CHAPTER II

Relation between Macroscopic Binding Constant and the Anticancer Efficacy of the BSA-Quercetin Derivatives Against Drug-Sensitive and Drug-Resistant Cells

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Relation Between Macroscopic Binding Constant and the Anticancer Efficacy of the BSA-Quercetin Derivatives Against Drug-Sensitive and Drug-Resistant Cells

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Abstract

This study aimed to investigate the role of BSA on increasing the solubility and suitable carrier of quercetin derivatives related to the anticancer efficacy against drug-sensitive and drug-resistant cells. The macroscopic (K_D) and microscopic (k_d) binding constant of the complexation and the cellular partition of molecules were analyzed using FRET and HPLC method, respectively. The K_D values reflex the stability of complexes was in the order of rutin > quercetrin > quercetin. BSA is a suitable carrier of quercetin ($K_D = 1.68 \times 10^5\ M^{-2}$) which spontaneously release the molecule into solutions and cells. The substitution of rhamnoside ($K_D = 1.37 \times 10^5\ M^{-2}$) and rutinoside ($K_D = 5.0 \times 10^4\ M^{-2}$) at C3 yielded an increase in stability of the complexes. Rutin was tiltly bound to BSA resulting in the changes in mode of action, probably mediated its cytotoxic via an interaction with the extrinsic pathway, activated by pro-apoptotic receptor signals at the cellular surface.

Key words: Serum albumin, Fluorescence resonance energy transfer (FRET), quercetin derivatives, multidrug resistance phenomenon, chemotherapy

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Introduction

Quercetin and its glycoside derivatives present great interest as potential anticancer and anticarcinogenic molecules are increasingly receiving interests due to their a variety of pharmacological activities including anti-inflammatory (1-2), cardioprotective (3-8) and cancer apoptosis induction activities (9-14). Despite the greate interest in these compounds over the recent years, there is little data dealing with the physicochemical properties of molecule. In addition, the molecular based mechanisms of anticancer and its limiting step remains to be elucidate. Recently, Tungjai *et al.* reported the speciation of the molecule in physiological like solutions and determined the log P value as well as the mean rate passive diffusion through the lipid bilayer membrane (15). These findings suggested that quercetin was passively diffused throughout the plasma membrane into the cytosolic site and the avialable cytosolic concentration of quercetin should be responsible for its specific anticancer action.

In fact these compounds are poorly soluble in water which limited their use in preclinical studies. It was known that lipids and proteins can considerable increase in the solubility of various drugs (16-18). Many research groups have studied the *in vivo* consequences of binding of drugs and their metabolites to serum albumins (19-20). These researchers have examined the binding mechanisms of ligands with serum albumin by using absorption (21), fluorescence and voltammetric techniques (22-25). Based on these studies, the information on the binding processes of many exogenous ligands like long chain fatty acids, amino acids, metals, drugs, bilirubin etc. have been reported at the molecular level. It has also been considered that such binding can increase the solubility of ligands and thus increase its cytotoxicity, particular ligands like bilirubin decreased on bonding to albumins (16-20).

Serum albumins are abundantly found in blood plasma, circulated in the body several times and act as carriers for numerous exogenous and endogenous compounds (26-27). The most popularly studied albumins are bovine serum albumin (BSA) and human serum albumin (HSA). In general, albumins are characterized by low tryptophan and high cystine contents. BSA and HSA mainly differ in tryptophan content. Except for this, the amino acid compositions of the two proteins are nearly the same. HSA has one tryptophan group at the 214 position, while BSA has two tryptophan groups at 134 and 212 position. Primarily, three domains and six principal binding sites have been identified for several important biomolecules. Both BSA and HSA have very high conformational adaptability to a great variety of ligands. HSA has one tryptophan group at the 214 position, while BSA has two tryptophan groups at 134 and 212 position (28-29).

The two serum albumin (HSA and BSA) have been shown to strongly bind quercetin and other structurally related flavonoids *in vitro* (22-25). Dangles et al. quantitatively measured the the binding constant (K_D) of quercetin and its glycoside derivatives to BSA by using fluorescence spectroscopy, the determined K_D values was equal to 8.6 ($\pm$ 1.1) x 10^5 M^{-1} and 14.5 ($\pm$ 2.8) x 10^5 M^{-1} for rutin and isoquercetrin, respectively (23, 30). However, the correlation among the K_D values, cellular partition and the anticancer activity of flavonoids has not been studied elswhere.

In this study we have applied the FRET analyzed the complexation of BSA and quercetin derivatives. The macroscopic dissociation rate constant (K_D) reflexs the stability of complexes was in the order rutin > quercetrin > quercetin. BSA can serve as a carrier bywhich spontaneously release quercetin into solutions and cells. The substitution of rhamnoside and rutinoside at C3 yielded an increase in stability of the complexes. Rutin was tiltly bound to BSA resulting in the changes in mode of action, probaly mediated its cytotoxicic via an interaction with the extrinsic pathway, activated by pro-apoptotic receptor signals at the cellular surface.

A preliminary report of this work has been presented elsewhere (33).

Materials and methods

Quercetin (3,5,7,3′,4′-Pentahydroxyflavone), quercetin-3-rhamnoside or quercetrin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl] oxychromen-4-one) and quercetin-3-rutinoside or rutin (3-[[6-O-(6-Deoxy-alpha-l-mannopyranosyl)-beta-d-glucopyranosyl]oxy]-2-(3,4-dihydroxy-phenyl)-5,7-dihydroxy-4H-1-benzo-pyran-4-one) were purchased from Sigma. BSA-bovine fraction V (biotechnology grad) was from Amresco.

Pirarubicin stock solutions were prepared in water just before used. Concentrations were spectrophotometrically determined by diluting stock solutions in water to approximately 10 μM and using ε_{480} = 11500 $M^{-1}cm^{-1}$.

HEPES-Na^+ buffer consists of 20 mM HEPES buffer plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$ and 1.5 mM $MgCl_2$, pH 7.25 at 37 °C.

The absorption spectra were recorded on a Hewlett Packard HP 8435 spectrophotometer. Experiments were conducted in a 1-cm quartz cuvette containing 2 ml of solution under continuous stirring. The temperature was controlled at 37 °C using a peltier temperature control, cell holder model 89090A.

Cell lines, cell culture and cytotoxicity assay

The human erythromyelogenous leukemia cell line (K562) and its DOX-resistant, P-glycoprotein-overexpressing K562/adr subline, (10, 15, 31-32) and the human small cell lung carcinoma cell line (GLC4) and its DOX-resistant, MRP1-overexpressing GLC4/adr subline (10, 15) were routinely cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum in a humidified atmosphere with 5% CO_2 at 37 °C. For the cytotoxicity assays, cells were plated at a density of 5×10^5 cell.mL^{-1} and used 24 h later (8×10^5 cell . mL^{-1}) so that cells were in exponential growth phase. Cell viability was assessed by Trypan blue exclusion, and cell number was determined with a haemocytometer.

The cytotoxicity assays were performed as follows: cells (5×10^4 mL^{-1}) were incubated in the presence of various concentrations of drugs. The number of cells was then determined by flow cytometry. The concentration of drug required to inhibit cell growth by 50% after 72 h (IC_{50}) was determined by plotting the percentage of cell growth inhibition versus the drug concentration. The resistance factor (RF) was defined as the IC_{50} of resistant cells divided by the IC_{50} of the corresponding sensitive cells.(10-11, 31)

Cellular partition of compounds: cells (4×10^6 mL^{-1}) were incubated in the presence

of 40 μM of quercetin, quercetrin or rutin in in RPMI 1640 supplemented with 10% (v/v) fetal calf serum or HEPES-Na^+ buffered solution without or with 9 mg/mL BSA pH 7.3 at 37°C. The supernatant and cell pellets were separated at 1, 3 and 6 hours after incubation.

Sample preparation: Supernatant and pellets of cells were extracted twice with 5 mL of DMSO–Acetonitrile (1:4 v/v) then centrifuged (NÜVE NF400, Turkey) at 4,400×g for 10 minutes. The acetonitrile fraction was collected and pooled together then dried using rotary evaporator and stored until HPLC analysis.

High performance liquid chromatography (HPLC): The samples were analyzed using A SPD-M20A High Performance Liquid Chromatography PDA detector (Shimadzu).

Spectrofluorometric measurements

Albumin of stock solution 1 mM was dissolved in a HEPES-Na^+ buffer pH 4 and 7.3 at 37°C. Steady-state fluorescence was recorded using a Perkin Elmer LS 50B spectrofluorometer. All studies were performed at 37 °C using 2.5 nm and 5 nm excitation and emission slit widths. The emission signal of BSA was collected from 310 to 580 nm (excited at 310 nm). Both of excitation-and emission-spectra of complexes were recorded at equilibrium state, a few minutes after addition the precised concentrations of flavonoids into BSA solution; the emission signal was collected from 460 to 580 nm (excited at 310 nm).

Results

1. Cellular uptake and metabolism of quercetin and its glycoside derivatives

Due to our long experiences on the cytotoxicty studied of flavonoids, it was observed that after adding varied concentration of quercetin ranging from 0.5 to 300 μM quercetrin and rutin into the HEPES-Na^+ buffered solution pH 7.3 at 37°C, then the solutions were visaulized under light microscope. At the concentration ≥ 100 μM, we observed crystalins homogeneously distributed through out the bottom of well while the same did not observe in the presence of 9 mg/mL BSA. We also observed that the presence of quercetin and its glycoside derivatives in the 24 well-plate contained culture medium and cells appeared yellow-green color. The supernatant of cells was collected and spectrophotmetrically identified. The supernatants possesed an absorption spectrum of between 320 nm to 500 nmwith the maximum absorbance at 410 nm. This absoption spectrum does not correspond to those of querctin, quertrin and rutin in 20 mM HEPES-Na^+ buffer, pH 7.25 at 37 °C. In fact, this differenc was caused by the presence of plasma proteins since the supernats were separated using 10% acrilamide gel electrophoresis and read by using FRET clearly demonstrated a band corresponding the BSA-flavonoid complex.

The uptake of the three molecules by K562, K562/adr, GLC4 and GLC4/adr cell were thus performed both the RPMI 1640 culture meidum completed with 10% fetal calf serum and 1% penicilin-streptomycin (exactly the same conditions with the cytotoxicity assay) and in HEPES-Na^+ buffer sotion.

The typical results of quercetin partitionned into the cell pellets as a function of time was shown Figure 1. As can be seen in in Figure 1a, the pattern of the amount of quercetin partitionned into the drug-sensitive cell was similar to

those of drug-resistant cell pellets. The cell-quercetin increased with incremental incubation time in RPMI 1640 medium. The cell-quercetin concentration was 15 ± 7 nmole (per 4 x10^6 cells) at 6 hours after incubation in the four cell lines. It should be noted that an increase in quercetin concentration in cell pellets as a function of incubation time was found in drug sensitive cells while an increase following a decrease quercetin content was clearly demonstrated in both drug resistant cells (Figure 1b).

The results of quercetrin partitionned into the cell pellets as a function of time was shown Figure 1c and d. Quercetrin was slightly partitonned into the cells susspended in RPMI 1640 medium while significaty increased in those cells suspended in HEPES-Na^+ buffered solution. The similar results were obtained with rutin (Figure 1e and f). It should be noted that both quercetrin and rutin were rapidly partitioned into the cells in both of cell suspended in RPMI 1640 and HEPES-Na^+ buffered solution. The concentration of quercetrin and rutin measured in cell pellets of

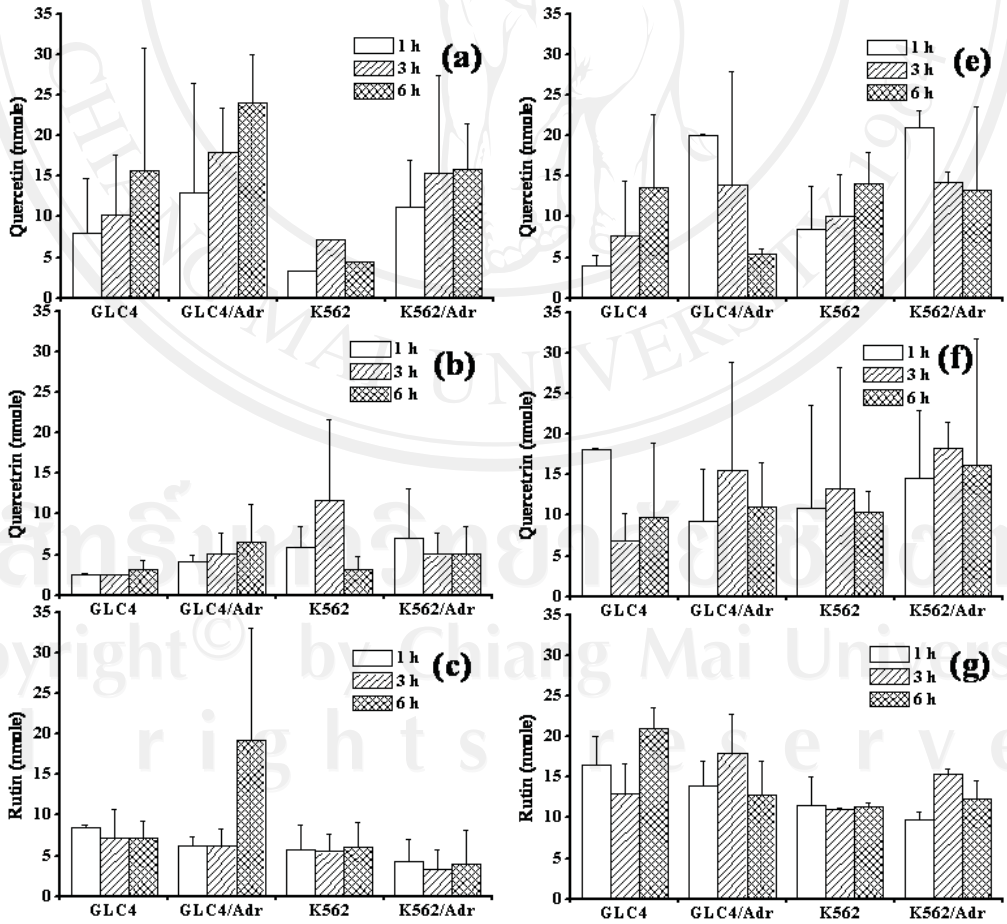

Figure 1. The partition of quercetin, quercetrin and rutin into K562, K562/adr, GLC4 and GLC4/adr cells at the indicated incubation time. Cells (4 × 106 mL-1) were

suspended in 4 mL RPMI 1640 supplemented with 10% (v/v) fetal calf serum or HEPES-Na+ buffered solution without BSA pH 7.3 at 37°C. The final concentration of molecules was 40 mM. The supernatant and cell pellets were separated at 1, 3 and 6 hours after incubation. Data were mean ± SD, n = 3.

HEPES-Na^+ was 12 ± 5 nmole (per 4 $x10^6$ cells) medium, about 2.5 time higher degree than in the RPMI 1640 medium. It should be also noted that these molecules were found intact and stable in both extracellular and intracellular compartments during the experimental time period of study (at least 6 hours).

2. Cytotoxicity of quercetin, quercetrin and rutin

Quercetin similarly exhibited anticancer activity against K562, GLC4 and their corresponding multidrug resistant cells with IC_{50} values varied from 20 μM to 26 μM (Table I). The rhamnoside (quercetrin) and rutinoside (rutin) substituted molecule mediated higher efficacy than its parent compound in both K562 and GLC4 cells.

Table I. IC_{50} of quercetin and its glycoside derivatives against K562 and GLC4 cells. RF is resistance factor defined as the IC_{50} of drug-resistant cell devided by that of its corresponding drug-sensitive cells.

Compounds	IC_{50}(K562), mM	RF	IC_{50}(GLC4), mM	RF
Quercetin	**23.0 ± 3.0**	**1.0**	**18.0 ± 8.5**	**1.0**
Quercetrin	**11.0**	**1.9**	**12.0 ± 2.5**	**4.8**
Rutin	**3.0 ± 0.2**	**11.6**	**4.0**	**14.8**

Particularly, the rutinoside substitution exhibits 5-fold anticancer activity higher than quercetin. Contrary these substituted molecules caused lower sensitivity in multidrug resistant cells. As can be seen when H-atom at C3 of ring C of quercetin was substituted by rutinoside the RF values were increased from 1 to 12 in K562/adr cells and from 1 to 15.

3. Serum albumin protein is potential carrier and spontaneously and constantly releases flavonoids

The previous results indicated that BSA should act as binder of flavonoids thus influenced on their uptake by cells. We thus applied the spectrophotometric and FRET measurments for determining the microscopic and macroscopic rate constant of the complexation of

BSA-molecules in our experimental conditions .

3.1 Spectroscopic properties of BSA, quercetin, quercetrin and rutin

BSA is completely dissolved in HEPES-Na^+ buffered solution pH 4 at 25 °C upto 10^{-2} M. It possesses an emission spectrum with maximum fluorescence intensity at 340 nm when excited at 310 nm (Figure 2a). However, the width at half height of the emission spectral shape become smaller and the maximum fluorescence intensity was 1.7 fold increase and this wavelength was shifted from 340 nm to 350 nm when the measurement was performed in the solution pH 7.3 (Figure 2b).

The absorption spectra of quercetin in HEPES-Na^+ buffered solution pH 4 and 7.3 at 37 °C were shown in Figure 1a and b. In HEPES-Na^+ buffered solution pH 4, quercetin possessed an absorption spectrum between 250 nm to 500 nm composed of two absorption bands which has maximum absorbance at 274 nm and 370 nm, respectively. It should be noted that the absorption spectral shape was changed when the measurement were performed in HEPES-Na^+ buffered solution pH 7.3; the width at half height of the peak became larger, the absorbance at 370 nm decreased and the maximum absorbance was shifted from at 370 nm to 390 nm (Figure 2a and b). These signified that quercetin was ionized and should be presented in neutral and positive charge form (see Tungjai et al. 2008). Similar results were obtained for quercetrin and rutin (Figure 3).

3.2 Binding studies of quercetin, quercetrin and rutin with BSA

The binding of quercetin and its glycoside to BSA can be easily analysed by using fluorescence resonance energy transfer (FRET). The overlay of an emission spectrum of albumin and an absorption spectrum of quercetin was indicated in Figure 2 a and b. It is clearly demonstrated that BSA and quercetin can be served as donnor and acceptor, respectively. BSA is excited at 310 nm, by a long-range dipole-dipole coupling mechanism. This excited state is then nonradiatively transferred to quercetin or its derivative molecules. Thus BSA returns to the electronic ground state. The yield of FRET depends on two physical parameters including (1) the spectral overlab integral ($J_{(\lambda)}$) between BSA emission spectrum and quercetin absorption spectrum and (2) the critical transfer distance (R_0).

The typical results of the spectral overlap integral ($J_{(\lambda)}$) between the BSA emission spectrum and the quercetin absorption spectrum of the solution pH 4 and 7.3 were indicated in Figure 3a and b, respectively. The $J_{(\lambda)}$ can be calculated by using equation (1);

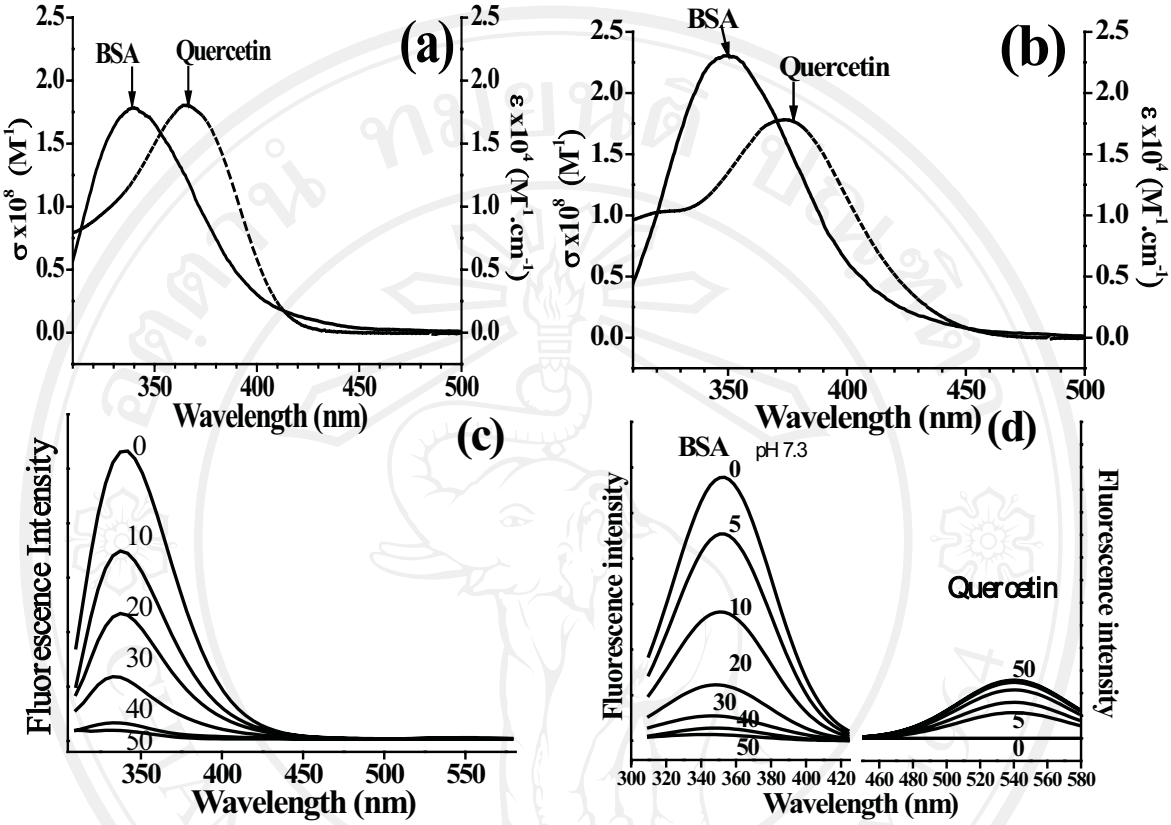

Figure 2. The emission spectra of BSA and absorption spectra of quercetin measured in the HEPES-Na^+ buffered solution pH 4 (a and c) and pH 7.3 (b and d). BSA 3μM was dissolved in 2 mL HEPES-Na^+ buffered solution, pH 4 or 7.3 in 1-cm quartz cuvette placed in the sample holder thermostated control 37°C. Steady-state fluorescence was recorded at after adding the indicated concentration (μM) into the BSA solution at 37 °C using 10 nm and 10 nm or 2.5 nm and 5 nm excitation and emission slit widths, respectively.

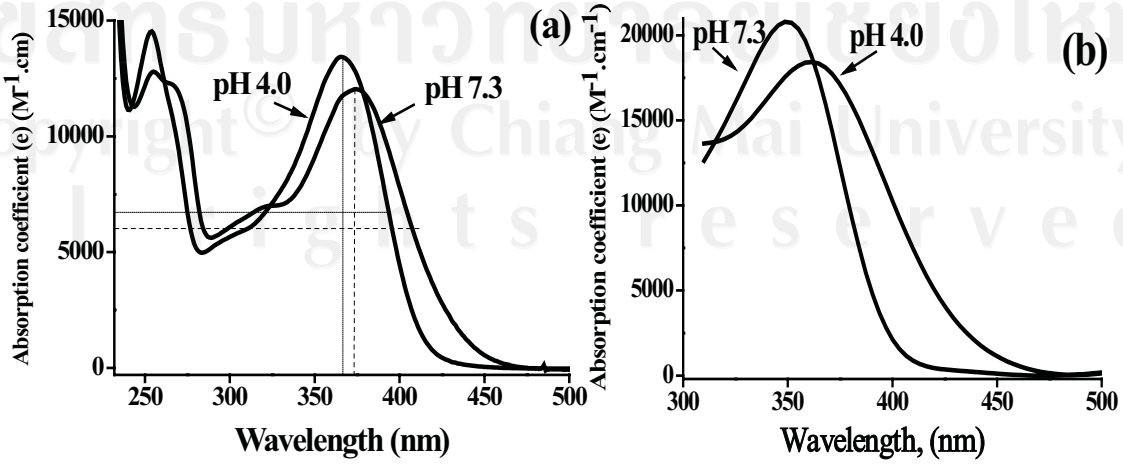

Figure 3. The absorption spectra of quercetrin (a) and rutin (b). BSA (3μM), quercetrin or rutin was dissolved in HEPES-Na+ buffered solution.

$$J(\lambda) = \frac{\int_0^{\infty} F_D \varepsilon_A \lambda^4 d\lambda}{\int_0^{\infty} F_D d\lambda} \qquad (1)$$

where $F_D(\lambda)$ and $\varepsilon(\lambda)$ are represent the non-linear fit curve of fluorescence intensity from pure BSA , and molar extinction coefficient of the quercetin respectively at the wavelength λ.

After integrating the equation 1; by varying the wave length (λ) between 310 nm to 580 nm, the J(λ) was determined equal to 1.49 x 10^{-14} $M^{-1}.cm^3$ in aqueous solution at pH 4, and 2.39 x 10^{-14} $M^{-1}.cm^3$ at pH 7.3.

Figure 2c and 2d showed the emission fluorescence spectra when excited at 310 nm recorded immediately after addition of varied concentrations of quercetin into the solution of BSA. All series of FRET experiments, the concentration of BSA was fixed to 3 and 5 μM while the concentrations of quercetin were varied from 0.5 to 50 μM. The complexation of BSA-quercetin can be clearly characterized by measuring the FRET fluorescence intensity at 540 nm (Figure 2d) or extinction fluorescence intensity at 350 nm (Figure 2c). As can seen in Figure 2c and 2d the FRET phenomenon can be measured only when the reactions were performed in the HEPES-Na^+ buffered solution pH 7.3 in the presence of 3 μM BSA. However, the extinction of BSA fluorescence intensity without appearance of FRET band was observed for the same reaction performed in HEPES-Na^+ buffered solution pH 4 but the FRET can be measured in these conditions of experiments when increased in concentration of BSA such as 5 μM. As can be seen in Figure 2d; in the buffered solution pH 7.3 contained 3 μM BSA, the FRET can easily be measured. In our conditions of experiments, the maximum of reaction product was obtained when 50 μM quercetin was used. These samples were used to estimate the FRET parameters between BSA and quercetin.

R_o is defined as critical transfer distance at which the transfer efficiency equals 50% or the fluorescence of donor is quenched by 50%. R_o is calculated by using the equation (2),

$$R_0 = 9790(\kappa^2 \phi_0 J n^{-4})^{\frac{1}{6}} \qquad (2)$$

where

κ^2 is the orientation factor between the emission of the dipole of the donor and the absorption dipole of the acceptor, which is generally 2/3 for isotropic donor and acceptor;

ϕ_0 is the quantum yield of the donor = 0.101 (29);

n is the refractive index of the medium (phosphate buffer pH 7.3, n = 1.32 , (29);

J is the spectral overlap integral between the donor emission spectrum and the acceptor absorption spectrum.

The R_o of BSA-quercetin system determined was equal to 55.38 Å at pH 4.0 and equal to 59.93 Å at pH 7.3.

Figure 2d also shows that the fluorescence intensity at 540 nm (excited at 310 nm) was immediately measured after addition of quercetin into the BSA solution. These signified that the complexation was immediately done and

cannot be measured the kinetics of reaction by using our technique. The fluorescence intensity at 540 nm was increased then reached a pseudo plateau when the concentration of quercetin increased (Figure 4). The data were fitted using Hill's equation yielding the Hill's number closed to 2 as indicated in Table 2, signifying a cooperative binding behavior and BSA has two binding sites for quercetin. The proposed mechanisms of BSA-quercetin complexation were done two steps as following:

$$BSA + Q_1 \rightleftharpoons BSA\text{-}Q_1 \quad \text{----(1)}$$

$$BSA\text{-}Q_1 + Q_2 \rightleftharpoons Q_2\text{-}BSA\text{-}Q_1 \quad \text{----(2)}$$

The first quercetin (***Q1***) binds to the low affinity binding site with the microscopic binding constant (k_{d1}). This yields a conformation change of the second binding site, causing an increase in affinity to the second quercetin (***Q2***), with the microscopic binding constant (k_{d2}). The experimental data should represent the fractional degree of saturation of the available protein binding sites for quercetin which can be expressed as $v = \frac{F}{C}$ where F is the molar fluorescence intensity of complex and C is total

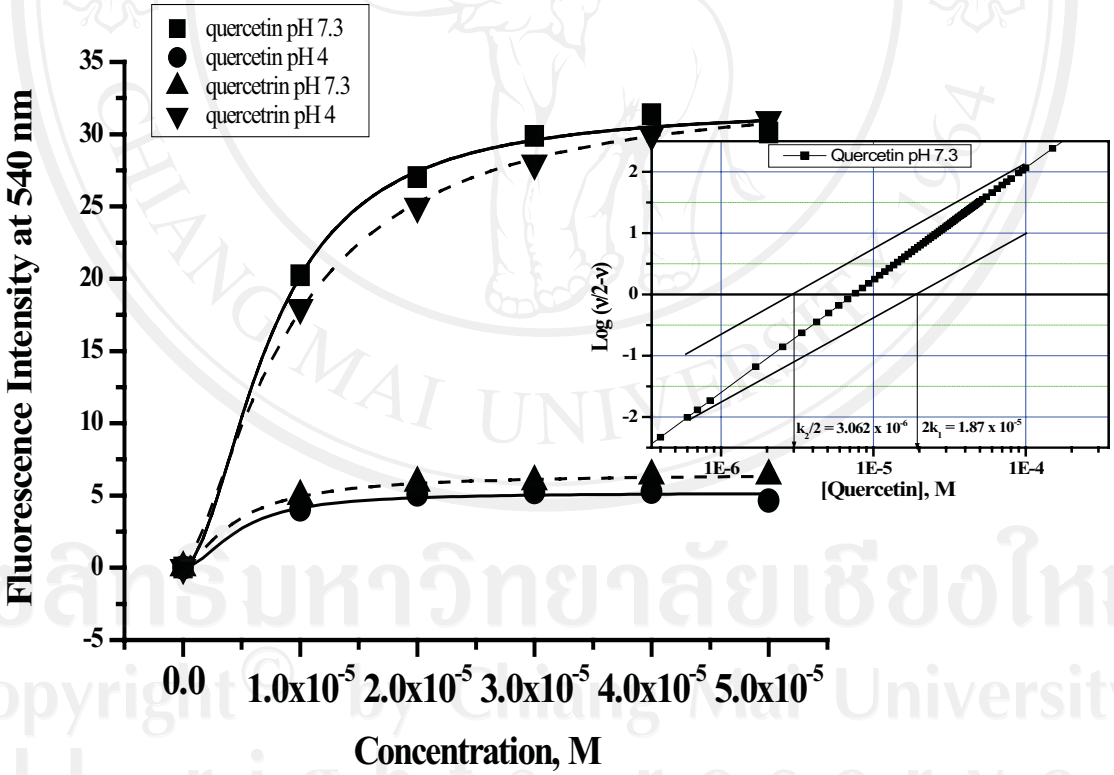

Figure 4. FRET analysis of BSA-Quercetin complex as a function of the quercetin concentration [Results were obtained from the series of experiments with same conditions as Figure 2].

In set of Figure 4. Hill's plot of log [ν/(2-ν)] as a function of log [Quercetin] for binding of quercetin to BSA with two identical binding sites. The curves correspond to independent binding (---------) or cooperative binding (——) to the two sites with the indicated binding constants.

concentration of BSA. When $\nu = 0$ means that all BSA is in free form whereas a value of $\nu = 2$ indicates that all binding sites of BSA are occupied with quercetin. In case of the stoichiometric complexation is 1 mole of BSA to 2 mole quercetin, the cooperative binding behavior can be characterized as the ratio of occupied sites (***υ***) to un-occupied sites (***2-υ***) which is given by

$$\frac{(v_2)}{(2-v_2)} = \frac{(k_{d2}C+2C^2)}{(2k_{d1}k_{2+}kd_2C)}.$$

If we now plot the log [ν/(2-ν)] versus the log (C) we obtain the Hill's plot from which several parameters of the cooperative binding interaction can be determined and which is shown in inset of Figure 4 and the k_{d1} and k_{d2} were determined as reported in Table II.

Figure 3c and 3d also allows calculating the efficiency of energy transfer (E) from the BSA to the quercetin by using equation (3);

$$E = 1 - \frac{F}{F_0} \qquad (3)$$

where F is fluorescence intensity of the BSA in the presence of 50 μM quercetin;

F_0 is fluorescence intensity of the BSA in the absence of FRET.

The E of BSA-quercetin system determined was equal to 0.94 at pH 4.0 and equal to 0.97 at pH 7.3.

In fact the efficiency of energy transfer (E) is related to the distance r (Å) between the donor and the acceptor as indicated in the equation (4);

$$E = \frac{R_0^6}{R_0^6 + r^6} \qquad (4)$$

where r is the distance between donor and acceptor in Angstrom unit.

The r of BSA-quercetin system determined was equal to 34.98 Å at pH 4.0, and equal to 33.81 Å at pH 7.3.

The FRET analysis in the similar conditions of experiments was performed with quercetrin and rutin. The FRET and BSA-molecule complexation parameters were indicated in Table II.

Table II. The FRET and BSA-molecule complexation parameters measured by using FRET analysis in the HEPES-Na+ buffered solutions pH 4 and 7.3, the final concentration of BSA was fixed at 3 mM.

Compounds	pH = 4						
	J(l), $M^{-1}.cm^3$	R_0, Å	r, Å	k_{d1}, M	k_{d2}, M	K_D, M^2	n
Quercetin	$1.49x10^{-14}$	55.38	34.98	$3.5x10^{-4}$	$5.2x10^{-7}$	$1.75x10^5$	1.7
Quercetrin	$1.1x10^{-14}$	24.6	11.0	$1.2x10^{-4}$	3.1x10-7	$1.12x10^5$	1.6
Rutin	$4.66x10^{-14}$	31.13	19.05	-	-	$5.0x10^4$	1
Compounds	pH = 7.3						
	J(l) , $M^{-1}.cm^3$	R_0, Å	r, Å	k_{d1}, M	k_{d2}, M	K_D, M^2	n
Quercetin	$2.39x10^{-14}$	59.93	33.81	$3.3\ x10^{-4}$	$4.76\ x10^{-7}$	$1.66\ x10^5$	1.7
Quercetrin	$1.7x10^{-14}$	26.2	13.5	$1.4\ x10^{-4}$	$4.9\ x10^{-7}$	$1.37\ x10^5$	1.6
Rutin	$9.14x10^{-14}$	34.83	21.32	-	-	$5x10^4$	1.18

Discussion

Quercetin and its glycoside derivatives present great interest as potential anticancer and anti-carcinogenic molecules, unfortunately its very poor solubility and unstable in an aqueous solution that hampers the progression in clinical trials. This study clearly shows that BSA increased in the solubility of quercetin, quercetrin qnd rutin in both RPMI 1640 medium and HEPES-Na^+ buffer solution. BSA found in the culture medium can serve as a carrier and spontaneously and constantly release source of flavonoids particular quercetin *in vitro* cytotoxicity assay. It was clearly shown that the complexation of BSA with quercetin, quercetrin and rutin was immediately done after addition the compounds into the BSA solutions. We rigorouslly determined the crucial parameters including the macroscopic and microscopic rate constant of binding of the complex by using FRET analysis. The ratio of complexation was 1 mole of BSA to 2 moles of quercetin and quercetrin. At pH 7.3, the K_D value was 1.37×10^5 M^{-2} for BSA-quercetrin and 1.68×10^5 M^{-2} for BSA-quercetin. These results should interpreted as the BSA-quercetrin complex is more stability than the BSA-quercetin. As a consequence quercetrin was significantly found lower degree than quercetin in cell pellets of the series using RPMI 1640 meidum. The rutinoside substitution caused a change in the ratio of BSA-rutin complexation (1:1) signifying that the rutinoside affected the molecular steric of quercetin thus changed the behavior of interaction with BSA. Moreover, the K_D value of the BSA-rutin was equal to 5.0×10^4 M^{-1} . In fact among the compounds studied, the BSA-rutin is the most stable and the cell-rutin concentration is very low compared with quercetin and quercetrin.

The study clearly demonstrated that BSA affected the cellular uptake of quercetin glycoside. Since the cellular uptake of quertin in HEPES-Na^+ buffered and RPMI 1640 was almost the same, this signified that BSA can improve the dissolubility of quercetin and can act as a suitable carrier of quercetin by which spontaneously release the quercetin to the solution and cells. Let consider the effects of the glycoside moeity on the behavior and reactivity of the molecules. The subsitution of *3*-rhamnoside particularly the *3*-rutinoside rendering higher affinity to BSA and considerably increased in stability of the complexes. This should be the predominant cause of very low concentration of rutin found in the cell pellets.

The cytotoxicity results clearly showed that rhamnoside and rutinoside substituted molecules mediated higher efficacy than its parent compound in both K562 and GLC4 cells. Particularly, the rutinoside substitution exhibits 5-fold anticancer activity higher than quercetin. Indeed, it is known that quercetin mediated anticancer action at the mitochondrial level triggering particular various cancer cell types to be sucided the so-called “apoptosis” (9, 11-14). The efficacy of apoptosis induction of quercetin depends upon its intracellular target concentration. These results suggested that the BSA-quercetrin and BSA-rutin complexes might change the specific target and cellular distribution pattern. We hypothesized that both BSA-quercetrin and BSA-rutin complexes might mediated anticancer action via an interaction with the extrinsic pathway,

activated by pro-apoptotic receptor signals at the cellular surface.

Conclusion:

BSA can considerably improve the solubility of quercetin and its glycoside derivatives, in particularly these flavonoids have high affinity to BSA. Among the compounds studied, the BSA-rutin is the most stable and the cell-rutin concentration is very low compared with quercetin and quercetrin. The subsititution of rhamnoside and rutinoside at C3 in particular the rutinoside yields an increase in complex stability by ~29-fold compared with quercetin. The macroscopic binding constant was directly influenced on the cellular uptake of molecules. This study reported for the first time the was financially supported by a grant funded under the Strategic Scholarships for Frontier Research Network for the Government, the Office of the Higher Education Commission (OHEC), Ministry of Education Thailand.

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suitable range of binding constant ($K_D \geq 10^5$ M^{-2}) of the carrier-drug by which the carrier can be useful for increasing the solubility of drug and also spontaneously release the drug into the solution and cells. As a consequence the complexation did not change in the mode of action of molecule. Contrary, an increase in complex stability by 29-fold caused dramatically decreased in the cellular concentration such as the case of rutin. It should be expected that the available cytosolic concentration might not be enough to mediate any biological effects but the complex might directly interact with the pro-apoptotic receptor signals at surface membrane of cells.

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