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Enhancement of quercetin water solubility with steviol glucosides and the studies of biological properties

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ABSTRACT:

Background: Quercetin, a flavonol contained in various vegetables and fruits, has numerous biological activities which include anticancer, antiviral, anti-diabetic, and anti-oxidative properties. However, quercetin also has low oral bioavailability, due to its insolubility in water. Thus, the bioavailability of quercetin administered to human beings in a capsule form was reported to be less than 1%, with only a small percentage of ingested quercetin getting absorbed in the blood. This leads to certain difficulties in creating highly effective medicines.

Methods: Quercetin-rubusoside and quercetin-rebaudioside were prepared. The antioxidant activities of quercetin and Q-rubusoside were evaluated by DPPH radical scavenging method. Inhibition activities of quercetin and Quercetin-rubusoside were determined by measuring the remaining activity of $3CL^{pro}$ with 200 μM inhibitor. The inhibition activity of quercetin, rubusoside and quercetin-rubusoside were determined by measuring the activity of human maltase, which remains at 100 μM rubusoside or quercetin-rubusoside. The mushroom tyrosinase inhibition was assayed with the reaction mixture containing 3.3 mM L-DOPA in 50 mM potassium phosphate buffer (pH 6.8), and 10 U mushroom tyrosinase/ml with or without quercetin or quercetin-rubusoside.

Results: With 10% rubusoside treatment, quercetin showed solubility of 7.7 mg/ml in water, and its solubility increased as the concentration of rubusoside increased; the quercetin solubility in water increased to 0.83 mg/ml as rubusoside concentration increased to 1 mg/ml. Quercetin solubilized in rubusoside solution showed DPPH radical-scavenging activity and mushroom tyrosinase inhibition activity, similar to that of quercetin solubilized in dimethyl-sulfoxide. Quercetin-rubusoside also demonstrated 1.2 and 1.9 folds higher inhibition activity against $3CL^{pro}$ of SARS and human intestinal maltase, respectively, than those of quercetin in DMSO.

Conclusions: Quercetin can be solubilized in water with rebaudioside or rubusoside treatment. As Ru concentration increases, the solubility of quercetin in water increases. The solubilization of quercetin in Ru solution did not reduce its biological functions such as the DPPH radical-scavenging and mushroom tyrosinase activity. Additionally, quercetin-rubusoside increased the inhibition activity against the $3CL^{pro}$ of SARS and human intestinal maltase, when compared with the activity of quercetin in DMSO. Therefore, rubusoside and rebaudioside are promising compounds which enhance the solubility of poorly water soluble compounds.

Keywords: rubusoside, rebaudioside, flavonol, quercetin, human maltase, $3CL^{pro}$

INTRODUCTION:

Flavonoids are polyphenolic secondary metabolites in plants, with a common diphenylpropane (C6-C3-C6) skeleton categorized into chalcones, flavonols, flavones, flavan-3-ols, flavanones, isoflavones, and anthocyanins [1]. Quercetin (3,3',4',5,7-pentahydroxylflavone, Figure 1) is the major representative of the flavonoids subclass of flavonols that are found in abundance in various fruits and vegetables. For example, tea, apples, onions and berries [2]. The average intake of flavonols in the Netherlands [3], United states [4], and Japan [5] is estimated to be around 20 mg/day, of which more than 50% consists of quercetin. Quercetin has demonstrated an excellent free-radical scavenging antioxidant activity [6], antidiabetic activity [7], inhibitory activity toward SARS-CoV (severe acute respiratory syndrome-associated coronavirus) $3CL^{pro}$ [8] or viral replication [9], and it has been proposed to be a chemopreventive and anticancer agent [10-12]. However, due to its chemical structure, the solubility of quercetin is poor. As a result, the bioavailability of quercetin administered to human beings in a capsule form was reported to be less than 1% [13], with only a small percentage of ingested quercetin getting absorbed in the blood [14]. This leads to certain difficulties in creating highly effective medicines, since solubility is one of the major biopharmaceutical characteristics that largely determines the drug's bioequivalence, in addition to the possibility of creating drug forms which have effective dosage, absorption rate, and completeness [15]. Therefore, various attempts have been made to improve the water solubility of quercetin, such as making quercetin loaded nanostructured lipid carriers [16], encapsulation on poly-D,L-lactide nanoparticles [17], or capsulation using β-cyclodextrin [18].

Steviol glycosides are natural sweeteners, approximately 200, 300, and 114 times sweeter than sucrose, present in rebaudioside (Reb) A, stevioside (Ste) and rubusoside (Ru, Figure 1), respectively [19-21]. Reb and Ste are diterpenoid glycosides found in high concentration levels

in the leaves from the *Stevia rebaudiana* (bertoni) plant commercially cultivated in Japan, Singapore, Taiwan, South Korea, China, Israel, India, Brazil, Australia, and Paraguay [22]; Ru is the main component of the leaves of *Rubus suavissimus* S. Lee (Rosaceae) which is widely grown in southwestern China and used for Chinese sweet tea. In addition to its use as a sweetener, Chinese sweet leaf has also been used to treat various diseases such as hypertension, diabetes, atherosclerosis, maintaining healthy kidneys, and relieving coughs [23]. Recently, it has been discovered that Ru showed enhanced solubility of curcumin [24], etoposide A [25], liquiritin and teniposide [26], and paclitaxel [27].

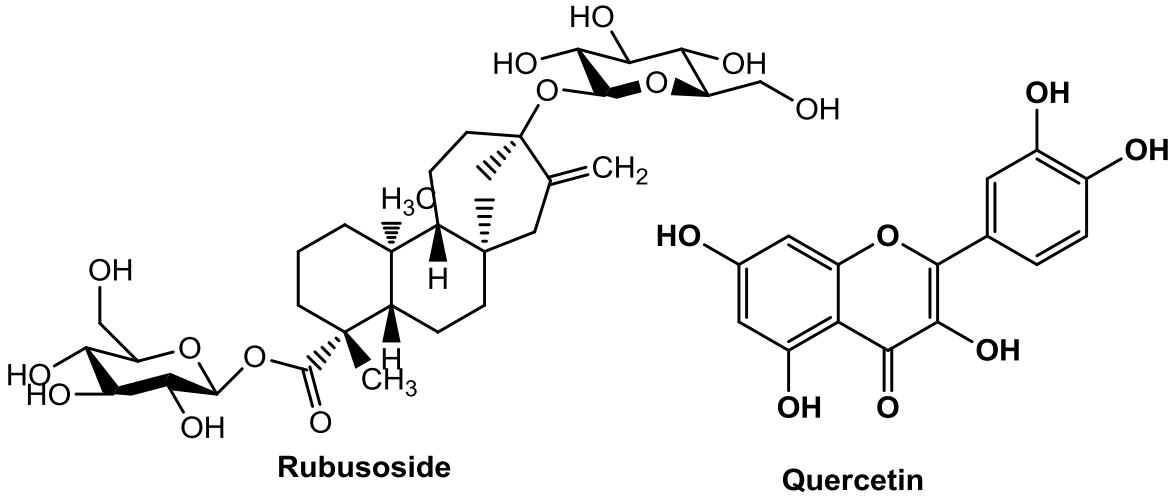

Figure 1. Chemical structures of quercetin and rubusoside

In this study, we found that quercetin solubility was improved in Ru and Reb. The biological properties of quercetin in Ru (Q-Ru), such as antioxidant activity, inhibition activities against $3CL^{pro}$ of SARS, human intestinal maltase and tyrosinase were studied. We discovered that they were comparable with those of quercetin solubilized in dimethyl-sulfoxide (DMSO).

MATERIALS AND METHODS:

Preparation of quercetin in steviol glucoside

Quercetin and Reb were purchased from Sigma, and Ru was prepared as reported earlier [26]. Q-Ru and quercetin-rebaudioside (Q-Reb) were prepared as reported previously [26], with slight modification. Solubility experiment was carried out as follows: 100 mg of Ru or Reb, were mixed with 10 mg of quercetin. In a tightly sealed tube, 1 ml of absolute ethanol was added to each mixture, and then vortexed for 15 min to form a clear ethanol solution. The mixture solution was transferred to an eppendorf tube, and centrifuged at 12,000 rpm for 10 min; the resultant supernatant was transferred to fresh tube. The ethanol was evaporated and the resulting powder was dissolved in 1 ml water. It was then centrifuged at 12,000 rpm for 10 min and filtered through a 0.20 µm membrane (Agilent, Santa Clara, CA, USA). Each compound in the filtrate was analyzed using thin layer chromatography with one ascent of acetonitrile/water 85:15 (v/v). Quercetin on TLC plate was visualized with UV 254 nm, and by dipping the TLC plate into a solvent mixture of 0.5 (w/v) N-(1-naphthy)ethylenediamine dihydrochloride and 5% (w/v)

sulfuric acid in methanol, followed by heating at 120^{o}C for 10 min or 1% ferric chloride in methanol [6].

The solubility test of quercetin with Ru or Reb was carried out as follows: 10 mg of quercetin was mixed with 0.5% (w/v), 1%, 2%, 3%, 4%, 5%, 7.5%, and 10% of each Ru or Reb. For the detection of quercetin in the mixture, 1% ferric chloride in methanol was used. The amount of quercetin solubilized in the Ru and Reb solution was calculated using the AlphaEaseFC 4.0 program (Alpha Inotech, San Leandro, CA, USA). The standard solution of each Ru and Reb in DMSO was prepared using concentrations ranging from 1 mg/ml to 60 mg/ml.

Antioxidant effect of quercetin solution

The antioxidant activities of quercetin and Q-Ru were evaluated by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging method as described previously [28]. Quercetin was dissolved in DMSO, or rather the Q-Ru and Ru were dissolved in water. Each solution was mixed with a 100 μM DPPH solution to give final concentrations ranging from 0.5 to 200 μM. After 30 min at room temperature in total darkness, the absorbance of each mixture was measured at 517 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). DPPH radical-scavenging activity (SC) was converted into percentage of antioxidant activity, as follows:

% SC = (Absorbance of control - Absorbance of test sample) × 100/Absorbance of control

A linear regression curve was established in order to determine the SC_{50} values, which is the amount of sample necessary to decrease the absorbance of DPPH by 50%. All the analyses were carried out in triplicate. A value of $p < 0.05$ was considered statistically significant.

Inhibition activity of quercetin solution against 3CL^{pro} of SARS

The method for the preparation and assay of SARS-3CL^{pro} activity was used in the same way as previous methods [8]. Inhibition activities of quercetin and Q-Ru were determined by measuring the remaining activity of 3CL^{pro} with 200 μM inhibitor, as previously reported [29]. Ru or Q-Ru was dissolved in water, and quercetin was dissolved in DMSO as a 10 mM stock solution. The enzyme reaction digest (100 μl) was composed of 3 μg enzyme, 16 μM FRET substrate, and test compound (25 to 200 μM) in 20 mM Tris buffer (pH 7.5). Reactions were run for 20 min at 25^{o}C, with continuous monitoring of fluorescence with a SpectraMax Gemini XPS (Molecular Devices, Sunnyvale, CA, USA) at excitation and fluorescence emission wavelengths of 355 nm and 538 nm, respectively. The inhibition was calculated using the following formula (a):

$$\% \text{ inhibition} = 100 - \text{remaining activity } (\%), \text{ where}$$
$$\text{remaining activity } (\%) = [(S - S_o)/ (C - C_o)] \times 100$$

C is the fluorescence of the control (enzyme, buffer, and substrate). After 18 min incubation, C_o is the fluorescence of the control at time zero. After 20 min incubation, S is the fluorescence of the test sample (enzyme, test sample solution, buffer and substrate). And S_o is the fluorescence of the test sample at time zero.

The 50% inhibitory concentration (IC_{50}) was defined as the concentration of $3CL^{pro}$ inhibitor necessary to reduce $3CL^{pro}$ activity by 50%, relative to a reaction mixture containing $3CL^{pro}$ enzyme without inhibitor.

Inhibition activity of quercetin solution against human intestinal maltase

The preparation and activity assay of human intestinal maltase (HMA) were conducted as reported previously [30]. The inhibition activity of quercetin, Ru and Q-Ru were determined by measuring the activity of HMA which remains at 100 μM Ru or Q-Ru. Ru and Q-Ru were dissolved in water, and quercetin was dissolved in DMSO, as 10 mM stock solutions. The reaction mixture composed of 0.04 U of enzyme, 5 mM maltose, test compound at different concentrations ranging from 0.001 mM to 1 mM in 50 mM potassium phosphate buffer (pH 6.5), maintained at 37^{o}C for 30 min. The control reaction was carried out by following the same inhibition assay protocol, except that buffer was used instead of the test compound. The reaction was stopped by adding 0.2 ml of 2M Tris-Cl (pH 8.0). Enzymatic activity was measured by the glucose oxidase-peroxidase (GOP) method using a Glucose-E kit (BMI, Sungnam, Korea). The 50% inhibition concentration (IC_{50}) was defined as the concentration of HMA inhibitor necessary to reduce HMA activity by 50%, relative to a reaction mixture containing HMA enzyme without inhibitor.

Inhibition activity of quercetin solution against mushroom tyrosinase

The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma.

The reaction mixture contained 3.3 mM L-DOPA (L-3,4-dihydroxypheylalanine) in 50 mM potassium phosphate buffer (pH 6.8), and 10 U mushroom tyrosinase/ml with or without Q or Q-Ru (0.001 to 1 mM). The reaction was run for 15 min using 96-well plate, and monitored using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 475 nm. The inhibition was calculated using following formula (b):

$$\% \text{ inhibition} = 100 - \text{remaining activity } (\%), \text{ where}$$
$$\text{remaining activity } (\%) = [(S - S_o)/ (C - C_o)] \times 100$$

C is the absorbance of the control (enzyme, buffer, and substrate) after 15 min incubation, C_o is the absorbance of the control at time zero, S is the absorbance of the test sample (enzyme, inhibitor, buffer and substrate) after 15 min incubation, and S_o is the absorbance of the inhibitor at time zero.

The 50% inhibition concentration (IC_{50}) was defined as the concentration of inhibitor necessary to reduce the mushroom tyrosinase activity by 50%, relative to a reaction mixture containing mushroom tyrosinase without inhibitor.

RESULTS AND DISCUSSION:

Solubility of quercetin in rubusoside or rebaudioside

Quercetin (Figure 1) is a phytochemical belonging to the flavonol subgroup, and is the most ubiquitous dietary flavonoid. Apples and onions are primary sources in the Western diet. Other foods containing quercetin include citrus fruits, berries, red grapes, red wine, broccoli, bark roots, flowers and tea [31]. Recently, quercetin has drawn attention for its remarkable scope of health

benefits, which make quercetin a leading compound for developing new and effective functional foods or medicines [14, 32]. However, orally administered quercetin is poorly absorbed [13, 14]. Many techniques have been used to improve quercetin solubility in water, such as solid lipid nanoparticle, poly-D,L-lactide nanoparticle, β-cyclodextrin [16-18], and biosynthesis of quercetin-4′-*O*-α-D-glucopyranoside by using dextransucrase [6], or quercetin-3-*O*-glucoside by using naringinase [33]. Previous studies have reported the solubility of quercetin is a crucial factor for its bioavailability. For example, quercetin dissolved in DMSO/polyethylene glycol 200 has up to 1.3 fold bioavailability in rats compared with that suspended in 0.7% CMC solution [34]. Bioavailability of quercetin was increased 5.7 fold by administration using a solid lipid nanoparticle as an oral delivery carrier, compared with those administered as a quercetin suspension [16]. In this study, quercetin was shown to be soluble in Reb and Ru solutions (Figure 2). The solubility of quercetin was detected under UV light and with ferric chloride staining detecting the phenol ring (Figure 2, lane 2, 3).

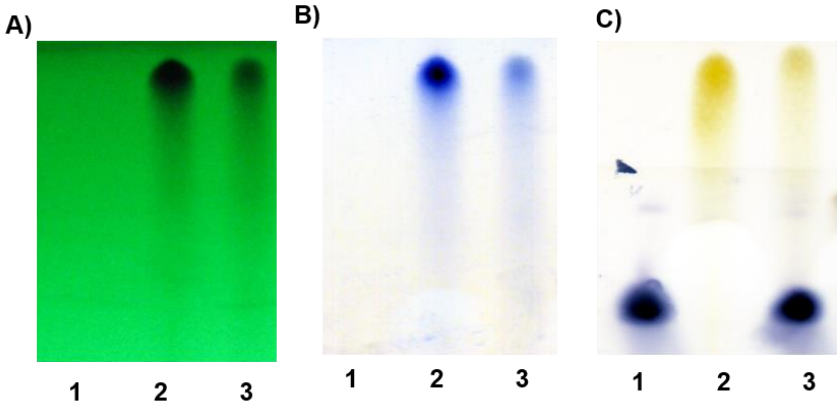

Figure 2. TLC analyses of solubilization of quercetin in water reconstituted from Q-Ru, under UV light at 254nm (A), ferric chloride staining (B), sulfuric acid in methanol (C). Lane 1: Ru; lane 2: 10 mg/ml of quercetin in DMSO; lane 3: 6 mg/ml of quercetin in Ru.

The quercetin solubility increased when the Reb or Ru concentrations increased. Quercetin was dissolved up to 0.6 mg/ml in the presence of 5% (w/v) Reb (Table 1). As Reb increased to 7.5% and 10% (w/v), the amount of quercetin in water also increased to 0.9 mg/ml and 1.5 mg/ml, respectively (Table 1). A linear relationship was found between the concentrations of quercetin and Reb, which was Y = 0.18 x A - 0.37 [where Y (quercetin concentration, mg/ml) and A (Reb concentration, mg/ml); R^2 = 0.95]. Thus, every mg/ml Reb would result in 0.18 mg/ml additional solubility increase of quercetin in water. In the presence of 2% (w/v) of Ru, quercetin was solubilized to 1.1 mg/ml (Table 1). As Ru increased to 2%, 3%, 4%, 5%, 7.5% and 10% (w/v), soluble quercetin in water also increased to 1.1, 1.9, 2.6, 3.8, 7.0 and 7.7 mg/ml, respectively (Table 1). A linear relationship was found between the concentrations of quercetin and Ru, which was Y = 0.83 x B - 0.58 [where Y (quercetin concentration in mg/ml) and B (Ru concentration in mg/ml); R^2 = 0.98]. As a result, every mg/ml Ru would result in 0.83 mg/ml additional solubility increase of quercetin in water. Because quercetin showed stronger solubility

in Ru than Reb, Q-Ru was selected for further studies of the biological properties of quercetin, and compared with those of quercetin in DMSO.

Table 1. The solubility of quercetin at different concentrations of rebaudioside and rubusoside

Rebaudoside or Rubusoside added (%)	Rebaudioside	Rubusoside
	Quercetin solubilized in solution (mg/ml)	
0.5	ND	ND
1.0	ND	ND
2.0	ND	1.1 ± 0.4
3.0	ND	1.9 ± 0.3
4.0	ND	2.6 ± 0.3
5.0	0.6 ± 0.1	3. 8 ± 0.5
7.5	0.9 ± 0.1	7.0 ± 0.9
10	1.5 ± 0.1	7.7 ± 0.5

10 mg/ml quercetin was used. ND: Not detected in solution

Antioxidant activity of quercetin in solution

For DPPH radical scavenging activity, quercetin or Q-Ru were prepared and tested at concentrations ranging from 0.5 to 200 µM. The SC_{50} of quercetin solubilized in DMSO (37.4 µM) was similar with that of quercetin in Ru solution (37.3 µM) (Table 2, Figure 3). This result indicates that the solubilization of quercetin in Ru does not reduce its antioxidant activity. Therefore, when considering both antioxidant activity and solubility, Q-Ru would be better than quercetin-4′-*O*-α-D-glucopyranoside synthesis, since it has solubility in water of 3.9 mg/ml but its SC_{50} was 3.9 times higher than SC_{50} of quercetin in DMSO [6].

Table 2. Biological properties of quercetin solubilized in DMSO and in rubusoside

Biological properties	**Rubusoside in water**	**Quercetin solubilized in**	
		DMSO	**Rubusoside solution**
DPPH radical-scavenging activity [SC_{50} (µM)]	-	37.4 ± 1.4	37.3 ± 1.3
$3CL^{pro}$ of SARS inhibition activity [IC_{50} (µM)]	12.6×10^3	89.7 ± 1.4	75.4 ± 1.1
HMA inhibition activity [IC_{50} (µM)]	530 ± 16	74.0 ± 1.5	38.5 ± 0.5
Mushroom tyrosinase inhibition activity [IC_{50} (µM)]	-	78.9 ± 1.7	78.6 ± 3.0

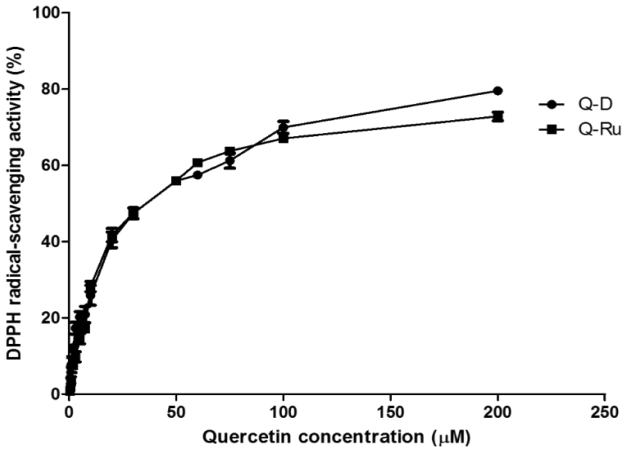

Figure 3. DPPH radical scavenging activities of quercetin solubilized in DMSO and of quercetin solubilized in rubusoside solution. Quercetin in DMSO (●), Quercetin in rubusoside (■). Each sample (0.50 ~ 200 μM) was mixed with a 100 μM DPPH, and kept in dark at room temperature for 30 min. The absorbance was monitored at 517 nm. Each value is mean ± SEM (n=3).

Inhibitory effect of quercetin against 3CL^{pro} activity of SARS coronavirus

The 3C-like protease (3CL^{pro}) of SARS-CoV is one of the most promising targets for discovery of drugs against SARS, because of its critical role in the life cycle of the virus [29, 35]. Quercetin is known to be active against herpes simplex, adeno-, respiratory syncytial, Rous sarcoma, pseudorabies, and parainfluenza viruses [36, 37], and the SARS coronavirus [8, 38], due to its mechanism which inhibits replication. The inhibitory activity of Q-Ru was compared with DMSO-solubilized quercetin using Dabcyl-KTSAVLQSGFRKME-Edans fluorogenic peptide as a substrate. Ru showed IC_{50} of 12.6 x 10^3 μM against 3CL^{pro} (Table 2, Figure 4). The IC_{50} of quercetin in DMSO and Ru solution was 89.7 μM and 75.4 μM, respectively (Table 2). Therefore, the combined inhibitory effect of quercetin with Ru in water increased 1.2 fold against the 3CL^{pro} activity.

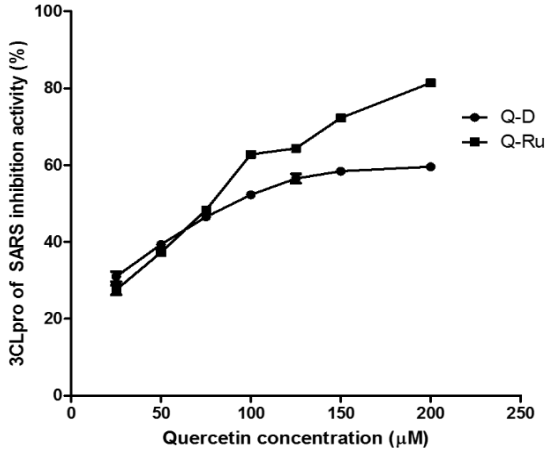

Figure 4. The inhibitory activity of quercetin solubilized in DMSO (Q-D) and quercetin solubilized in rubusoside (Q-Ru) solution against 3CL^{pro} of SARS. The reaction mixture was composed of 3 μg enzyme, 16 μM FRET substrate, each test compound (25 ~ 200μM) in 20 mM Tris buffer (pH 7.5). Reactions were run for 20 min at 25^{o}C, with continuous monitoring of fluorescence using a SpectraMax Gemini XPS apparatus, at excitation and fluorescence emission wavelengths of 355 nm and 538 nm, respectively.

Inhibitory effect of quercetin in Ru solution against human intestinal maltase

HMA hydrolyzes linear α-1,4-linked oligosaccharide substrates, plays a crucial role in the production of glucose in the human lumen, and acts as an efficient drug target for type 2 diabetes and obesity [39, 40]. The HMA inhibitory activity of Ru, quercetin in DMSO, and Q-Ru at different concentrations (0.001 to 1 mM) were investigated (Figure 5). Q-Ru showed higher HMA inhibition than that of quercetin solubilized in DMSO. Ru also showed inhibition against HMA activity with IC_{50} of 530 μM. Quercetin in DMSO and Ru showed inhibition of HMA activity with IC_{50} of 74.0 μM and 38.5 μM (Table 2, Figure 5), respectively. As a result, the quercetin in Ru increased the HMA inhibition by 1.9 folds.

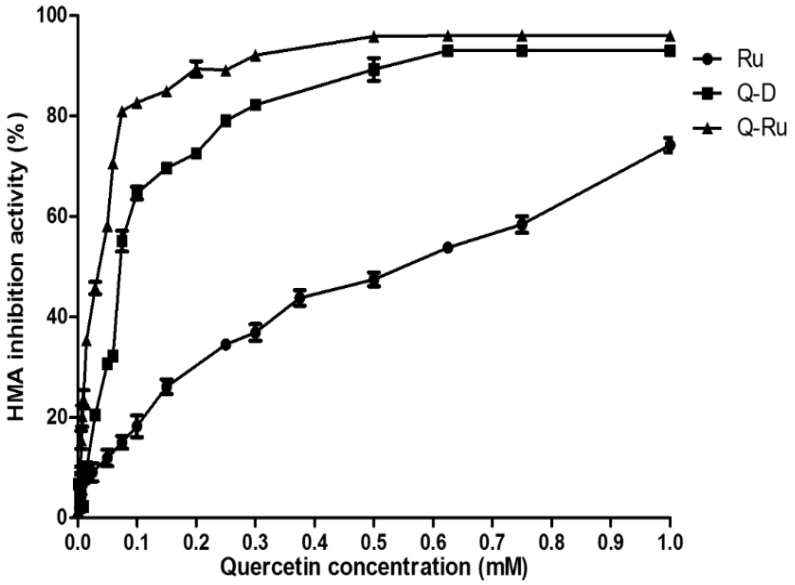

Figure 5. The inhibitory activities of rubusoside (Ru), quercetin solubilized in DMSO (Q-D) and quercetin solubilized in rubusoside (Q-Ru) solution against HMA. The reaction mixture composed of 0.04 U of enzyme, 5 mM maltose, each test compound (0.001 mM ~ 1 mM) in 50 mM potassium phosphate buffer (pH 6.5), and was kept at 37^{o}C for 30 min. Each value is mean ± SEM (n=3).

Inhibitory effect of quercetin in Ru solution against mushroom tyrosinase

Tyrosinase (monophenol, dihydroxyl-L-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing enzyme, which is widely distributed in microorganisms, animals and plants. It is a key enzyme in melanin biosynthesis, and plays a crucial role in determining the color of mammalian skin and hair [41]. During melanoma, the uncontrolled tyrosinase activity results in increased melanin synthesis [42]. The inhibition of mushroom tyrosinase was evaluated using different concentrations of quercetin in DMSO and Q-Ru (0.001 to 1.0 mM). The inhibition of mushroom tyrosinase by quercetin in DMSO and Q-Ru is shown in Figure 6. Q-Ru demonstrated similar tyrosinase inhibition activity as quercetin solubilized in DMSO, with an IC_{50} of 78.6 and 78.9 μM, respectively (Table 2, Figure 6).

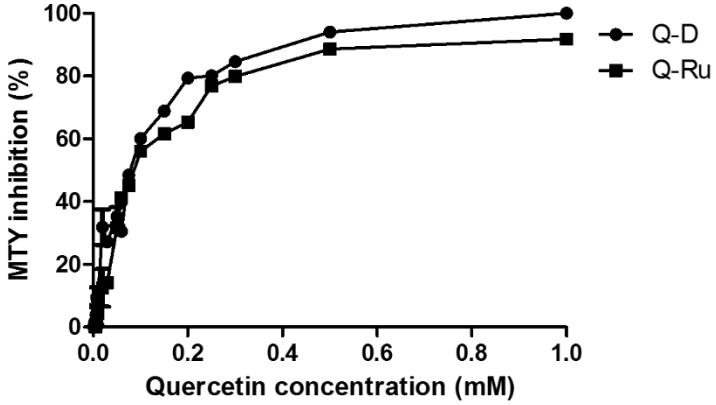

Figure 6. The inhibitory activity of quercetin solubilized in DMSO (Q-D) and of quercetin solubilized in rubusoside (Q-Ru) solution against mushroom tyrosinase. The reaction mixture contained 3.3 mM L-DOPA (L-3,4-dihydroxypheylalanine), 10 U/ml mushroom tyrosinase, different concentration of each test compound (1.0 ~ 1000 μM) in 50 mM potassium phosphate buffer (pH 6.8), with or without quercetin and Q-Ru; reaction was for 15 min, and the absorbance was monitored at 475 nm. Each value is mean ± SEM (n=3).

CONCLUSIONS:

The present study demonstrated that quercetin can be solubilized in water with rebaudioside or rubusoside treatment. As Ru concentration increases, the solubility of quercetin in water increases. Interestingly, the solubilization of quercetin in Ru solution did not reduce its biological functions such as the DPPH radical-scavenging and mushroom tyrosinase activity. Additionally, Q-Ru increased the inhibition activity against the $3CL^{pro}$ of SARS and human intestinal maltase, when compared with the activity of quercetin in DMSO. Therefore, Ru and rebaudioside are promising compounds which enhance the solubility of poorly water soluble compounds (including quercetin, rutin and astragallin). Studies pertaining to the biological functions of the solubilized quercetin in Ru and Reb are currently in progress.

Competing Interests:

The authors have no financial interests or conflicts of interest.

Authors' Contributions:

All authors contributed to this study. D.K. and J.P. designed experiments. T.T.H.N., S.Y., J.K., E.A. and K.H. performed experiments. D.K., T.T.H.N. and J.P. wrote the manuscript.

Abbreviation:

Q-Ru, quercetin solubilized in rubusoside; DMSO, dimethyl-sulfoxide; DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; SARS-CoV, severe acute respiratory syndrome-associated coronavirus; $3CL^{pro}$, 3C like protease; Reb, rebaudioside A; Ste, stevioside, Ru, rubusoside; SC, DPPH radical-scavenging activity; FRET, fluorescence resonance energy transfer; HMA, human intestinal maltase; GOP, glucose oxidase-peroxidase; L-DOPA, L-3,4-dihydroxypheylalanine; ND, not detected in solution.

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