Food Chemistry 141 (2013) 266–273

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Enzymatic de-glycosylation of rutin improves its antioxidant and antiproliferative activities

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ARTICLE INFO

Article history:
Received 3 June 2012
Received in revised form 18 February 2013
Accepted 23 February 2013
Available online 14 March 2013

Keywords:
Quercetin-3-glucoside
Rutin
Antioxidant
Antiproliferative
α-L-Rhamnosidases
Hydrolysis

ABSTRACT

Bioavailability and biological properties of flavonoid glycosides can be improved after the enzymatic hydrolysis of specific glycosyl groups. In this study, we evaluate the antioxidant and antiproliferative potential of rutin after enzymatic hydrolysis performed by α-L-rhamnosidases (hesperidinase from *Penicillium sp.* and naringinase from *Penicillium decumbens*) previously heated at 70 °C for 30 min to inactivate the undesirable β-D-glucosidase activity. The highest *in vitro* antioxidant activity determined by DPPH radical scavenging was achieved with rutin hydrolyzed by hesperidinase. Rutin was predominantly bioconverted into quercetin-3-glucoside. There was no statistical difference between xanthine oxidase inhibition by rutin before and after hydrolysis. However, *in vitro* inhibitory activity against ten human tumor cell lines showed that hydrolyzed rutin exerted a more potent antiproliferative effect than quercetin and rutin on various cancer cell lines, specially glioma, and ovarian and breast adenocarcinomas. These results indicate that quercetin-3-glucoside could be a promising functional derivative obtained by rutin hydrolysis.

1. Introduction

Rutin, also called rutoside, quercetin-3-O-rutinoside and sophorin, is a flavonoid glycoside consisting of the aglycone form, quercetin bound at the C-3 position (on ring C) to a disaccharide molecule, rutinose ($C_{12}H_{22}O_{10}$), which is composed of one molecule of rhamnose and one of glucose (Aherne & O'Brien, 2002). Rutin is found in the fruit of *fava d'anta* tree (*Dimorphandra mollis*) native to the Cerrado vegetation of Brazil, fruit rinds (especially citrus fruits, such as orange, grapefruit, lemon and lime) and berries such as mulberry, ash tree fruits and cranberries. It has been reported that rutin has several pharmacological functions such as antioxidant (Boyle et al., 2000), cytoprotective (Potapovich & Kostyuk, 2003), vasoprotective (Tang et al., 2011), antiproliferative (Santos et al., 2011), antithrombotic (Sheu, Hsiao, Chou, Shen, & Chou, 2004) and cardioprotective activities (Ziaee, Zamansoltani, Nassiri-Asl, & Abbasi, 2009). Quercetin is also an important dietary flavonoid with antioxidant, anti-inflammatory and antiproliferative properties (Nijveldt et al., 2001) in addition to being an effective inhibitor of xanthine oxidase (Day, Bao, Morgan, & Williamson, 2000). Xanthine oxidase catalyzes the oxidation of hypoxanthine and xanthine to uric acid, generating superoxide radicals, which are involved in many pathological processes such as inflammation, atherosclerosis, cancer, and aging (Paravicin & Touyz, 2008).

Previous work has shown that enzymatic hydrolysis of specific glycosyl groups or the conversion of flavonoid glycosides to aglycones increases anti-inflammatory activity of naringin (Amaro et al., 2009) and antioxidant activity of kaempferol (Park, Rho, Kim, & Chang, 2006), besides improving the bioavailability of hesperidin (Nielsen et al., 2006) and of flavonoid glycosides in fruit juices and green tea (Gonzáles-Barrio et al., 2004). The use of enzymes to modify the structure and improve the physicochemical and biological properties of flavonoids has been of great scientific and industrial interest due to their wide availability, high selectivity, low cost and their promotion of efficient reactions with few by-products.

α-L-Rhamnosidases [E. C. 3.2.1.40] are glycosyl hydrolases which cleave terminal α-L-rhamnose from natural glycosides. Only two commercial preparations of α-L-rhamnosidases (naringinase and hesperidinase) are available, and both are from fungal sources.

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0308-8146
http://dx.doi.org/10.1016/j.foodchem.2013.02.127

Hesperidinase is obtained from species of *Penicillium* and *Aspergillus niger* and naringinase is obtained from *Penicillium decumbens*. All these preparations also show significant β-D-glucosidase activity that catalyzes the hydrolysis of terminal non-reducing residues with glucose release (Yadav, Yadav, & Yadav, 2010). Thus the activity of the α-L-rhamnosidases in the rutin substrate produces two derivatives: quercetin-3-glucoside (isoquercetrin) and quercetin, in proportions that depend on the reaction conditions.

Although there is a structural similarity to rutin, quercetin-3-glucoside and quercetin, there are some noticeable differences in physical, chemical and biological properties. Quercetin glycosides show higher solubility in water than quercetin due to the hydrophilicity of the sugar moieties (Aherne & ÓBrien, 2002). In comparison with rutin and quercetin, quercetin-3-glucoside is better absorbed, suggesting that conjugation with glucose enhances quercetin absorption in small intestine (Arts, Sesink, Faassen-Peters, & Hollman, 2004). Indeed, previous reports have shown that quercetin-3-glucoside has a more potent antiproliferative effect than quercetin or rutin (You, Ahn, & Ji, 2010). Thus, the synthesis of mono-glycosylated quercetin from rutin by the enzymatic hydrolysis method seems to be a good alternative for obtaining compounds with enhanced functional properties. Hesperidinase or naringinase with inactivated β-D-glucosidase activity and expressing α-L-rhamnosidase activity allow the production of very expensive flavonoid glycosides, quercetin-3-glucoside, in an easy and cheap bioprocess starting from rutin. In the present work, the enzymatic hydrolysis of rutin by two commercial heat-treated glycosyl hydrolases (hesperidinase and naringinase) was investigated in order to obtain partially hydrolyzed rutin with enhanced functional properties. Antioxidant properties using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·), β-carotene bleaching and xanthine oxidase (XO) inhibition activity were evaluated before and after enzymatic treatment. Antiproliferative activity of quercetin and rutin before and after bioconversion were compared using nine different cancer cell lines including glioma, chronic myeloid leukemia and breast, ovarian, prostate, kidney, colon, and lung cancer cells.

2. Materials and methods

2.1. Enzymes and reagents

Hesperidinase from *Penicillium sp.*, naringinase from *Penicillium decumbens*, xanthine oxidase from bovine milk, rutin (95% min.), DPPH·(2,2-diphenyl-1-picril-hydrazyl radical), *p*-Nitrophenyl α-L-rhamnopyranoside (4-NRP), *p*-nitrophenyl β-D-glucopyranoside (4-NGP), β-carotene (95%) rutin, *quercetin-3-glucoside* and quercetin standards were purchased from the Sigma–Aldrich Chemical Co. All solvents and other reagents were of analytical, spectrometric or chromatographic grade.

2.2. Inactivation kinetics

The activity of α-L-rhamnosidases naringinase and hesperidinase was evaluated using 0.20 mM of *p*-nitrophenyl α-L-rhamnopyranoside in 20 mM citrate buffer at pH 4.0, while the activity of β-D-glucosidase was determined using 0.20 mM *p*-nitrophenyl β-D-glucopyranoside in 20 mM citrate buffer at pH 4.0. An enzyme concentration of 50 mg L^{-1} in 0.05 M acetate buffer pH 4.0 was used in these experiments. The concentration of free *p*-nitrophenol produced after hydrolysis was evaluated spectrophotometrically at $\lambda = 340$ nm, using calibration curve of each compound. In order to study β-D-glucosidase and α-L-rhamnosidase inactivation kinetics of hesperidinase and naringinase, a temperature range of 50–80 °C was used. The reaction was carried out in isothermal conditions with shaking at 100 rpm for 30 min. Inactivation was stopped by boiling (100 °C) for 30 min. The control was enzyme sample not submitted to thermal inactivation. One unit of enzymatic activity (U) was defined as the amount of enzyme that liberated 1 μmol of free *p*-nitrophenol per minute under the above assay conditions (U/min). The ratio of α-L-rhamnosidase activity to β-D-glucosidase activity (Rha/Glu) was used to describe the inactivation kinetics for β-D-glucosidase and α-L-rhamnosidase activities.

2.3. Bioconversion reaction

Hesperidinase and naringinase solutions (50 mg L^{-1}in 0.05 M acetate buffer pH 4.0) were heated at 70 °C for 30 min to inactivate glucosidase activity. The reaction mixtures containing 100 μL of enzyme preparation (50 mg L^{-1}) and 4 mL of a 1% m/v rutin solution (dissolved previously in 1 mL of methanol) were mixed and incubated for 2, 4, 8 and 12 h with shaking (130 rpm) at 40 °C. The reactions were stopped by boiling (100 °C) for 30 min, and samples were subsequently freeze-dried and stored at −80 °C prior to extraction and analysis. The assays were performed in triplicate. Rutin incubated in the same conditions without adding any enzyme and with unheated enzymes were used as controls, aiming to evaluate the effects of heat on enzyme activity.

2.4. UPLC–ESI-MS quantification

After the conversion reactions, the freeze-dried products of hydrolysis were dissolved in methanol, filtered through a 0.22 μm syringe filter and analyzed by ultra-high performance chromatography – mass spectrometry (UPLC–MS). Three μL of each sample were analyzed on an Acquity UPLC system (Waters, Milford,MA, USA) using a UPLC BEH column (2.1 × 50 mm, 1.7 μm particle size) at a temperature of 30 °C. A gradient of (A) deionized purified water with 1% formic acid and (B) methanol (Tedia, Brazil) starting with 5% B and ramping to 100% B at 8 min, maintained till 8.50 min, then returning to initial conditions and stabilizing by 10 min. Detection in negative ion modes was achieved on an Acquity TQD mass spectrometer (Micromass Waters, Milford, MA, USA) with capillary – 3000 V, Cone – 30 V, source temperature 150 °C; desolvation temperature 350 °C.

2.5. Antioxidant activity

2.5.1. DPPH· radical-scavenging activity

The antioxidant activity of samples was assessed on the basis of scavenging activity of the stable 2, 2-diphenylpicrylhydrazyl free radical (DPPH·). 750 μL of a methanolic solution of DPPH (0.02 mg/L) were added to 0.1 μmol of test samples in acetate buffer 0.3 M, pH 3.8 or 120 μL of methanol, in the case of control. Flasks were incubated at 25 °C for 25 min and absorbance was determined at 517 nm. All assays were performed in triplicate. A solution of rutin in acetate buffer 0.3 M, pH 3.8 (0.75 mg/mL) was used to calibrate the equipment. The scavenging capacity of DPPH radical was calculated using the following equation:

$$\text{DPPH}^{\cdot}\ \text{scavenging effect}\,(\%) = (A_c - A_s/A_c) \times 100,$$

where A_c and A_s are absorbance values of control reaction and test samples, respectively.

2.5.2. β-Carotene bleaching method

The effect on lipid peroxidation inhibition was determined in a β-carotene-oleic acid system according to the Miller (1971) method with adaptations. A mixture containing 50 μL of β-carotene (2 mg/mL in chloroform), 40 μL of oleic acid standard, 1 mL of chloroform and 400 mg of Tween 40 was prepared. Chloroform was

removed under nitrogen atmosphere and 50 mL of aerated redistilled water were added. The mixture was then subjected to vigorous shaking. In screw-top glasses, 225 μl of β-carotene/oleic acid solution was added to 0.1 μmol of samples in acetate buffer (0.3 M, pH 3.8) or 100 μL of methanol (blank assay). Absorbance was read at 470 nm, at 0 min (immediately after emulsion addition) and after 120 min of incubation at 45 °C (induction of thermal oxidation). Peroxidation leads to the bleaching of the β-carotene molecule, so the higher the absorbance the higher the antioxidant activity. The degradation rate was calculated according to zero order reaction kinetics. Inhibition of lipid peroxidation was calculated according to the following equation:

$$\% \text{ inhibition} = [1 - (A_0 - A_1)/(A'_0 - A'_1)] \times 100,$$

where A_0 is the absorbance of sample at zero time, A_1 is the absorbance of sample after incubation (120 min) at 45 °C, A'_0 is the absorbance of control at zero time and A'_1 is the absorbance of control after incubation (120 min) at 45 °C.

2.5.3. Determination of xanthine oxidase (XO) inhibition

Xanthine oxidase activity was determined by measuring the formation of uric acid from xanthine. Xanthine in phosphate buffer 0.1 M, pH 7.4 (1.3 μmol) was incubated with 100 μL of ethanol and test samples (90 μM). The mixtures were pre-incubated at 37 °C, for 10 min. XO (0.1 U/mL) was then added to the mixtures and the flasks were incubated at 37 °C for 20 min. Enzymatic reaction was stopped by adding 25 μL of 3.2% hydrochloric acid. Absorbance was determined at 290 nm. Phosphate buffer (0.1 M, pH 7.4) was used as blank and a solution containing xanthine and xanthine oxidase, at same conditions previously described, as used as reaction control. All assays were performed in duplicate. Xanthine oxidase inhibition (XO inhibition) was calculated as follows:

$$\text{XO inhibition}\,(\%) = (1 - A_s/A_c) \times 100,$$

where A_c and A_s are the absorbance values of reaction control and tested samples, respectively.

2.6. Determination of antiproliferative activity

Nine human cancer cell lines [U251 (glioma, CNS), MCF-7 (breast), NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance), 786–0 (kidney), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-03 (ovarian), HT-29 (colon adenocarcinoma) and K-562 (chronic myeloid leukemia)] were kindly provided by Frederick Cancer Research & Development Center – National Cancer Institute – Frederick, MA, USA. Stock cultures were grown in 5 mL of RPMI-1640 (GIBCO BRL) supplemented with 5% fetal bovine serum (FBS, GIBCO BRL). Penicillin: streptomycin mixture 1000 U/mL:1000 μg/mL (1 mL/L RPMI-1640) was added to experimental cultures. Cells in 96-well plates (100 μL cells/well) were exposed to different concentrations of samples (0.25, 2.5, 25 and 250 μg/mL) in DMSO/RPMI/FBS 5% at 37 °C, 5% CO_2, for 48 h. Final DMSO concentration did not affect cell viability. Cells were then fixed with trichloroacetic acid solution (50%, v/v) and cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay (Monks et al., 1991). Doxorubicin (DOX; 0.025–25 μg/mL) was used as a positive control. Three measurements were obtained: at the beginning of incubation (T_o) and 48 h post-incubation for compound-free (C) and tested (T) cells. The choice of 48 h incubation was based on the NCI60 protocol, proposed by NCI/EUA for antiproliferative screening. Cell proliferation was determined according to the equation $100 \times [(T - T_o)/C–T_o]$. Cytostatic effect was observed when $T_o \leqslant T < C$ while cytocidal effect occurred when $T < T_o$. From the concentration–response curve for each cell line, TGI value (concentration that produces 0% cell growth or totally cytostatic effect) was determined through nonlinear regression analysis using the software Origin 8.0® (OriginLab Corporation). The experiments were done in triplicate.

2.7. Statistical analysis

All the experiments were performed in triplicate, and the data were expressed as means ± the standard deviation. The statistical significance of the analytical results was assessed by ANOVA, and the differences identified were pinpointed by an unpaired Student's t-test. An associated probability (p value) of less than 5% was considered significant.

3. Results

3.1. Effects of heat on enzyme activity

The heat stability of α-L-rhamnosidase and β-D-glucosidase were investigated at 50, 60, 70 and 80 °C for 30 min (Fig. 1). β-D-glucosidase activity decreased more rapidly than α-L-rhamnosidase activity with heat treatment. Although heating at 70 °C for 30 min leads to a significant reduction on α-L-rhamnosidase activity of hesperidinase and naringinase compared to the activity at 50 °C (from 4.2 to 2.6 and 3.7 to 2.1 U/min, respectively), the ratio of α-L-rhamnosidase activity to β-D-glucosidase activity (Rha/Glu) was highest at 70 °C for both enzymes. Once β-D-glucosidase had been selectively inactivated at 70 °C for 30 min, the residual α-L-rhamnosidase activity of the enzymes was used for the production of mono-glycoside flavonoids starting from rutin.

3.2. Analysis of the reaction products by UPLC–MS

Quercetin-3-glucoside was detected by UPLC–MS in negative ion mode, as a peak at 5.4 min (m/z 463) which corresponded to its deprotonated ion $[M - H]^-$, and confirmed by comparison of the retention time and MS/MS fragments of a standard of quercetin-3-glucoside. UPLC–MS analysis of hydrolyzed rutin after 2h-hesperidinase reaction furnished a conversion of 48% of rutin into quercetin-3-glucoside (Fig. 2 and Fig. 3A) while after 4h-reaction, the conversion increased to 69.5% of quercetin-3-glucoside and 7.5% of quercetin (Fig. 2 and Fig. 3B). More than 4 h hydrolysis did not increase quercetin-3-glucoside production. The Fig. 3C show MS profiles in the negative mode of quercetin-3-glucoside produced after 4 h of treatment enzymatic with hesperidinase. Only 34.5% of quercetin-3-glucoside was detected after 4 h of reaction using naringinase in identical conditions (data not shown), which explains the lower values for antioxidant activity obtained after bioconversion using naringinase.

3.3. The influence of rutin bioconversion on antioxidant capacity

In order to investigate the efficiency of enzymatic treatment of rutin in its antioxidant capacity, *in vitro* methods were used as summarized in Table 1. The $DPPH^{\bullet}$ method was used to evaluate hydrogen donating ability, and the antioxidant capacity was expressed as the percentage of $DPPH^{\bullet}$ radical-scavenging activity as compared to the control. The antioxidant capacity of the rutin increased by approximately 30% after 4 h of treatment with hesperidinase, while with the use of naringinase, only a slight increase was observed (approximately 10%). The values obtained show a pattern similar to that of quercetin, well-known for its powerful antioxidant properties. These results suggested that rutin bioconversion catalyzed by hesperidinase was more effective than that promoted by naringinase. Hydrolyzed rutin produced by

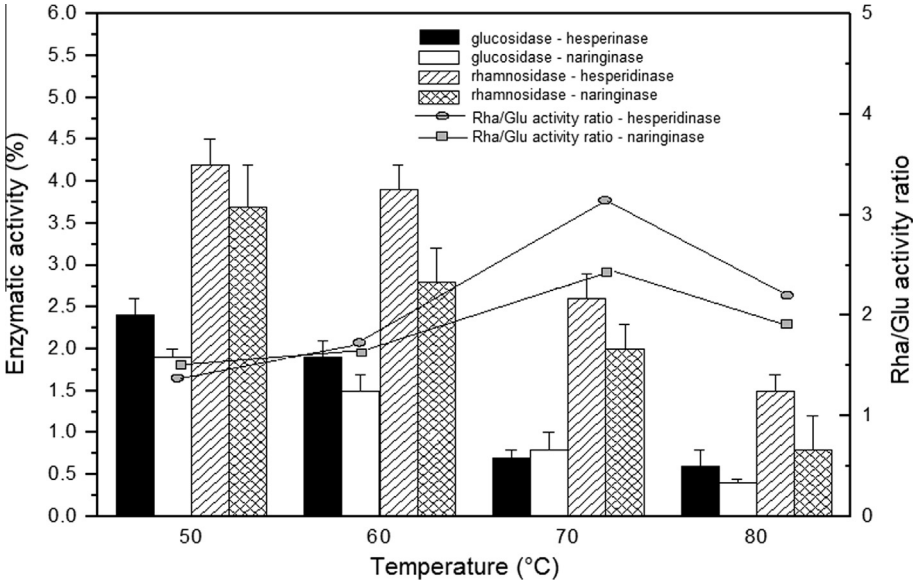

Fig. 1. Effect of temperature on β-D-glucosidase and α-L-rhamnosidase activities of naringinase and hesperidinase. The ratio of the two enzyme activities is represented as Rha/Glu.

hesperidinase bioconversion was subsequently selected for further investigation.

The β-carotene bleaching method is widely used since it does not require high temperatures use, and antioxidant capacity of heat-sensitive flavonoids can be readily determined and quantitatively evaluated. The determination of the antioxidant activity of samples is based on their capacity to inhibit the β-carotene bleaching caused by free radicals generated during linoleic acid peroxidation. There was no significant increase in the antioxidant capacity of the hydrolyzed rutin after the reaction catalyzed by both enzymes, when evaluated using β-carotene bleaching method. However the quercetin standard showed a higher percentage of oxidation inhibition, possibly due to its more hydrophobic nature.

The XO inhibitory capacity of hydrolyzed rutin (after 4, 8 and 12 h of hydrolysis with hesperidinase) was not statistically different from rutin, which could be considered a weak inhibitor of XO. Quercetin, on the other hand, exhibited the strongest inhibitory activity, as shown in Table 1.

3.4. Antiproliferative activity in vitro

The antiproliferative properties of the samples before and after bioconversion were assessed using nine human cancer cell lines, and the chemotherapeutic drug, doxorubicin, as a positive control (Fig. 4 and Table 2). A horizontal line at 0% was traced to visualize Total Growth Inhibition (TGI) that represents the concentration required to completely inhibit cell growth (total cytostatic effect) (Table 2). For all cell lines tested, rutin hydrolyzed by hesperidinase displayed a moderate antiproliferative activity with selectivity for OVCAR-3 (ovarian, TGI = 1.5 μg/mL), MCF-7 (breast, TGI = 2.3 μg/mL) and U251 (glioma, TGI = 3.6 μg/mL) while quercetin presented a weak activity with selectivity for U251 (glioma, TGI = 31.4 μg/mL), MCF-7 (breast, TGI = 31.9 μg/mL), 786–0 (renal, TGI = 42.7 μg/mL) and NCI-ADR/RES (ovarian expressing multidrug resistance, TGI = 44.0 μg/mL). Rutin did not inhibit cell proliferation of any of the cancer cell lines tested.

4. Discussion

Flavonoid glycosides production of by removing rhamnose from rutinosides can be performed through controlled enzymatic catalysis. In the present study, we were able to define a good condition of β-D-glucosidase inactivation for hesperidinase and naringinase, while keeping a high level of α-L-rhamnosidase activity. After 4 h of enzymatic reaction catalyzed by hesperidinase, previously heated at 70 °C for 30 min, significant amounts of quercetin-3-glucoside (approximately 70%) were obtained. Hesperidinase hydrolyzed rutin more efficiently than naringinase. Hydrolyzed rutin produced by bioconversion using hesperidinase was subsequently selected for further investigation.

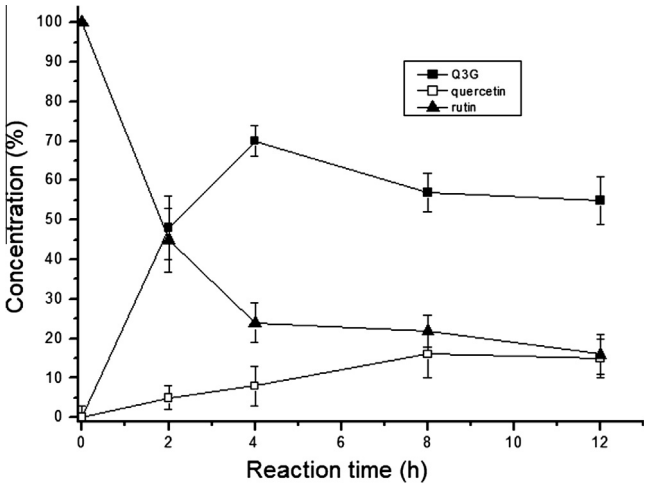

Fig. 2. Concentrations of rutin, quercetin-3-glucoside and quercetin after enzymatic treatment with hesperidinase. Data are mean ± SD (n = 3).

Vila-Real, Alfaia, Bronze, Calado, and Ribeiro (2011) performed a similar procedure to produce flavonoid monoglycosides, including quercetin-3-glucoside, from rutinosides, using naringinase from *Penicillium decumbens* as biocatalyst. The authors reported that a selective inactivation of β-D-glucosidase activity of naringinase was achieved at 81.5 °C and pH 3.9, keeping a very high residual activity of α-L-rhamnosidase (78%). Similarly, You et al. (2010) reported that β-D-glucosidase activity of crude enzyme extract of *Aspergillus niger* was completely inactivated by treatment for 30 min at 70 °C while the α-L-rhamnosidase activity was decreased by only 50%.

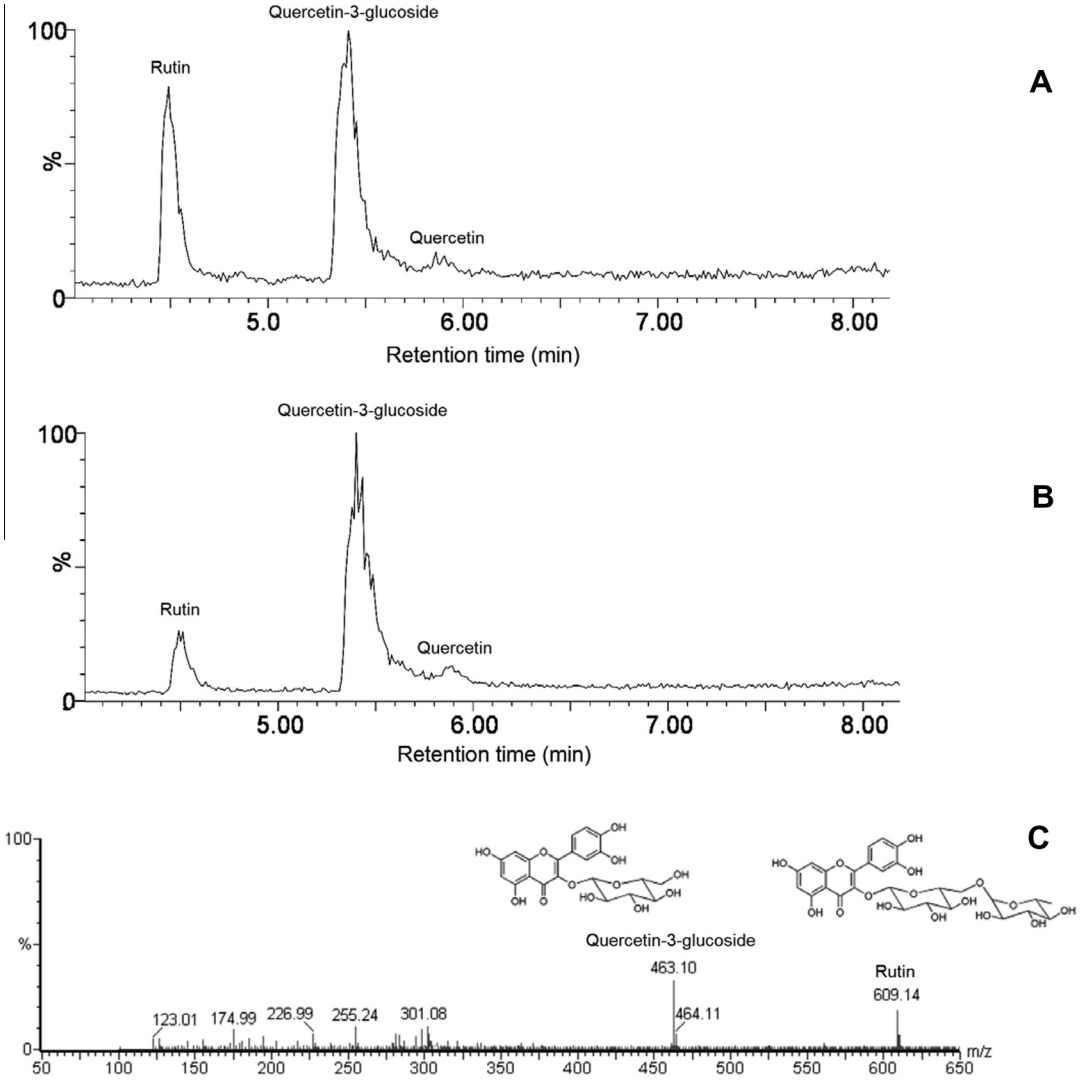

Fig. 3. UPLC–MS chromatogram (A and B) of rutin after of 2 and 4 h of enzymatic treatment with heat-treated hesperidinase. Negative ion mode MS of quercetin 3-glucoside (C) produced after 4 h of enzymatic treatment with hesperidinase.

Table 1
Antioxidant activity of rutin control, hydrolyzed rutin (bioconversion with hesperidinase and naringinase) and standard quercetin determined by DPPH and β-carotene-linoleic acid method and xanthine oxidase inhibition of rutin control, hydrolyzed rutin (bioconversion with hesperidinase) and standard quercetin.

Samples	Time of bioconversion reaction (h)	DPPH$^{\bullet}$ scavenging effect (%)	β-Carotene (% oxidation inhibition)	Xanthine oxidase inhibition (%)
Rutin (control)		62.6 ± 6.9[a]	49.4 ± 2.7[a]	16.7 ± 8.7[a]
Hydrolyzed rutin (bioconversion with hesperidinase)	4	81.4 ± 2.3[b]	47.1 ± 7.5[a]	21.8 ± 8.6[a]
	8	72.4 ± 6.6[a]	50.1 ± 3.9[a]	29.5 ± 8.0[a]
	12	79.3 ± 1.3[b]	57.2 ± 7.9[a]	29.7 ± 11.0[a]
Hydrolyzed rutin (bioconversion with naringinase)	4	70.3 ± 3.9[a]	50.5 ± 5.4[a]	
	8	68.4 ± 3.6[a]	50.4 ± 4.6[a]	
	12	70.3 ± 5.2[a]	52.2 ± 3.9 [a]	
Quercetin (standard)		89.8 ± 2.5[b]	78.1 ± 2.7[b]	79.3 ± 7.2[b]

The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to rutin before bioconversion ($p < 0.05$).

The difference in the Rha/Glu activity ratio between hesperidinase and naringinase (Fig. 1) could explain the slight increase in DPPH scavenging (Table 1) observed for hydrolyzed rutin obtained by bioconversion with heat-treated hesperidinase in comparison with hydrolyzed rutin obtained by bioconversion with heat-treated naringinase. On the other hand, using the β-carotene bleaching method both hydrolytic products afforded a similar result to that of rutin (Table 1).

Many *in vitro* and *in vivo* studies related quercetin bioactivities and correlated with its glycosides, showing differences in effectiveness of these compounds. Our findings have shown that quercetin-3-glucoside, at the concentration obtained by heat-treated hesperidinase treatment, has an antioxidant activity comparable to that observed for quercetin and considerably greater than that of rutin when evaluated using DPPH method (Table 1). This is in line with other studies comparing the antiradical activity of quercetin and

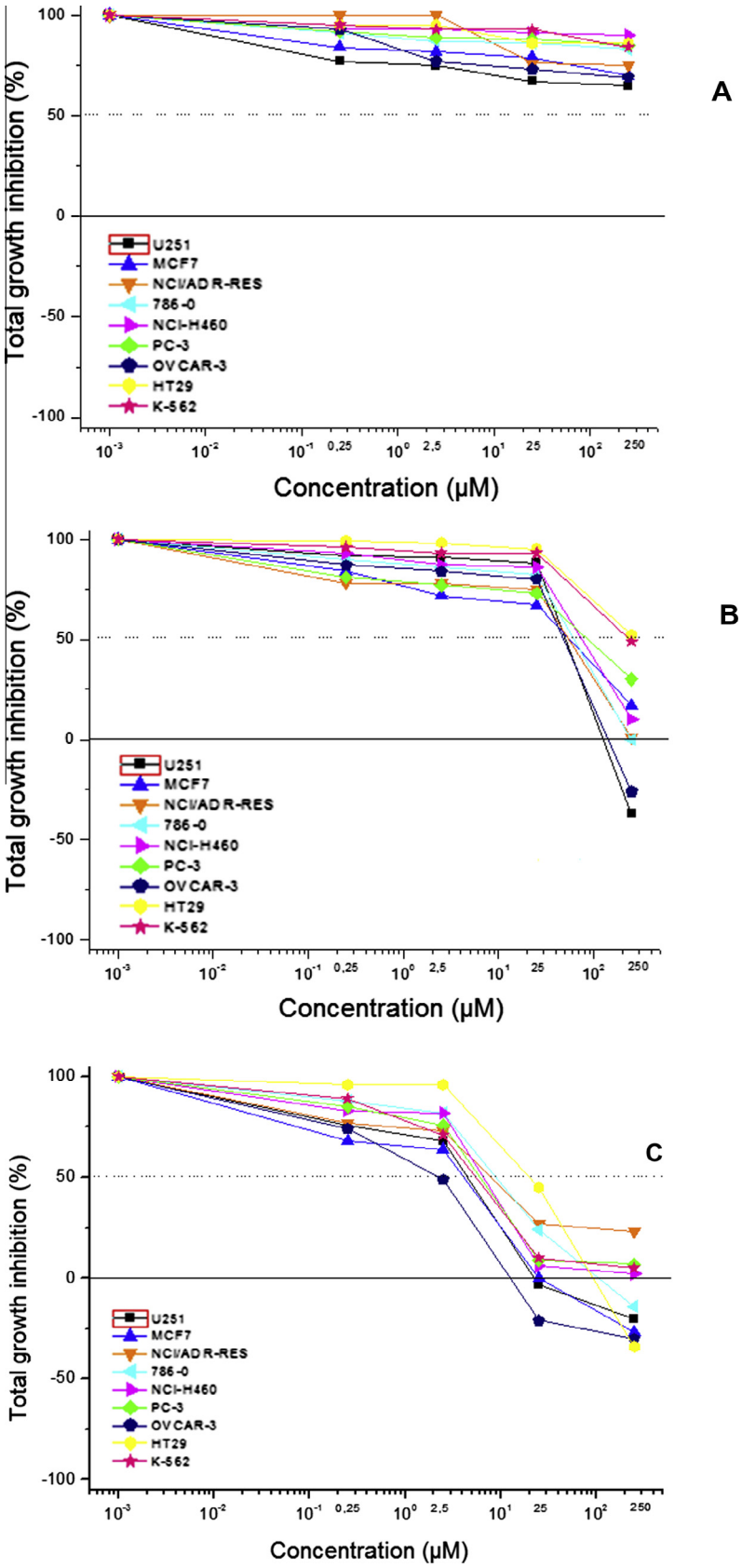

Fig. 4. Antiproliferative activity of rutin control (A), quercetin (B) and hydrolyzed rutin (C) obtained after 4 h of enzymatic treatment with hesperidinase, against nine cancerous cell lines: ■ U251 (glioma), ▲ MCF-7 (breast adenocarcinoma); ▼ NCI-ADR/RES (ovarian expressing multidrug resistance); ◄ 786-O (kidney adenocarcinoma); ► NCI-H460 (non-small cells lung adenocarcinoma); ♦ PC-3 (prostate adenocarcinoma); ⬟ NIH:OVCAR-3 (ovarian adenocarcinoma); ● HT-29 (colon adenocarcinoma) and ★ K-562 (chronic myeloid leukemia).

Table 2
Values of Total Growth Inhibition (TGI) (μg/mL) in each cell line tested for doxorubicin, rutin, quercetin and hydrolyzed rutin (obtained after 4 h of bioconversion with heat-treated hesperidinase).

	U251[a]	MCF-7[b]	NCI-ADR/RES[c]	786-0[d]	NCI-H460[e]	PC-3[f]	OVCAR-3[g]	HT-29[h]	K562[i]
Doxorubicin	0.025	<0.025	0.90	0.90	<0.025	0.069	0.033	0.091	0.032
Rutin (control)	>250	>250	>250	>250	>250	>250	>250	>250	>250
Hydrolyzed rutin	3.6	2.3	7.9	8.6	5.7	5.4	1.5	24.4	4.8
Quercetin	31.4	31.9	44.0	42.7	70.5	72.7	72.7	>250	>250

[a] Glioma.
[b] Breast adenocarcinoma.
[c] Ovarian expressing multidrug resistance.
[d] Kidney adenocarcinoma.
[e] Non-small cells lung adenocarcinoma.
[f] Prostate adenocarcinoma.
[g] Ovarian adenocarcinoma.
[h] Colon adenocarcinoma.
[i] Chronic myeloid leukemia.

its C(3)-OH and C(4′)-OH glycoside derivatives. Quercetin glycosylation at C(4′)-OH markedly decreased the H-donating ability (Goupy, Dufour, Loonis, & Dangles, 2003), while C(3)-OH derivatives of quercetin showed reducing potential comparable with that of free aglycone (Burda & Oleszek, 2001).

On the other hand, when evaluated by the β-carotene method, bioconversion failed to promote any enhancement on the antioxidant capacity, as only flavonols with a free hydroxyl group at the C-3 position of the flavonoid skeleton show high inhibitory activity to β-carotene oxidation. Furthermore, quercetin glycosides are more hydrophilic than quercetin. This modifies the coefficients of distribution between aqueous and lipid phases, which is of great significance in lipid systems such as TEAC or β-carotene emulsions (Burda & Oleszek, 2001).

Our results had also suggested that the enzymatic product obtained, containing almost 70% of quercetin-3-glucoside, showed no significant difference in xanthine oxidase inhibition when compared to rutin, while quercetin showed the highest inhibitory activity (Table 1). According to literature, rutin has a much lower activity in a xanthine/xanthine oxidase system despite a free C(4′)-OH group in the B-ring (Masuoka, Matsuda, & Kubo, 2012). In other model systems, quercetin derivatives had been also demonstrated displaying a lower antioxidant activity in comparison with free aglycone (Burda & Oleszek, 2001) suggesting that the lower antioxidant activity of quercetin derivatives could be mainly due to hydroxyl groups blocked by sugar or alkoxyl substituents. There is also evidence that XO inhibition by flavonoids depends on location and number of hydroxyl groups in molecule. The strongest contribution towards XO inhibition seems to be result from introduction of a 5-hydroxyl or 7-hydroxyl moiety into a flavone backbone, while 2′-hydroxyl and 3-hydroxyl groups result in negative contribution to XO inhibition (Nijveldt et al., 2001).

In vitro inhibitory activity against human tumor cells showed that hydrolyzed rutin exerted a more potent antiproliferative effect than quercetin and rutin on various cancer cell lines, showing higher activity for ovarian adenocarcinoma (OVCAR-3, TGI = 1.5 μg/mL), breast adenocarcinoma (MCF-7, TGI = 2.3 μg/mL) and glioma (U251, TGI = 3.6 μg/mL) (Fig. 4 and Table 2). The most potent antiproliferative effect observed for this quercetin-3-glucoside mixture might be related to the specific transport system, glucose transport carrier SGLT1. It was reported that glucose conjugation to a phenyl compound resulted in active absorption from mucosal side to serosal side by glucose transport system (Mizuma, Ohta, Hayashi, & Awazu, 1992). Experimental evidence has suggested that quercetin-3-glucoside and quercetin-4′-glucoside can be transported by sodium-dependent glucose transporter (SGLT1) and subsequently deglycosylated within enterocyte by cytosolic β-glucosidase (Wolffram, Blöck, & Ader, 2002). Quercetin itself was not transported by SGLT1 or GLUT2 (Kwon et al., 2007). Quercetin, but not rutin, has been shown to express anti-proliferative effects on the human colon cancer cell line (HT-29), in a dose-dependent and time-dependent manner (Kim, Bang, & Kim, 2005). You et al. (2010) recently related that quercetin-3-glucoside produced by removal of rhamnose sugar by crude enzyme extract of *Aspergillus niger* showed growth-inhibitory effects in colon (HT-29 and HCT 116), breast (MCF-7), hepatocellular (HepG2), and lung cancer (A549) cells with the IC_{50} value between 15 and 25 μM, while quercetin was greater than 80 μM. Quercetin-3-glucoside is not an abundant flavonol glycoside in foods; however, its bioavailability has been shown to be one and a half times greater than quercetin in rats (Morand, Manach, Crespy, & Remesy, 2000) and in dogs (Reinboth, Wolffram, Abraham, Ungemach, & Cermak, 2010). On the other hand, investigations of protective mechanism of quercetin and its derivatives against oxidative damage of *in vitro* rat C6 glioma cells showed that quercetin to be an active cell protector but not rutin or quercetin-3-glucoside (Chen, Jeng, Lin, Wu, & Chen, 2006).

5. Conclusion

The enzymatic reaction catalyzed by heated hesperidinase from *Penicillium sp.* efficiently converted rutin into its mono-glycoside form, quercetin-3-glucoside. Quercetin-3-glucoside and quercetin showed similar antioxidant capacity as evaluated by DPPH assay, but quercetin-3-glucoside showed lower inhibitory effects on xanthine oxidase and as antioxidant when evaluated by the β-carotene assay. However, this derivative exerted a more potent antiproliferative effect than quercetin or rutin on various cancer cell lines. The results obtained from this study indicate that quercetin-3-glucoside could be a promising functional derivative obtained by rutin hydrolysis and further *in vivo* evaluations are needed.

Acknowledgements

The authors gratefully acknowledge the financial support of FAPESP (Grant Proc. 09/09224-3; 2011/12394-8 and 2008/58035-6) and CAPES (Brazil).

References

Aherne, S. A., & O'Brien, N. M. (2002). Dietary flavonols: Chemistry, food content, and metabolism. *Nutrition, 18*, 75–81.
Amaro, M., Vila-Rela, H., Jr., Eduardo-Figueira, M., Mota-Filipe, H., Sepodes, B., & Ribeiro, M. H. (2009). Anti-inflammatory activity of naringin and the biosynthesized naringenin by naringinase immobilized in microstructured materials in a model of DSS-induced colitis in mice. *Food Research International, 42*, 1010–1017.
Arts, I. C., Sesink, A. L., Faassen-Peters, M., & Hollman, P. C. (2004). The type of sugar moiety is a major determinant of the small intestinal uptake and subsequent

biliary excretion of dietary quercetin glycosides. *British Journal of Nutrition, 91*, 841–847.
Boyle, S. P., Dobson, V. L., Duthie, S. J., Hinselwood, D. C., Kyle, J. A. M., & Collins, A. R. (2000). Bioavailability and efficiency of rutin as an antioxidant: A human supplementation study. *European Journal of Clinical Nutrition, 54*, 774–782.
Burda, S., & Oleszek, W. (2001). Antioxidant and antiradical activities of flavonoids. *Journal of Agricultural and Food Chemistry, 49*, 2774–2779.
Chen, T. J., Jeng, J. Y., Lin, C. W., Wu, C. Y., & Chen, Y. C. (2006). Quercetin inhibition of ROS-dependent and independent apoptosis in rat glioma C6 cells. *Toxicology, 223*, 113–126.
Day, A. J., Bao, Y., Morgan, M. R. A., & Williamson, G. (2000). Conjugation position of quercetin glucoronides and effect on biological activity. *Free Radical Biology & Medicine, 2912*, 1234–1243.
Gonzáles-Barrio, R., Trindade, L. M., Manzanares, P., De Graaff, L. H., Tomás-Barberán, F. A., & Espín, J. C. (2004). Production of bioavailable flavonoid glucosides in fruit juices and green tea by use of fungal α-L-rhamnosidases. *Journal of Agricultural and Food Chemistry, 52*, 6136–6142.
Goupy, P., Dufour, C., Loonis, M., & Dangles, O. (2003). Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *Journal of Agricultural and Food Chemistry, 51*, 615–622.
Kim, W. K., Bang, M. H., & Kim, E. S. (2005). Quercetin decreases the expression of ErbB2 and ErbB3 proteins in HT-29 human colon cancer cells. *The Journal of Nutritional Biochemistry, 16*, 155–162.
Kwon, O., Eck, P., Chen, S., Corpe, C. P., Lee, J. H., Kruhlak, M., et al. (2007). Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *The FASEB Journal, 21*, 366–377.
Masuoka, N., Matsuda, M., & Kubo, I. (2012). Characterisation of the antioxidant activity of flavonoids. *Food Chemistry, 131*, 541–545.
Miller, H. E. (1971). A simplified method for the evaluation of antioxidants. *Journal of the American Oil Chemists' Society, 48*, 91.
Mizuma, T., Ohta, K., Hayashi, M., & Awazu, S. (1992). Intestinal active absorption of sugar-conjugated compounds by glucose transport system: Implication of improvement of poorly absorbable drugs. *Biochemical Pharmacology, 43*, 2037–2039.
Monks, A., Scudeiro, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., et al. (1991). Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *Journal of the National Cancer Institute, 83*, 757–766.
Morand, C., Manach, C., Crespy, V., & Remesy, C. (2000). Quercetin 3-O-beta-glucoside is better absorbed than other quercetin forms and is not present in rat plasma. *Free Radical Research, 33*, 667–676.
Nielsen, I. L. F., Chee, W. S. S., Poulsen, L., Offord-Cavin, E., Rasmussen, S. E., Frederksen, H., et al. (2006). Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: A randomized, double-blind, crossover trial. *Journal of Nutrition, 136*, 404–408.
Nijveldt, R. J., van Nood, E., van Hoorn, D. E., Boelens, P. G., van Norren, K., & van Leeuwen, P. A. (2001). Flavonoids: A review of probable mechanisms of action and potential applications. *The American Journal of Clinical Nutrition, 74*, 418–425.
Paravicin, T. M., & Touyz, R. M. (2008). NADPH oxidases, reactive oxygen species, and hypertension: Clinical implications and therapeutic possibilities. *Diabetes Care, 31*, 171–180.
Park, J. S., Rho, H. S., Kim, D. H., & Chang, I. S. (2006). Enzymatic preparation of kaempferol from green tea seed and its antioxidant activity. *Journal of Agricultural and Food Chemistry, 54*, 2951–2956.
Potapovich, A. I., & Kostyuk, V. A. (2003). Comparative study of antioxidant properties and cytoprotective activity of flavonoids. *Biochemistry, 68*, 514–519.
Reinboth, M., Wolffram, S., Abraham, G., Ungemach, F. R., & Cermak, R. (2010). Oral bioavailability of quercetin from different quercetin glycosides in dogs. *British Journal of Nutrition, 104*, 198–203.
Santos, B. L., Silva, A. R., Pitanga, B. P. S., Sousa, C. S., Grangeiro, M. S., Fragomeni, B. O., et al. (2011). Antiproliferative, proapoptotic and morphogenic effects of the flavonoid rutin on human glioblastoma cells. *Food Chemistry, 127*, 404–411.
Sheu, J. R., Hsiao, G., Chou, P. H., Shen, M. Y., & Chou, D. S. (2004). Mechanisms involved in the antiplatelet activity of rutin, a glycoside of the flavonol quercetin, in human platelets. *Journal of Agricultural and Food Chemistry, 52*, 4414–4418.
Tang, D.-Q., Wei, Y.-Q., Gao, Y.-Y., Yin, X.-X., Yang, D.-Z., Mou, J., et al. (2011). Protective effects of rutin on rat glomerular mesangial cells cultured in high glucose conditions. *Phytotherapy Research, 25*, 1640–1647.
Vila-Real, H., Alfaia, A. J., Bronze, M. R., Calado, A. R. T., & Ribeiro, M. H. L. (2011). Enzymatic synthesis of the flavone glucosides, prunin and isoquercetin, and the aglycones, naringenin and quercetin, with selective α-L-rhamnosidase and β-D-glucosidase activities of Naringinase. *Enzyme Research*, 1–11. Article ID 692618.
Wolffram, S., Blöck, M., & Ader, P. (2002). Quercetin-3-glucoside is transported by the glucose carrier SGLT1 across the brush border membrane of rat small intestine. *Journal of Nutrition, 132*, 630–635.
Yadav, P. K., Yadav, S., & Yadav, K. D. S. (2010). α-L-rhamnosidase: A review. *Process Biochemistry, 45*, 1226–1235.
You, H. J., Ahn, H. J., & Ji, G. E. (2010). Transformation of rutin to antiproliferative quercetin-3-glucoside by *Aspergillus niger*. *Journal of Agricultural and Food Chemistry, 58*, 10886–10892.
Ziaee, A., Zamansoltani, F., Nassiri-Asl, M., & Abbasi, E. (2009). Effects of rutin on lipid profile in hypercholesterolemic rats. *Basic & Clinical Pharmacology & Toxicology, 104*, 253–258.