonesia.
Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: At present, there are many drugs used to manage diabetes including dipeptidyl peptidase-4 (DPP-IV) inhibitors which target insulin secretion. Abelmoschus manihot L. Medic, an endemic species of Minahasa, Indonesia, has been used as an antidiabetic herbal medicine.

AIM: In this study, we studied its metabolites activities, in silico and in vitro, as inhibitor for DPP-IV, thus regulating insulin secretion.

RESULTS: Of 38 identified metabolites, when docked into the catalytic site DPP-IV, 10 showed good binding energy within range of the standard gliptin drugs, that is, hibiscetin, gossypentin, gossypetin - 3-glucoside, myricetin, myricetin 3-glucoside, alpha spinasterol, quercetin, syringaresinol, stigmasterol, and isoquercetin. Three of those ten metabolites showed Ki within standard drugs values, that is, gossypetin, alpha spinasterol, and stigmasterol. The profile of molecular dynamic simulation, total energy and root mean square deviation of those metabolites were all similar with the standard gliptin drugs and predicted good stability of the complexes. The subsequent in vitro assay determining DPP-IV activity of the red Gedi leaves extract demonstrated that indeed the extract inhibited DPP-IV activity with IC50 860.67 µg/mL. Further studies are ongoing to prove the antidiabetic properties of the whole as well as isolated single compounds of the extract in particular gossypetin, alpha spinasterol, and stigmasterol as DPP-IV inhibitors.

CONCLSION: Our in silico studies showed that the compounds of ethanolic extract of red Gedi leaves potentially serve as DPP-IV inhibitors. Based on computed binding affinity, Ki, total energy, RMSD, and stability, the most potent compounds of the extract to inhibit DPP-IV activity are probably gossypetin, alpha spinasterol, and stigmasterol.

Introduction

Diabetes Mellitus type 2 (DM2), a chronic metabolic diseases worldwide, is characterized by hyperglycemia and imbalance between insulin levels and sensitivity [1], [2]. One of the recent drugs to manage DM2 is inhibitors of dipeptidyl peptidase-4 (DPP-IV) enzyme DPP-IV enzyme is responsible for fast degradation of incretin hormones, mainly glucagon such as peptide 1 (GLP-1) and *insulinotropic polypeptide* (GIP). GLP-1 secreted by intestine L cells facilitates blood glucose control by stimulating insulin secretion, suppressing apoptosis of and increasing proliferation of β cells. Inhibiting DPP-IV will potentiate endogenous GLP-1 and GIP, thus, increasing insulin secretion and decreasing glucagon secretion. DPP-IV reduces postprandial and fasting hyperglycemia.

The leaves extract of *Abelmoschus manihot* (L.) Medik, an endemic species of Minahasa, North Sulawesi, Indonesia, has been widely used as an antidiabetic herbal medicine [3], [4]. Our previous study identified 38 metabolites of ethanol extract of *A. manihot* (L.) Medik [5]. Based on an *in silico* study, although from different plant, stigmasterol and gossypetin may serve as candidates for DPP-IV inhibitor agents [6]. As now, there are not many studies addressing the antidiabetic pharmacology of the extract and its components [7], [8], [9], [10]. Phytochemicals are useful as templates or lead compounds and optimized further to generate novel, safe, and effective drugs. In this study, we predicted and determined the DDP-IV inhibitor activity of each 38 metabolites using *in silico* and *in vitro* assays. Computational molecular simulation or virtual screening aims to suggest new drug candidates by docking large libraries of small molecules into the binding site of the therapeutic target and using scoring functions to determine binding poses and estimate binding affinity. Such *in silico* studies make predictions about the binding affinity and selectivity of small molecules, important parameters in drug discovery and design [11]. The *in silico* studies have been applied and help in screening molecules for many pharmacological class of drug candidates, then the results must be assessed by *in vitro* assays

before the molecules selected and examined further employing *in vivo* approach in pre-clinical studies.

Materials and Methods

Plant material

The leaves of red Gedi *A. manihot* L. Medik were collected during August 2018 from North Tondano Plantation, North Sulawesi, Indonesia. Determination of the plants was performed at the Center for Plant Conservation Botanic Gardens, Indonesian Institute of Sciences, Bogor, Indonesia (Letter B-3177/IPH.3./KS/IX/2018 on September 18, 2018) [5].

Chemicals

Gly-pro-p-nitroanilide, tris buffer saline, sodium acetate buffer, and sitagliptin were all of pro-analytic quality from Sigma-Aldrich, and DPP-IV (derived from human placenta) from Biovision.

Extraction

Extraction of red leaves *A. manihot* (L.) Medik was performed as reported [5]. Fifty grams of dried red leaves *A. manihot* L. Medic extract were dynamically macerated in 96% ethanol solvent, then 500 mL 96% ethanol (1:10), stirred at 30–40°C, 200 rpm, for 6 h. The maserate was subsequently evaporated at 50°C and 80°C. The final thick extract was use for DPP-IV inhibitory assay or stored at refrigerator.

Protein and ligand preparation

Crystal structure of human DPP-IV (ID 4FFW complex control Sitagliptin) of X-Ray Diffraction with 2.9 Angstrom resolution as receptor was collected from PDB database (http://www.rcsb.org/). Identified binding sites of sitagliptin with target protein were used as standard for docking analyses. The 3D structure of 38 identified compounds of red Gedi was collected from PubChem database (https://pubchem.ncbi.nlm.nih.gov/search/) as previously reported [5].

Molecular docking

Docking analyses of red Gedi compounds and the standard gliptins as the ligands against DPP-IV protein were carried out by using Autodock Vina PyRx 9.5 and validated using PyMol 2.3.1. The interaction of the ligand and protein DPP-IV complex as well as the binding amino acid residues with each ligand were visualized using Discovery Studio Visualizer 2017 R2 Client dan LigPlot 2.3.1. Estimation of inhibition constants (Ki) against DPP-IV of each identified red Gedi metabolites were performed on Docking Server (https://www.dockingserver.com/) with grid box coordinates according to size and center of the protein. The parameterization of the grid box was as follow docking receptor = DPP-IV.pdbqt, exhaustiveness 8; num_modes 9; center_x = 20.0878578572; center_y = −8.83700476594; center_z = 54.9585865565; size_x = 15.1426866538; size_y = 11.979308064; size_z = 11.879307033; cpu = 7.

Molecular dynamics (MDs) simulation

The strength and stability of ligand-receptor complexes (red Gedi metabolites and DPP-IV) were predicted for red Gedi compounds which showed best docking results, binding affinity, and Ki. Gliptins (sitagliptin, saxagliptin, and/or vildagliptin) were used as standards for comparison. MD was also simulated for red Gedi compounds and the standard drugs using software YASARA with setting parameters 310K, pH 7.4, 1 atm, observed every 25 ps (from 0 to 1000 ps), AMBER03 force field [12], [13], [14] implemented on Intel Xeon. Subsequently, root mean square deviation (RMSD) and total energy were also calculated.

In vitro DPP-IV inhibitory assay

The assay of DPP-IV inhibition activity was performed in 96 well ELISA plates. In each well, 15 μL of DPP-IV enzyme, 20 μL of red Gedi extract (312.5; 625; 1250; 2500; 5000; and 10000 μg/mL) or standard sitagliptin (3.125; 6.25; 12.5; 25; 50; and 100 μg/mL) and 15 μL Tris-HCl buffer (50 mM, pH 7.5) were added. The mixture was incubated at 37°C for 10 min, followed by addition of 50 μL Gly-pro-p-nitroanilide as the substrate, and then incubated at 37°C for 30 min. The reaction was teminated with 25 μL sodium acetate buffer, pH 4.0. The amount of p-nitroanilide produced was measured at 405 nm using an ELISA plate reader. The nonenzymatic hydrolysis of substrate was corrected by measuring the increase in absorbance at 405 nm of control mixture without of DPP-IV enzyme. The DPP-IV inhibitory activity of each sample was calculated by the following equation: Inhibition ratio (%) = (1 – [Sample OD–Blank 2 OD]/[Control OD–Blank 1 OD]) × 100. For *in vitro* experiments, all analyses were carried out at least in triplicates.

Statistical analysis

Means ± standard deviations (SD) of data from *in vitro* DPP-IV inhibitory assay were calculated and transform into percent inhibition for each concentration tested; linear regression analyses were performed and used for IC_{50} calculations. All calculations were done using SPSS and MS Excel software.

Results

In silico assays

The red Gedi compounds tested in *in silico* docking were malic acid; p-coumaric acid; vanilic acid; ferulic acid; caffeic acid methyl ester; syringic acid; famesol; ficusol; hibiscone a; gmelofuran; hibiscone b; hibiscoquinone a; quercetin; moupinamide; myricetin; gossypetin; hibiscetin; α-spinasterol; stigmasterol; aquillochin; syringaresinol; quercetin -3-o- rhamnoside; hyperin; isoquercetin; myricerol; gossypetin -3-glucoside; myricetin-3-glucoside; hibiscetin-3-glucoside; daphniphyllum; kaempferitrin; sambicyanin; cyanidin-3-o-rutinoside; myriceric acid A; myriceric acid B; Boehmenan; erythro-carolignan E; hibicusin; and myriceric acid C. Among all the ligand compounds identified from red Gedi leaves extract, the binding affinity energy values between ligand and DPP-IV as receptor ranged from −3 kcal/mol predicted for myriceric acid B up to −8.2 kcal/mol for hibiscetin, whereas the control drugs were −7 kcal/mol for saxagliptin and vildagliptin and −8.6 kcal/mol for sitagliptin. Thus, based on predicted binding affinity, the top ten compounds showed binding energy close to standard drugs were hibiscetin, gossypentin, gossypetin -3-glucoside, myricetin, myricetin 3-glucoside, alpha spinasterol, quercetin, syringaresinol, stigmasterol, and isoquercetin (Table 1). The superimposed 3D diagram rendered by Pymol simulated binding site of those ten compounds and sitagliptin on the DPP-IV enzyme (Figure 1). Of those ten red Gedi metabolites, Ki values closest to that of standard drugs, Ki sitagliptin 0.48196 and vildagliptin 3.6 μM, were gossypentin 0.07741, alpha-spinasterol 1.86, and stigmasterol 2.1 μM (Table 1).

Table 1: Binding affinity and inhibition constant (Ki)

Ligands	Binding Affinity (kcal/mol)	Ki (μM)
Sitagliptin	−8.6	0.48
Hibiscetin	−8.2	541.64
Gossypetin	−8	0.77
Gossypetin 3-glucoside	−7.8	58.89
Myricetin	−7.7	199.95
Myricetin 3-glucoside	−7.7	32.95
Alpha Spinasterol	−7.5	1.86
Quercetin	−7.5	513.84
Syringaresinol	−7.2	115.65
Stigmasterol	−7	2.10
Vildagliptin	−7	3.60
Isoquercetin	−6.9	24.80

Docking results using AutoDock Vina visualized with Discovery Studio demonstrated the intermolecular interactions along with amino acid residues involved. Interaction of the red Gedi compounds with DPP-IV (Tables 2 and 3) occurred in the vicinity of the catalytic sites involving 2 extracellular domains of DPP-IV, Ser630-Asp708-His740 of pocket/site 1 (S1) and Glu205-Glu206-Arg125 of pocket/site 2 (S2) [15]. For instance, gossypetin bound the active site of DPP-IV through van Der Waals interaction at Glu203, Ile205 of S2 DPP-IV and Tyr632, Asn711 of S1 DPP-IV, pi-cation at Arg123 of S2 DPP-IV, and conventional hydrogen bond at Glu204 of S2 DPP-IV and Ser631-His741 of S1 DPP-IV. The LigPlot results showed similar interaction, particularly hydrogen bonds at Glu204 of S2 and Ser631, Asp709, His741 of S1 DPP-IV, and hydrophobic bonds at Arg123, Glu203 of S2. Both docking and visualization software showed similar prediction for chemical interactions and that the red Gedi leaves compounds interact with DPP-IV at sites important for enzyme activity. Such interactions present steric hindrance for the DPP-IV to bind its native ligands, the incretin hormones, thus red Gedi compounds may inhibit DPP-IV activity.

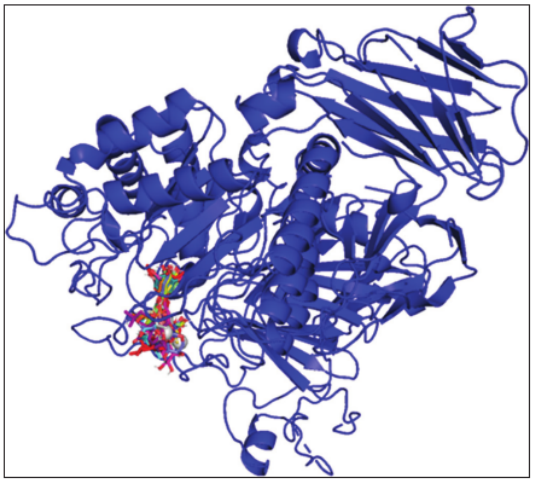
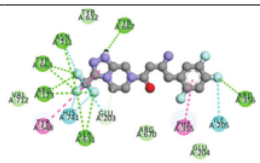
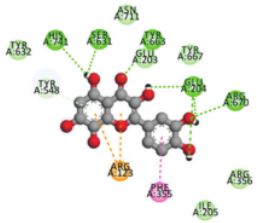
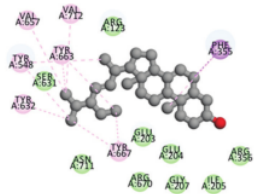
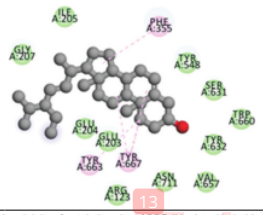


Figure 1: 3D superimposed diagram of ten bioactive compounds and sitagliptin binding sites on DPP-IV enzyme. All compounds bind to catalytic domain S1 and S2 of the enzyme (blue). DPP-IV: Dipeptidyl peptidase-4

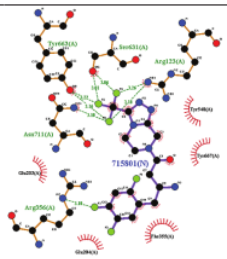
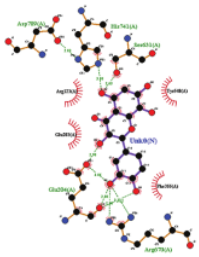
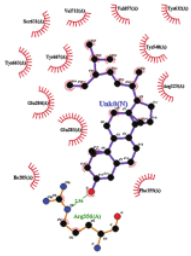
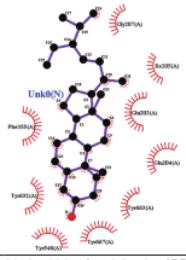
The analysis of interaction between red Gedi compounds and DPP-IV depicted in Tables 2 and 3 indicated that the studied compounds have a lot of van der Waals and hydrogen bonds. Such interactions suggested that the ligands were stable within the complex with DPP-IV. The MD simulations also supported the notion of complexes stability (Figures 2 and 3). The Gedi leaves compounds, whether it has high Ki like hibiscetin or low Ki like gossypetin, were predicted to have similar total energy and RMSD patterns. The calculated structure differences at certain time points, in respect with the initial structure at the start of simulation (RMSD), indicate the stability of a protein system. The smaller the differences, the more equivalents the structures, the more stable the system [16]. Approximately at 25 ps, the Gedi leaves compounds and the gliptins achieved the stable complex with DPP-IV at total energy -1.4 × 10^9 kJ/mol and remained stable for the duration of MD simulations (1000 ps) with good RMSD values between 0.9 and 1.7 Angstrom [17].

Table 2: Visualization using Discovery Studio for interactions of red Gedi compounds with DPP-IV established after docking

Ligand	Ligand-receptor interactions	Amino acids interactions*
Sitagliptin		Van Der Waals: Arg670, **Glu204**, Val712, Tyr632 Pi-Pi T-Shaped: *Tyr548*, **Phe355** Carbon Hydrogen Bond: **Glu203** Conventional Hydrogen Bond: **Arg123**, Arg356, **Ser631**, Tyr663, *Tyr667*, **Asn711**
Gossypetin		Van Der Waals: **Glu203**, **Ile205**, *Arg356*, **Tyr632**, *Tyr667*, **Asn711** Pi-Donor Hydrogen Bond: **Tyr548** Pi-Cation: **Arg123** Pi-Pi Stacked: *Phe355* Conventional Hydrogen Bond: **Glu204**, **Ser631**, Tyr663, Arg670, **His741**
Alpha Spinasterol		Van Der Waals: **Arg123, Glu203, Glu204, Ile205, Gly207,** Arg356, Ser631, Arg670, **Asn711** Pi-Sigma: **Phe355** Pi-Alkyl: *Tyr548*, Tyr632, Bal657, **Tyr663**, Tyr667, Val712
Stigmasterol		Van Der Waals: **Arg123, Glu204, Glu203, Ile205, Gly207**, *Tyr548*, **Ser631, Tyr632,** Val657, Trp660, **Asn711** Pi-Alkyl: **Phe355**, Tyr663, **Tyr667**

*Amino acids posit in the vicinity of catalytics site of DPP-IV printed in bold.

Table 3: Visualization using LigPlot interactions between red Gedi compounds with DPP-IV

Compound	Visualization	Hydrogen bond*	Hydrophobic bond*
Sitagliptin		**Arg123**, Arg356, **Ser631**, Tyr663, **Asn711**	**Glu203**, **Glu204**, Phe355, *Tyr548*, Tyr667
Gossypetin		**Glu204, Ser631**, Arg670 **Asp709, His741**	**Arg123, Glu203,** Phe355 **Tyr548**
Alpha Spinasterol		Arg356	**Arg123, Glu203, Glu204 Ile205,** Phe355, *Tyr548* **Ser631, Tyr632**, Val657 Tyr663, **Tyr667, Val712**
Stigmasterol			**Glu203, Glu204, Ile205 Gly207**, Phe355, *Tyr548* **Tyr632**, Tyr663, **Tyr667**

*Amino acids posit in the vicinity of catalytics site of DPP-IV or similar with sitagliptin printed in bold.
DPP-IV: Dipeptidyl peptidase-4

In vitro DPP-IV inhibitory assays

The ethanolic extract of red Gedi leaves *A. manihot* (L.) Medik was investigated for DPP-IV enzyme inhibitory activity using *in vitro* assays. Sitagliptin was used as the standard comparison. The experiments were replicated four times, with an excellent agreement for each replication, resulting in SD 0.00. The results showed that the extract inhibited DPP-IV activity similar with sitagliptin at 10 times higher concentration (Table 4). The calculated IC_{50} of the extract was 860.87 μg/mL, whereas IC_{50} of sitagliptin was 9.67 μg/mL. The different concentration between ethanolic extract of red Gedi leaves and sitagliptin was expected. Sitagliptin is a single active pharmaceutical ingredient whereas the extract is a mix of compounds. Thus, such difference of IC_{50} between sitagliptin and extract can be explained by the varied potency of the active compounds and percentage of each potent DPP-IV inhibitors within the extract. From *in silico* studies, the most potent compounds of the extract to inhibit DPP-IV activity are probably gossypetin, alpha spinasterol, and stigmasterol.

Discussion

The results of *in silico* analysis demonstrated that there were three compounds of red Gedi leaves extract may serve as DPP inhibitors, that is, gossypetin, alpha spinasterol, and stigmasterol. Their binding affinity values were favorable, Ki were below 20 μM [18] and closest to the values of gliptins. The interaction sites of those three compounds with DPP-IV involved amino acid residues important for recognition of native ligand and as active site of the enzyme Ser630-Asp708-His740 of pocket/site 1 (S1) and Glu205-Glu206-Arg125 of pocket/site 2 (S2). Additional interactions with

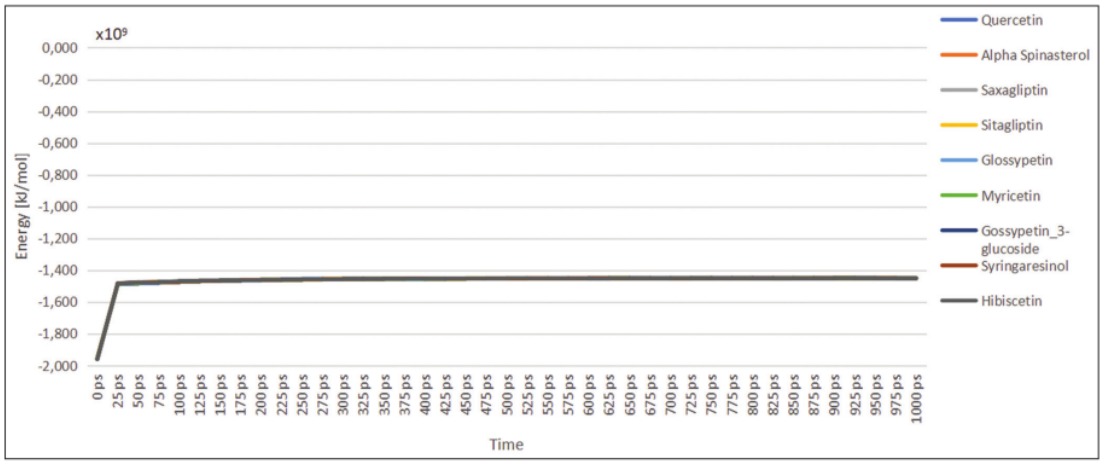


Figure 2: Total energy of red Gedi compounds with DPP-IV complexes estimated using molecular dynamic simulation. DPP-IV: Dipeptidyl peptidase-4

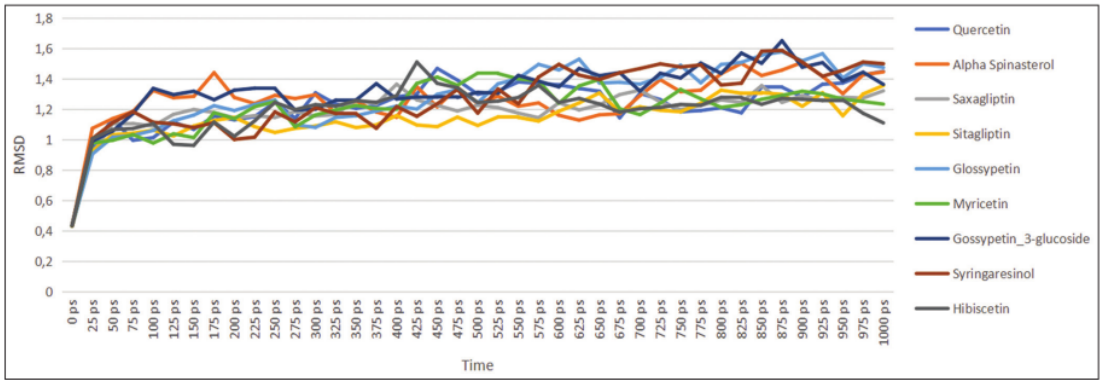


Figure 3: RMSD of red Gedi compounds with DPP-IV complexes estimated using molecular dynamic simulation at different time points. DPP-IV: Dipeptidyl peptidase-4, RMSD: Root mean square deviation

Table 4: Inhibitory effects of red Gedi leaves ethanolic exxtract on DPP-IV activity

S. No.	Ethanolic extract of red Gedi leaves		Sitagliptin	
	Concentration (µg/mL)	Maximum Inhibition ± SD (%)	Concentration (µg/mL)	Maximum Inhibition ± SD (%)
1.	312.5	29 ± 0.00145	3.125	26 ± 0.00057
2.	625	54 ± 0.00252	6.25	35 ± 0.00361
3.	1250	60 ± 0.00120	12.5	64 ± 0.00138
4.	2500	75 ± 0.00235	25	74 ± 0.00099
5.	5000	79 ± 0.0017	50	78 ± 0.00191
6.	10000	89 ± 0.0091	100	88 ± 0.00413
	860.67	Calculated IC_{50}	9.67	Calculated IC_{50}

DPP-IV: Dipeptidyl peptidase-4

other sites also noticed, which may increase DPP-IV inhibition. The other binding sites identified were in the vicinity of S2 ext. (S2 extensive) subsite constructed by Val207, Ser209, Phe357, Arg358, S2 subsite Arg125, Phe357, Arg358, Glu205, Glu206, Arg669, S1 subsite Ser630, Val656, Trp659, Tyr662, Tyr666, Val711, Asn710, S1′ subsite Phe357, Tyr547, Pro550, Ser630, Tyr631, Tyr666, and S2′ subsite Tyr547, Trp629, Ser630, His740 [19]. Based on such interactions, the red Gedi compounds may be develop further as DPP-IV inhibitors Class 3 like sitagliptin which form interactions with the S1, S2, and S2 extensive subsites [20].

Despite the promising results from *in silico* analyses showing comparable interactions and potency with sitagliptin, the *in vitro* assays demonstrated that the red Gedi leaves extract IC_{50} was 100 times higher than that of sitagliptin. Such discrepancy may be explained by the nature of the crude extract which contains many compounds with different concentrations. The mixture of the compounds may act antagonistically to each other in *in vitro* assay for DPP-IV activity, thus, resulted in higher concentration than the single compound like sitagliptin. Another explanation is probably due to amount of the metabolites in the extract. For instance, if the total flavonoids of the ethanol extract of red Gedi leaves are minimum 23.63 mg/g extract, stated as the minimum standard of Gedi extract [21], then within 860.67 µg of extract there will be 19.78 µg of total flavonoid. The amount of gossypetin, a type of flavonoid, may be <19.78 µg. Thus, the IC_{50} of gossypetin most probably

not so different with 9.67 μg/mL that of sitagliptin. At present, however, amount the total flavonoid and other metabolites within the ethanolic extract of red Gedi leaves have not been determined nor published, yet. Further studies are needed to clarify, whether any interaction among metabolites, or with DPP-IV, or simply due to amount of each metabolite within extract, or else, explain the IC_{50} of the extract. Nevertheless, the red Gedi leaves extract can inhibit DPP-IV activity.

Conclusions

Our *in silico* studies showed that the compounds of ethanolic extract of red Gedi leaves potentially serve as DPP-IV inhibitors. Based on computed binding affinity, Ki, total energy, RMSD, and stability, the most potent compounds of the extract to inhibit DPP-IV activity are probably gossypetin, alpha spinasterol, and stigmasterol. *In vitro* assay determining the DPP-IV inhibitory activity of the extract showed that IC_{50} of the extract was 860.87 μg/mL whereas IC_{50} of sitagliptin was 9.67 μg/mL. It will be interesting to test isolated compounds, especially gossypetin, alpha spinasterol and stigmasterol, to develop new DPP-IV inhibitors while progress into antidiabetic *in vivo* studies of the total extracts of red Gedi leaves.

Acknowledgments

The authors would like to thank Dr. Didik Widyatmoko, M.Sc. from Center for Plant Conservation Botanic Gardens – Indonesia Institute of Sciences, Bogor, Indonesia for determination of the red Gedi leaves, Didik H. Utomo, S.Si., M.Si., from Inbio, and Fitri Armania, S.Si. Laboratory of Biomedical Science at Faculty of Medicine of Universitas Brawijaya for enabling *in silico* and *in vitro* works done. This study was funded partly by DIPA for Development of Health Human Resources, Ministry of Health, Republic of Indonesia (JT).

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