Molecular and Cellular Biomedical Sciences, Vol.3 No.2, September 2019, p.67-74
Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v3i2.60

RESEARCH ARTICLE

MCBS
Mol Cell Biomed Sci. 2019; 3(2): 67-74
DOI: 10.21705/mcbs.v3i2.60

Antioxidant, α-Glucosidase Inhibitory Activity and Molecular Docking Study of Gallic Acid, Quercetin and Rutin: A Comparative Study

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Background: Plant-phenolics and flavonoids, including gallic acid, quercetin and rutin, are considered as safe inhibitors for α-glucosidase. This study aimed to compare antioxidant and α-glucosidase inhibitory activities of gallic acid (GA), quercetin (QUE) and rutin (RUT).

Materials and Methods: Pure compounds of GA, QUE, and RUT were used. Their antioxidant and inhibitory activity on α-glucosidase were investigated spectroscopically, including their kinetic analysis and interaction mechanism by docking simulation.

Results: All the tested compounds (GA, QUE, and RUT) showed good antioxidant activity better than the standards ascorbic acid (AA) and butylated hydroxytoluene (BHT), with QUE showing the highest antioxidant activity based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. Based on their reducing properties, the activities of the compounds follow the following order: AA > GA > BHT > QUE > RUT. Both GA and RUT induced a competitive type of inhibition, with activities stronger than acarbose (IC_{50} = 823 µg/mL), whereas QUE inhibited in a mixed type manner. The IC_{50} of GA, QUE, and RUT were 220.12, 65.52, and 224.55 µg/mL respectively. The results obtained from molecular docking indicate that all compounds have affinity in the active site pocket of α-glucosidase, with the hydrogen bond being the major force involved in each compound binding to the enzyme.

Conclusion: In conclusion, QUE has better antioxidant and α-glucosidase inhibitory activity than GA and RUT. This work provides insights into the interactions between GA, QUE, and RUT and α-glucosidase.

Keywords: docking, gallic acid, α-glucosidase, rutin, quercetin

Introduction

Plant products are potentially a good source for screening α-glucosidase inhibitors. Flavonoids and phenolics which are widely existed in various plants have been reported to exhibit inhibitory effect on α-glucosidase, such as luteolin[1], kaempferol[2] and gallic acid (GA)[3]. GA, quercetin (QUE) and rutin (RUT) are commonly used as standard compounds for

Date of submission: January 31, 2019
Last Revised: February 22, 2019
Accepted for publication: February 22, 2019

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total phenolic and/or total flavonoid measurements. Their inhibitory activities on α-glucosidase are not yet compared experimentally. In the present study, the antioxidant capacities and α-glucosidase inhibitory activities of these compounds, including their type of inhibitions and binding interactions with the enzyme were reported.

GA or 3,4,5-trihydroxybenzoic acid (Figure 1) is a plant-derived phenolic acid. GA exerts many biological effects such as antioxidant, anticancer, antiviral, cardioprotective and anti-diabetic effects.[4,5] GA showed strong antidiabetic activity both *in vitro* and *in vivo*[6] and its combination with acarbose was recommended for anti-diabetic therapy.[3] Moreover, derivates of GA *i.e.,* methyl gallate and propyl gallate showed strong α-glucosidase inhibitory activity.[3,7] GA extracted from plants have been shown to exert strong inhibition on α-glucosidase.[8,9]

QUE is a kind of plant flavonoid, whereas RUT is a glycoside of QUE (Figure 1). QUE and RUT belong to flavonol which are often found in onion, kale, broccoli, apples, cherries, berries, black tea, and mango.[10,11] Both were reported to possess anti-diabetic potentials.[12,13] Some studies proposed that QUE and RUT exerts its hypoglycemic effect through multiple actions including by increasing the proliferation of pancreatic β-cells, enhancing insulin sensitivity and stimulating insulin secretion.[12,14] Further, antidiabetic effect of QUE may also involve the protection of β-cells, as were shown by previous *in vivo* studies.[15] In addition, QUE and RUT were shown to strongly inhibit α-glucosidase.[16]

The aims of this study were to determine and compare inhibitory activities of GA, QUE and RUT on α-glucosidase and to investigate their binding interaction with the enzyme by in vitro and molecular docking studies. Their antioxidant capacities were also evaluated.

Materials and methods

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Activity Assay

Antioxidant activities of GA (Cat. No. #sc205704, Santa Cruz Biotechnology, Dallas, USA), QUE (Cat. No. #PHR1488, Sigma-Aldrich, St Louis, USA), RUT (Cat. No. #sc204897B, Santa Cruz Biotechnology) and standard compounds, ascorbic acid (AA) (Cat. No. #470300-286, VWR BDH Prolabo Chemicals, Lutterworth, UK) and butylated hydroxytoluene (BHT) (Cat. No. #B1378, Sigma-Aldrich) were determined based on reported method.[17] Solution of DPPH (Cat. No. #D9132, Sigma-Aldrich) (0.6 mM in ethanol) was prepared and 1 mL of this solution was added to 3 mL of tested compounds at various concentration. The reactions were incubated for 30 min in darkness at room temperature and the absorbance was read at 517 nm

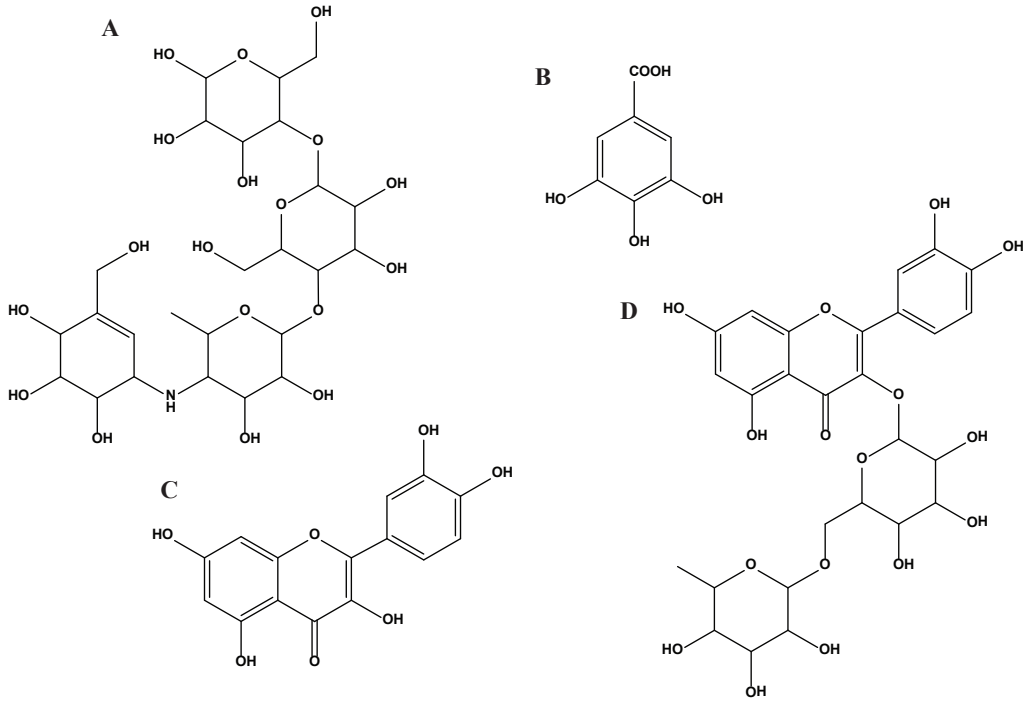

Figure 1. Molecular structures of acarbose (A), GA (B), QUE (C) and RUT (D).

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v3i2.60

with spectrophotometer Biochrom Libra S-22 (Biochrom, Cambridge, UK). Ethanol (3 mL) in place of extract was used as control. The percentage inhibition activity was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Where $A_{control}$: absorbance of control, A_{sample}: absorbance of sample. The percentage of inhibition was plotted against concentration to calculate the IC_{50} which is defined as the amount of antioxidant required to decrease the inhibition of DPPH radical by 50%. The IC_{50} values were expressed as µg/mL and compared with the standards.

Reducing Power Activity Assay

Reducing power capacity of GA, QUE, and RUT was determined according to reported method.[18] The procedure was based on the reduction of Fe(III) to Fe(II) reaction. Different concentrations of compounds or standards (ascorbic acid and BHT) in water (50, 100, and 200 µg/mL) were prepared. In a test tube, 1 mL of each sample solution was mixed with 2.5 mL $K_3Fe(CN)_6$ (Cat. No. #104971, Merck, Darmstadt, Germany) solution (1% w/v), followed by 2.5 mL phosphate buffer (200 mM, pH 6.6). The mixture was incubated in a water bath for 20 minutes at 50°C. Into the mixture, trichloroacetic acid (Cat. No. #T6399, Sigma-Aldrich) solution (2.5 mL, 10% w/v) was added and centrifuged for 10 min at 3000 rpm. The upper layer of the solution (2.5 mL) was taken out and mixed with water (2.5 mL) and $FeCl_3$ (Cat. No. #sc215192, Santa Cruz Biotechnology) solution (0.5 mL, 0.1% w/v). The absorbance of each sample was read at 700 nm by spectrophotometer and compared with the standards.

Total Antioxidant Activity/Phosphomolybdate Assay

Total antioxidant activity of GA, QUE, and RUT was determined according to a phosphomolybdate method reported previously[19]. Phosphomolybdate reagent contained sulfuric acid (0.6 M), sodium phosphate (28 mM), and ammonium molybdate solution (4 mM). Into 3 mL of this solution was added 0.3 mL compound solution of standards (ascorbic acid and BHT) in water (50, 100, 200 and 400 µg/mL) placed in capped tubes. The reaction mixture was incubated in water bath at 95°C for 1.5 hours and let to cool at ambient temperature. The absorbance was measured at 695 nm using a spectrophotometer and the reading was compared with the standards.

α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory activities of GA, QUE, RUT, and acarbose (Cat. No. #1000521, USP, North Bethesda, USA) as standard compound were carried out according to previous study.[20] Briefly, a 50 µL of compound solution at different concentrations was mixed with 50 µL phosphate buffer (50 mM, pH 6.8) and 50 µL of α-glucosidase (Cat. No. #G5003, Sigma-Aldrich) (0.5 unit/mL). After pre-incubating for 5 min at 37°C, substrate (1 mM p-nitrophenol-α-D glucopyranoside) (Cat. #N1377, Sigma-Aldrich) was added to the reaction mixture and incubated for 20 min at 37°C. The reaction was stopped by adding 750 µL Na_2CO_3 (100 µM). The absorbance was read at 405 nm. For the control solution, all procedures were followed except that sample was replaced by buffer. The percentage of inhibition was calculated using the following equation:

$$\text{α-Glucosidase inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Where $A_{control}$: absorbance of control, A_{sample}: absorbance of sample. The α-glucosidase inhibitory activity was expressed as IC_{50} values (µg/mL) and was determined from the graph plotted against the percentage inhibition. Values were compared with the positive control acarbose, the antidiabetic medicine.

Enzyme Kinetic Assay

GA, QUE, RUT and the positive control, acarbose were evaluated for their inhibitory kinetics on α-glucosidase activity according to a method described previously.[18] The inhibition type was determined based on the effect of different concentrations of each compound on increasing concentration of 4-Nitrophenyl-β-D-glucopyranoside (p-NPG) (0.25-1.25 mM) as substrate. The study of the inhibition type (competitive, uncompetitive, non-competitive or mixed) was performed using the nonlinear regression Michaelis-Menten enzyme kinetics and the corresponding Lineweaver-Burk double reciprocal plots (1/Vmax vs 1/[S], as y and x axis, respectively) for each concentration of the inhibitor and substrate. The Ki values were calculated with Molecular Operating Environment Software.

Preparation of Ligands

GA, QUE, RUT, and acarbose were compounds to be used for molecular docking simulations on α-glucosidase. All of the two dimensional (2D) structures of the compounds

were retrieved from PubChem database as an .sdf file and were imported into MOE 2010.10 and save as a .pdb file. The three dimensional (3D) structures of all ligands were obtained from PubChem database with PubChem CID 370 (GA), 41774 (acarbose), 5280343 (QUE), and 5280805 (RUT). The optimization of these ligands was performed using a Merck molecular force field MMFF94x in MOE.

Molecular Modelling (of α-Glucosidase, GA, QUE, RUT, and Acarbose)

The 3D structures of *Saccharomyces cerevisiae* α-glucosidase are not reported yet. However, the crystal structure that has been isolated for Saccharomyces cerevisiae is of manosyl-oligosaccahride glucosidase. The amino acid sequence of this protein was obtained from NCBI data base (http://www.ncbi.nlm.nih.gov/protein/) and the 3D model was built using SWISS-MODEL server and the predicted template of 3D model was obtained from Protein Data Bank. The 3D structures of the protein were validated using Ramachandran plot.

Molecular Docking of GA, QUE and RUT with α-Glucosidase

Before the molecular docking was performed, the following preparations were conducted, such as addition of hydrogen atoms and/or partial charges to the model and minimization of unwanted steric effect by keeping heavy atoms fixed. The target receptors model of α-glucosidase energy was optimized using CHARMM in MOE. Molecular docking between ligand and target receptors was performed on the active site generated from the MOE-Alpha Site Finder module using a wall constraint with radius 7Å. The molecular docking itself was carried out using Triangle Matcher placement method and a force field refinement. Docking poses were scored using the Affinity ΔG scoring method of MOE. Selection of lead compound was performed by analyzing the residue interactions, hydrogen bonds, and electrostatic interactions generated from molecular docking.

Statistical Analysis

All experiments were carried out in triplicates. Results were reported as mean±standard deviation (SD). Regression method was used to calculate IC_{50} and enzymatic kinetic.

Results

Three different methods were used to evaluate the antioxidant activity of GA, QUE and RUT. For each method, ascorbic acid and BHT as standards were also assessed to compare the activity.

DPPH assay was the method used to assess the radical scavenging activity of the compounds. Scavenging free radicals is an important mechanism involving antioxidant pathway. The results for radical scavenging activities (IC_{50}) are shown in Table 1. QUE was the best scavenger/antioxidant among other compounds (GA, RUT, AA and BHT). In addition to DPPH assay, the antioxidant activity of the compounds was also evaluated based on reducing power and total antioxidant activity. In this case, an electron is donated from the antioxidant compounds to reduce Fe(III) to Fe(II) or Mo(VI) to Mo(V) for reducing power and total antioxidant activity assay, respectively. Results are shown in Figure 2. Based on these methods, generally, the activities of the compounds showed the following order: AA > GA > BHT > QUE > RUT.

The α-glucosidase inhibition activity of the compounds was investigated, and the results are shown in Table 2. All the tested compounds, GA, QUE and RU, showed excellent inhibition activity on α-glucosidase compared to that of the standard acarbose, as indicated by their IC_{50} (μg/mL). Their

Table 1. Comparative DPPH radical scavenging activities of GA, QUE, RUT and standards (IC_{50}).

Concentration (μg/mL)		Inhibition (%)	IC_{50} (μg/mL)
Gallic Acid	10	20.12 ± 0.01	58.82 ± 0.37
	15	24.14 ± 0.09	
	25	33.31 ± 0.09	
	43.75	39.05 ± 0.22	
	62.5	52.79 ± 0.16	
Quercetin	1	18.02 ± 0.19	2.71 ± 0.04
	2	29.36 ± 4.11	
	2.5	39.32 ± 0.43	
	3	69.71 ± 0.20	
	3.75	74.20 ± 0.61	
Rutin	5	25.42 ± 0.14	8.53 ± 0.01
	6.25	33.22 ± 0.05	
	7.5	41.17 ± 0.08	
	10	62.76 ± 0.16	
	12.5	77.33 ± 0.10	
Ascorbic acid			53.24 ± 0.82
BHT			21.36 ± 0.80

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v3i2.60

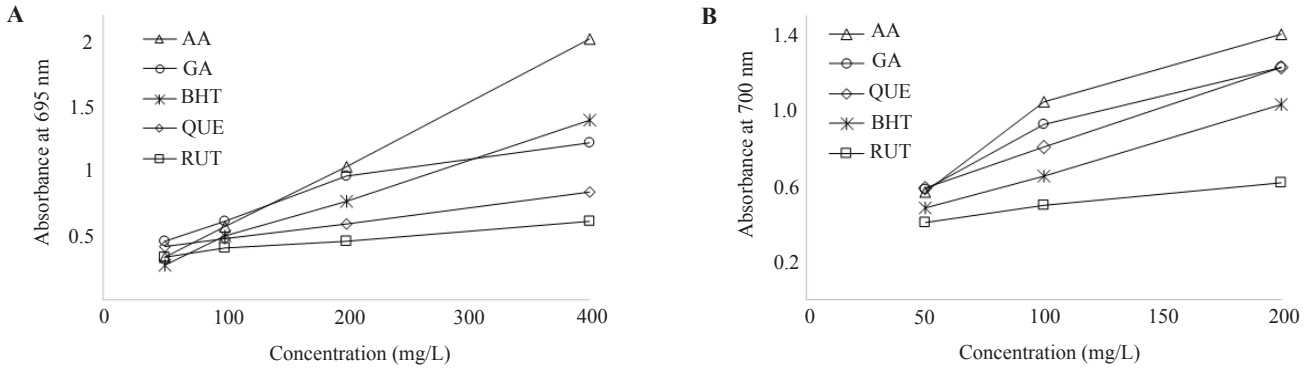

Figure 2. Antioxidant activity measured by phosphomolybdate (A) and reducing power assay (B).

IC_{50} values were in the range of 65.52 to 224.55, lower than IC_{50} of acarbose of 823.00 (Table 2). It was evident that QUE was the most active inhibitor.

To characterize the type of inhibition of the compounds on α-glucosidase, *i.e.,* competitive, noncompetitive, uncompetitive, or mixed, the Linewaver–Burk double reciprocal plots were generated. The results were shown in Figure 3. The LB plots of GA, RUT, and acarbose gave straight lines which had different intersection on the X-axis. These results suggest a competitive inhibition on α-glucosidase by these compounds. Different result was observed for QUE. In the presence of increasing concentrations of QUE, the Lineweaver–Burk plot generated straight lines which had a point of intersection in the second quadrant, indicating that the inhibition was of the mixed competitive and non-competitive type.

Table 2. IC_{50} of α-glucosidase inhibition by GA, QUE and RUT.

Concentration (µg/mL)		Inhibition (%)	IC_{50} (µg/mL)
Gallic Acid	200	16.34 ± 2.00	220.12 ± 5.05
	210	25.84 ± 5.58	
	225	74.98 ± 1.76	
	250	89.91 ± 10.84	
Quercetin	25	19.46 ± 6.71	65.52 ± 2.88
	50	32.99 ± 0.44	
	75	72.22 ± 2.78	
	125	84.07 ± 1.60	
Rutin	200	26.99 ± 3.94	224.55 ± 5.48
	225	50.7 ± 9.96	
	250	75.57 ± 2.54	
	275	92.81 ± 2.54	
Acarbose			823 ± 0.06

The molecular docking studies were conducted in order to understand the binding interaction between inhibitors and the enzyme α-glucosidase. Table 3 shows the binding energy of four different ligands and the receptor, together with the kind of interaction and bond lengths. The docking simulations are shown in Figure 4.

Discussion

According to Niedowicz, oxidative stress may contribute to the incidence of oxidative related complications in diabetes.[21] Long term hyperglycemia may induce increased production of reactive oxygen species (ROS) via non-enzymatic glucose autoxidation, glycation, and alterations in poly-ol pathway activity.[22] Therefore, in the present study, the antioxidant properties of GA, QUE, and RUT were evaluated and compared. Phytochemicals that possess antioxidant properties were reported to exert their antioxidative mechanism by way of reducing oxidative species.[23] The current study evaluated the reducing properties of the compounds by their ability to reduce Fe(III) to Fe(II) and Mo(VI) to Mo(V). These assays reflect the reducing capability of antioxidant compounds and may serve as a significant indicator for antioxidant activity.[24] In both assays, the results revealed that GA possessed the strongest reducing capacity compared to QUE and RUT. Furthermore, the current study also evaluated antioxidant properties of the tested compounds based on their free radical scavenging ability. The result found that QUE and RUT (flavonoids) were stronger radical scavengers than GA (a phenolic compound). The different results obtained

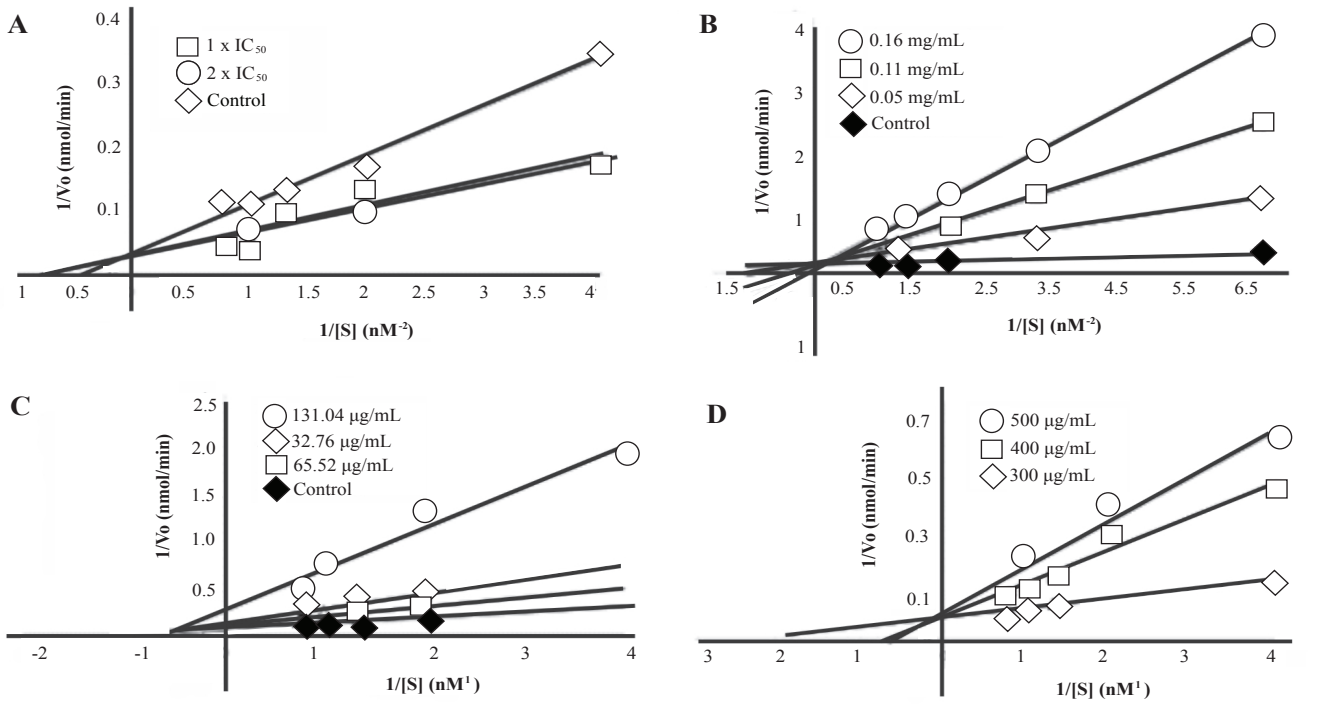

Figure 3. Lineweaver-Burk plot of GA (A), Acarbose (B), QUE (C), and RUT (D) on α-glucosidase at different concentration of p-NPG.

in reducing power and DPPH assays could be due to the difference in antioxidant mechanism that took place in the assays. According to Celik and Arinç, quercetin expressed higher antioxidant capacity than its flavonoids derivatives, like rutin and naringenin.[25] Heijnen, *et al*., reported that the presence of multiple hydroxyl groups of flavonoid act as vigorous scavengers not only for ROS but also for RNS.[26] The hydroxyl groups dominate the capability of flavonoids as antioxidant.[10.]

Diabetes mellitus is characterized by postprandial hyperglycemia over a prolonged period, which may be due to resistance to insulin action and/or inadequate

Table 3. Energy calculation and H-bond parameter.

	ΔG (kcal/mol)	K_i (M)	Interaction	Residue	Distance (Å)
Quercetin	-6.6711	12.8	H-donor	Glu 411	3.23
			H-donor	Asp 215	2.88
Rutin	-8.7832	0.361464	H-donor	Asp 242	2.74
			H-donor	Ser 311	2.78
			H-donor	Leu 313	3.01
			H-donor	Gln 279	2.79
			H-donor	Glu 411	2.98
Gallic Acid	-4.4458	548.76	H-acceptor	Ser 240	3.3
			H-acceptor	Ser 241	3.1
			H-pi	Tyr 158	3.49
Acarbose	-5.668	69.664	H-donor	Asp 242	2.91

Print ISSN: 2527-4384, Online ISSN: 2527-3442
DOI: 10.21705/mcbs.v3i2.60

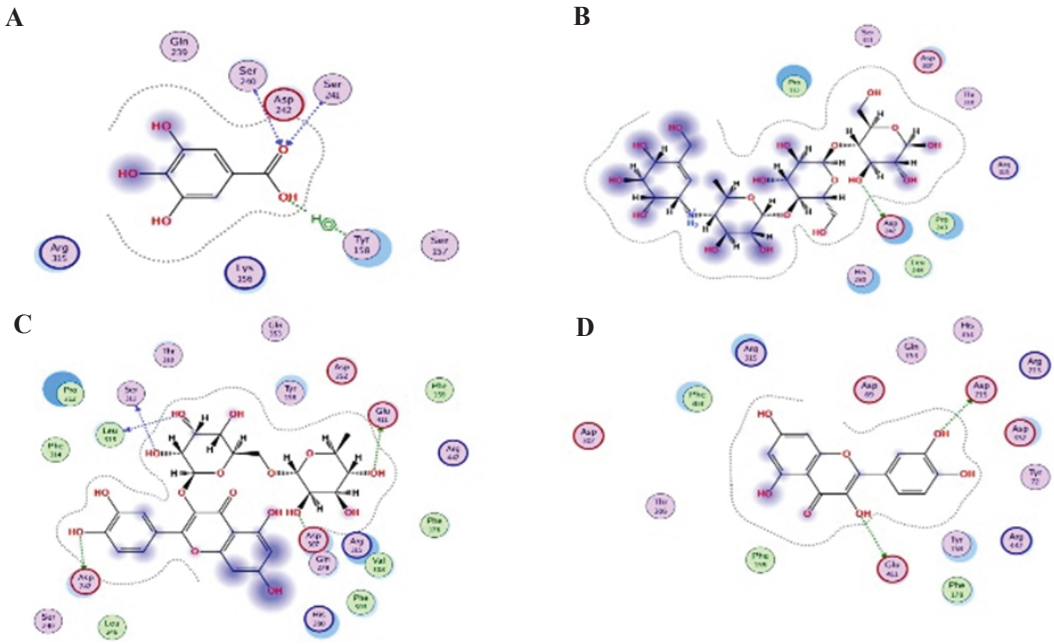

Figure 4. Two-dimension (2D) simulation by docking analysis for Acarbose (A), GA (B), QUE (C) and RUT (D).

insulin secretion.[27] Inhibition of digestive enzymes (such as α-glucosidase and α-amylase) that hydrolyze dietary polysaccharide into glucose is crucial for controlling glucose blood level. Therefore, inhibitors that target α-glucosidase serve as a key strategy in the treatment of diabetes mellitus. In the present work, the potential inhibition of the compounds on α-glucosidase activity was evaluated.

All compounds showed significant inhibition on α-glucosidase compared to acarbose, with QUE almost 13 times more active than acarbose, whereas GA and RUT almost 4 times stronger than acarbose. Through the enzyme kinetic study, GA and RUT were shown to act as competitive inhibitors on α-glucosidase, similar to that found for acarbose. These results suggest that GA, RUT, and acarbose inhibited α-glucosidase by binding with free enzyme in a manner that prevents substrate binding. These findings are in accordance with previous studies by Zhang, *et al*, and Proença, *et al.* [28,29] On the other hand, QUE inhibited α-glucosidase in a mixed type of competitive and noncompetitive inhibition. This observation indicates that QUE may form enzyme-inhibitor complex and form substrate – inhibitor to interrupt enzyme-substrate intermediate. It is likely that the mixed type inhibition enables QUE to inhibit α-glucosidase in a broader binding sites compared to GA, RUT and acarbose as competitive inhibitors, giving rise to the lowest IC_{50} value observed for QUE.

In order to further understand the binding mechanism at molecular level, molecular docking analysis was performed between α-glucosidase and each compound. In all ligand-receptor models, the hydrogen bonds are the main drivers involved for the interaction of each docked ligand to α-glucosidase. At α-glucosidase binding site, GA showed two H-interactions with Ser240 and Ser241, and one interaction of H-pi with Tyr158. Two H-bonds were observed between QUE and active site residues Glu411 and Asp215. Five H-bonds were formed between RUT with the amino acid residues Asp242, Ser311, Leu313, Gln279 and Glu411, whereas the competitive inhibitor acarbose showed one H-bond interaction with Asp242. It is worth pointing that GA, RUT and acarbose occupy the same active sites at α-glucosidase to avoid the entrance of the substrate pNPG. The binding sites observed by docking simulations supported the competitive type of inhibition obtained from kinetic experimental assay for these compounds.

The docking energy calculated for all the tested compounds further supported the experimental results. The predicted free energy calculated for GA, QUE, and RUT were lower than acarbose. This result confirms the stronger inhibitory activities of these compounds on α-glucosidase when compared with acarbose. In addition, the predicted Ki values for QUE and RUT (Ki = 12.8 and 0.36, respectively) were also lower than acarbose (Ki = 69.66), indicating

that QUE and RUT have more affinity for α-glucosidase compared to acarbose.

Conclusion

The present study found that flavonoids like QUE and RUT are good antioxidants. QUE and RUT are also good inhibitors for α-glucosidase activity, with inhibition activities better than the standard compound acarbose, thus can be effective in reducing post-prandial hyperglycemia (PPHG). These findings may facilitate further research for the development and application of these compounds as α-glucosidase inhibitors and ingredients in functional food.

Acknowledgement

We are grateful to the Faculty of Medicine and the Research Institute of Krida Wacana Christian University, for supporting this project financially and institutionally.

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