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Conversion of Rutin to Quercetin by Acid Treatment in Relation to Biological Activities

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ABSTRACT: De-glycosylation could be an important process for enhancing the biological activities of flavonoids. In this study we investigated de-glycosylation of rutin by acid treatment by comparing hydrolysis of rutin to quercetin with two different solvents and acid concentrations. Antioxidant activity was measured using chemical methods and biological activities were examined in cell-based systems. Rutin hydrolysis occurred more rapidly when 80% ethanol was used as the reaction solvent (as compared to water), and the rate of hydrolysis accelerated as acid concentrations increased. In reactions of rutin with 0.5 M HCl in 80% ethanol for 3 h, almost all the rutin was converted into quercetin. 2,2-Diphenyl-1-picrylhydrazyl, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical scavenging activities, and reducing powers were correlated with conversion rate. Protective activity in HepG2 cells, anti-inflammatory activity in RAW264.7 cells, and anti-adipogenic activity were increased with increased conversion of rutin to quercetin. This study suggests that de-glycosylation of glycoside flavonoids may increase physiological activity and, therefore, enhance its use in various fields.

Keywords: conversion, rutin, quercetin, acid treatment, biological activity

INTRODUCTION

Rutin (quercetin-3-*O*-rutinose), which is composed of one molecule of quercetin (3,3',4',5,7-pentahydroxyflavone) as aglycone and rutinose, is widely distributed as secondary metabolites in apple trees, onion plants, and buckwheat (Wang et al., 2011). Rutin and its aglycone (quercetin) possess numerous biological activities including anti-inflammatory (Guardia et al., 2001), anti-carcinogenic, and anti-oxidant activities (Burda and Oleszek, 2001). The molecular structures of rutin and quercetin are similar, however quercetin does not possess rutinoside at C3. The antioxidant properties of flavonoid glycosides are reported to decline as the number of glycosidic moieties increase (Xiao, 2017). The aglycone is likely to have a greater biological effect than the glycoside flavonoids (Williamson et al., 1996). Aglycones are readily absorbed by the small intestine, whereas flavonoid glycosides must be converted into aglycone forms (Hollman et al., 1999). De-glycosylation of flavonoids could therefore be important for increasing their biological activities.

Microorganisms isolated from human intestines are used in conversion methods for flavonoid de-glycosylation (Braune and Blaut, 2011). The bioactivity of citrus flavonoids has been reported to significantly increase after enzymatic conversion (da Silva et al., 2013). Other studies have reported use of quercetin produced from separation of enzymes capable of hydrolyzing rutin in tartary buckwheat (*Fagopyrum tataricum*) to enhance biological activities (Cui and Wang, 2012; Yasuda and Nakagawa, 1994). Disadvantages of these biological conversion methods include dependence on substrate specificity and that the conditions must be optimized for each reaction (Yu et al., 1998).

Acid treatment can be used to convert flavonoid glycosides to aglycones for flavonoid analysis in foods (Crozier et al., 1997; Hertog et al., 1992). Chemical methods using acid can overcome the disadvantages of biological methods through their simple and fast reaction times. In this study, effects of different concentrations of HCl and

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solvents on the conversion of rutin to quercetin were evaluated, and the biological activities of hydrolyzed rutin during the reaction were measured.

MATERIALS AND METHODS

Chemicals

Rutin, quercetin-3-glucoside, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and Trolox® were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade water, ethanol, *O*-phosphoric acid, and acetonitrile used in HPLC analysis were purchased from Burdick and Jackson (Muskegon, MI, USA).

Acidic hydrolysis of rutin

About 60 mg of rutin was weighed and dissolved in 80 mL acidic solvent (80% ethanol with 0.0, 0.1, 0.5, and 1.0 M HCl in water). Solutions were shaken in a water bath shaker at 75°C for 0 to 5 h with a reflux condenser. When the reaction was complete, reactants were cooled in cold water. The crude hydrolysis products were made up to a volume of 100 mL using solvents. The solvent of reactants was eliminated by use of a vacuum evaporator and lyophilization. The residue was dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C until use.

Analytical method for flavonoids and antioxidant

Rutin and quercetin were quantified using a HPLC-UV system (Shin et al., 2016). DPPH and ABTS radical scavenging activities were estimated according to the method described by Kong et al. (2008) with some modifications. DPPH in methanol (1 mL, 0.2 mM) were mixed with 50 μL of reactants. Mixtures were vigorously shaken and left to stand for 30 min in the dark. The absorbance at 520 nm was measured against water as a blank. For ABTS radical scavenging activity assays, stock solutions comprised of 7.4 mM $ABTS^+$ solution and 2.6 mM potassium persulfate solution, and reactions were allowed to react for 12 h at room temperature in the dark. Solution was then diluted by mixing $ABTS^+$ solution with water to obtain an absorbance of 1.00±0.02 units at 735 nm using a spectrophotometer. The ABTS working solution (1 mL) was mixed with 50 μL of the reaction solution. The mixtures were vigorously shaken and left to stand for 60 min in the dark. The absorbance at 735 nm was measured using water as a blank. The reducing power was determined according to the method described by Lue et al. (2010). Briefly, hydrolyzed rutin solution (250 μL), sodium phosphate buffer (250 μL, 200 mM, pH 6.6) and 1% potassium ferricyanide (250 μL) were mixed and incubated in a water bath at 50°C. After 20 min, 250 μL of 10% trichloroacetic acid (w/v) was added to the mixture and centrifuged at 1,000 rpm (240 g) for 10 min. The supernatant (500 μL) was then mixed with equal volume of distilled water and ferric chloride solution (0.1 %, w/v). The absorbance was measured at 700 nm using a spectrophotometer.

Cell culture

RAW264.7 macrophages and 3T3-L1 adipocytes were cultured as previously described. Cell viability, nitric oxide (NO) content, and lipid accumulation assays were carried out according to the method by Namkoong et al. (2017). NO production was determined by measuring the amount of nitrite, a relatively stable NO oxidation product. Briefly, RAW264.7 cells (1×10^5 cells/well) were seeded in 96-well plates and incubated for 6 h. Cells were treated with or without lipopolysaccharide (LPS, 1 μg/mL) for 18 h. Culture medium of each sample (100 μL) was then mixed with the same volume of Griess reagent (Sigma-Aldrich Co.) and incubated at 37°C for 10 min. Absorbance was measured at 550 nm. NO concentrations were determined using dilutions of sodium nitrite as standards. Intracellular lipid accumulation was measured using the Oil red O staining method. 3T3-L1 adipocytes treated for 6 days. In brief, cells were washed twice with cold phosphate-buffered saline (PBS), fixed in 10% neutral formalin for at least 20 min at room temperature, and then washed twice with PBS. Lipid droplets in cells were then stained with 0.3% Oil red O. Cells were washed exhaustively with distilled water and the staining dye was extracted with isopropyl alcohol; the absorbance was then measured at 490 nm. Viability of human liver hepatoma cells (HepG2) was measured according to the method by Noh et al. (2018), using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays. Cells were seeded in 96-well plates and allowed to adhere overnight. After 24 h, culture medium containing samples was added to each well and the cells were incubated for 24 h; untreated cells were used as a control. Culture media was then replaced with MTT (0.5 mg/mL), and samples were incubated in the dark for 4 h. Violet formazan crystals were dissolved in DMSO and absorbance was quantified using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 550 nm. Cell viability (%) was calculated by comparing absorbance of the samples with that of the control.

Statistical analysis

Results are expressed as mean±standard error (SE) and are representative of three or more independent experiments. Statistical analysis of the results was performed using the general linear model procedure from SAS version 9.0 (SAS Institute Inc., Cary, NC, USA). $P<0.05$ was used to indicate statistical significance.

RESULTS AND DISCUSSION

The effects of solvent, HCl concentration, and reaction time on rutin deglycosylation

Multiple studies have reported that rutin has weaker anti-inflammatory and antioxidant activities than quercetin, which may be attributed to the presence of a glycoside moieties (Nguyen et al., 2013; Moretti et al., 2012). Rutin de-glycosylation can be used as an important method to increase its biological activities (de Araújo et al., 2013; You et al., 2010). In this study, we used acidic hydrolysis to break the glycosidic bonds of rutin. The effects of various concentrations of HCl in two reaction solvents, water and 80% ethanol, are shown in Fig. 1. The conversion rate of rutin to quercetin was very slow when reactants were treated with weak organic acids such as citric acid, acetic acid, and malic acid (data not shown). It has been previously shown that strong acids and high temperatures lead to rapid rutin hydrolysis (Wang et al., 2011). We showed that rutin de-glycosylation was faster in 80% ethanol than water. It is predicted that flavonoid solubility affects the rate of reaction; rutin and quercetin both show higher solubility in 80% ethanol than water (Zi et al., 2007). When 1.0 M HCl was diluted in water, almost all the rutin was hydrolyzed to quercetin within 3 h, whereas, with when 1.0 M HCl was diluted in 80% ethanol, most of the rutin was converted to quercetin within 1 h. Therefore, we demonstrate that 1.0 M HCl diluted in 80% ethanol provides the most efficient conditions for converting rutin to quercetin.

Change of antioxidant activities during rutin acid treatment

Some studies have reported that aglycone flavonoids are stronger antioxidants than their corresponding glycosides (Gao et al., 1999; Heim et al., 2002). Therefore, conversion of rutin to quercetin is an important strategy to increase antioxidant activity. This study examined changes in antioxidant activities during the acidic hydrolysis of rutin. The effect of different concentrations of HCl diluted in water and 80% ethanol on the antioxidant activities of hydrolyzed rutin are shown in Fig. 2. Radical scavenging activities and reducing powers were slightly increased in conditions of 0.1 M HCl in water, and antioxidant activities increased with HCl concentration in a

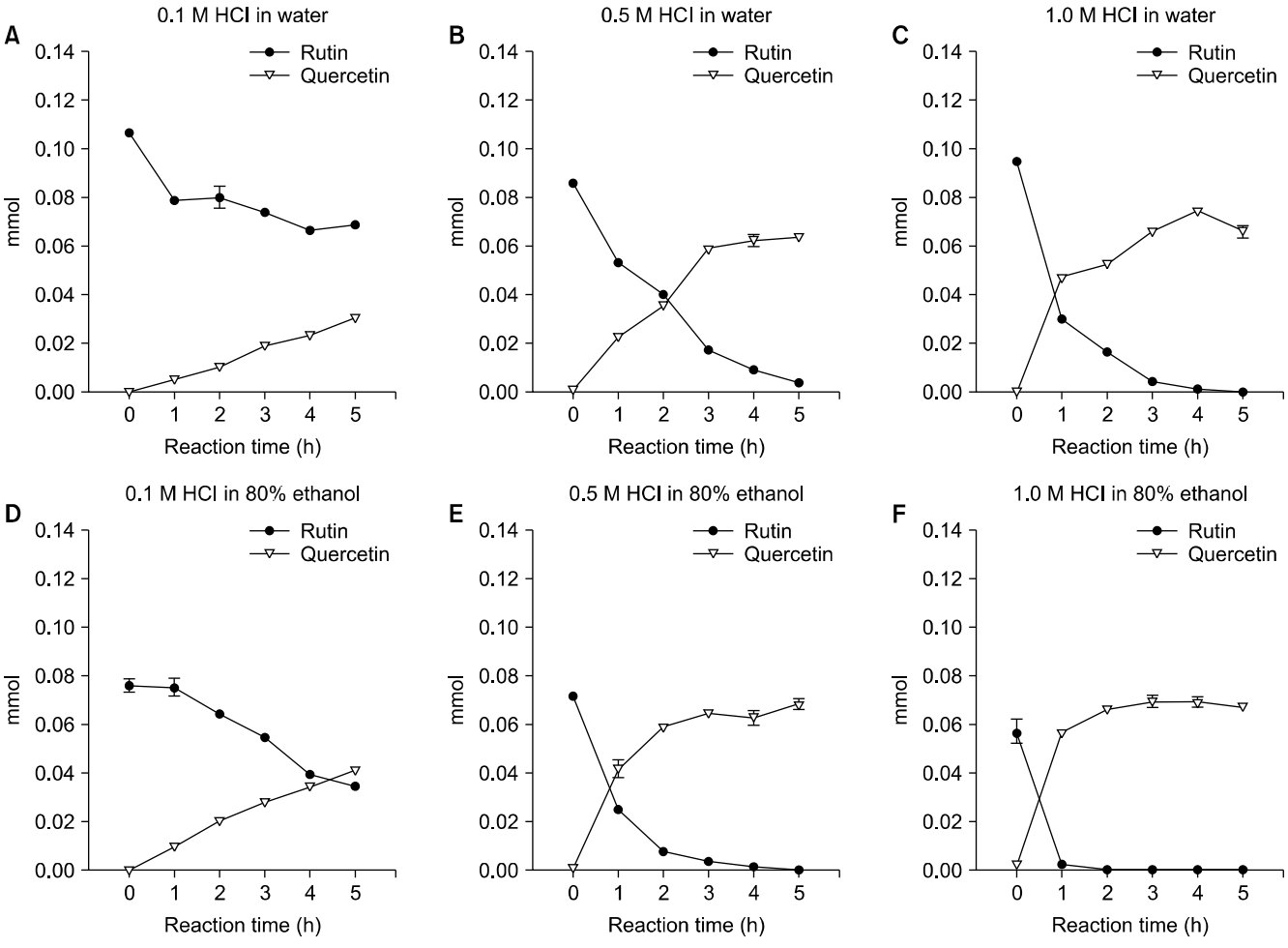

Fig. 1. The effects of solvent, HCl concentration, and reaction time on rutin deglycosylation. (A) 0.1 M HCl, (B) 0.5 M HCl, and (C) 1.0 M HCl in water, and (D) 0.1 M HCl, (E) 0.5 M HCl, and (F) 1.0 M HCl in 80% ethanol. The de-glycosylation reaction was conducted at 75°C for 0~5 h.

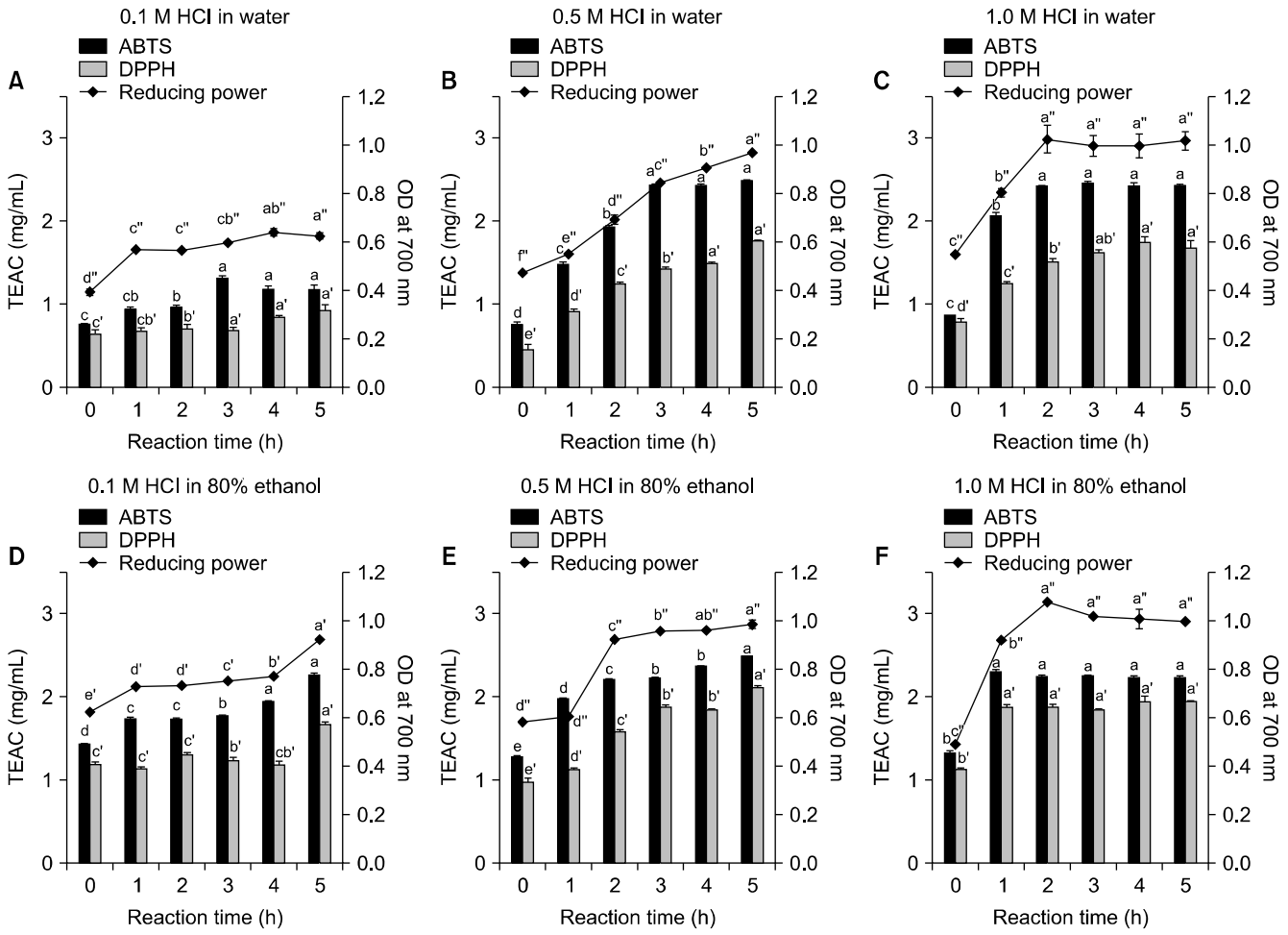

Fig. 2. The 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities, and reducing power of rutin reacted in water or 80% ethanol with various concentration of HCl and reaction time. (A) 0.1 M HCl, (B) 0.5 M HCl, and (C) 1.0 M HCl in water, and (D) 0.1 M HCl, (E) 0.5 M HCl, and (F) 1.0 M HCl in 80% ethanol. The used concentration of hydrolyzed rutin is 30 μg/mL. The radical scavenging activities were expressed as Trolox equivalent antioxidant capacity (TEAC) and reducing power was expressed as absorbance at 700 nm. Each data represents the mean of triplicates. Error bars indicate the standard error (n=3). Mean values with different letters (a-f) are significantly different (P<0.05).

dose-dependent manner. These results indicate that conversion of rutin to quercetin is crucial for increasing its antioxidant activities. In conditions of 0.5 M HCl in 80% ethanol, rapid increases in ABTS radical scavenging activities and reducing powers were reached within 2 h. However, when using the same concentrations of HCl in water, highest ABTS radical scavenging activities and reducing powers were achieved after 3 h and 5 h, respectively. These results indicate that the antioxidant activities of hydrolyzed rutin can be more rapidly altered in reactions containing 80% ethanol.

The protective effect of acid-treated rutin on oxidative stress

Multiple studies have demonstrated that aglycone flavonoids show higher hepatoprotective activities than glycoside flavonoids in cell systems (Heim et al., 2002; Ratty and Das, 1988). Naringin is a flavonone glycoside found in fruits. The aglycone of naringin and naringenin, is attached to rutinose, and both naringin and naringenin show strong antioxidant activities in cell systems. However, naringin is reportedly less potent than naringenin due to steric hindrance of sugar moieties of the scavenging group (Alam et al., 2014). Hesperidin and its aglycone and hesperetin, are flavonoids from citrus species that have various biological properties, particularly hepatoprotective properties involved in preventing related diseases (Roohbakhsh et al., 2015). Hesperetin has been reported to exhibit higher hepatoprotective effects than hesperidin by increasing the activities of various antioxidant enzymes (Parhiz et al., 2015). Accordingly, conversion of rutin to quercetin is expected to be effective for protecting against oxidative stress in HepG2 liver cells. This study examined changes in the protective effect of hydrolyzed rutin against oxidative stress induced by *tert*-butyl hydroperoxide (TBHP) in HepG2 liver cells. We showed that hydrolyzed rutin (15 μg/mL) does not affect viability of HepG2 liver cells (Fig. 3). When using both solvents, the protective effect increased as acid concentration and reaction time increased. At a concentration of

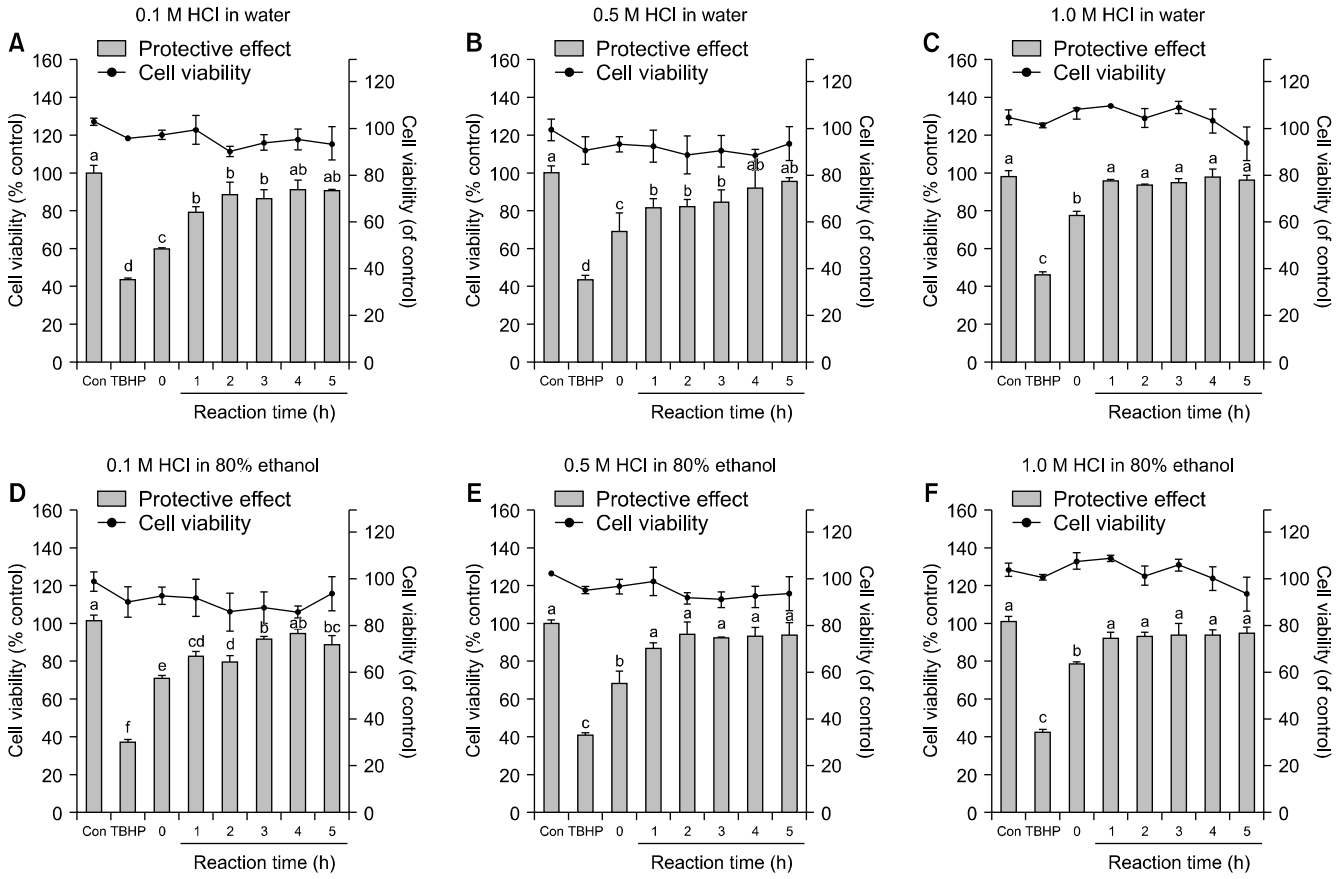

Fig. 3. The protective effect of rutin hydrolyzed in water or 80% ethanol with various HCl concentrations and reaction time on *tert*-butyl hydroperoxide (TBHP, 500 μM) induced cytotoxicity. (A) 0.1 M HCl, (B) 0.5 M HCl, and (C) 1.0 M HCl in water, and (D) 0.1 M HCl, (E) 0.5 M HCl, and (F) 1.0 M HCl in 80% ethanol. The used concentration is 15 μg/mL. Cell viability was expressed as percent of the control group. Each data point represents the mean of triplicates. Error bars indicate standard error (n=3). Mean values with different letters (a-f) are significantly different (P<0.05).

0.1 M HCl, 80% ethanol showed a higher protective effect than water over the same reaction period. These results demonstrate that acid hydrolysis both increases its radical scavenging activity of rutin and increases protective effects against oxidative stress induced by TBHP in cell systems.

The inhibitory effect of acid-treated ruin on NO production

NO is a mediator of inflammatory responses and is considered a harmful molecule during inflammatory processes (Nathan, 1992). Several flavonoids, including apigenin, hesperidin, luteolin, and quercetin, reportedly show anti-inflammatory effects by inhibiting NO production (Kumar and Pandey, 2013); these flavonoids are aglycones. Moreover, querceitin, gossypetin, and naringenin show greater inhibitory effects on inflammation than their respective glycoside flavonoids by inhibiting cyclooxygenases, key enzymes for inflammatory reactions (Moroney et al., 1988). Many studies have reported that quercetin has greater anti-inflammatory effects than rutin in cell model systems (Chen et al., 2001; Hämäläinen et al., 2007). Therefore, conversion of rutin to quercetin is one strategy to increase its anti-inflammatory effects. In this study, we examined the change in the anti-inflammatory effects during rutin acid hydrolysis reactions by inhibiting NO production in RAW264.7 macrophages. The cytotoxicity of hydrolyzed rutin in RAW264.7 macrophages was assessed using MTT assay; we showed that 20 μg/mL did not affect the macrophage viability (Fig. 4). Upon LPS (1 μg/mL) treatment, nitrate concentrations increased by about 18-fold. After a 3 h rutin hydrolysis, NO production was inhibited by 54.62%, 64.84%, and 82.22% compared to LPS when incubated with 0.1 M, 0.5 M, and 1.0 M HCl in water, respectively. For rutin hydrolysis in 80% ethanol, NO production was inhibited by 54.51%, 82.52%, and 88.98% with 0.1 M, 0.5 M, and 1.0 M HCl, respectively. Consistent with the previous results, the inhibitory effect of NO production during rutin hydrolysis was greater when 80% ethanol was used as the reaction solvent in comparison to water.

The inhibitory effect of acid-treated ruin on lipid accumulation

Some studies have reported anti-obesity effects of rutin and quercetin, and others have reported that quercetin,

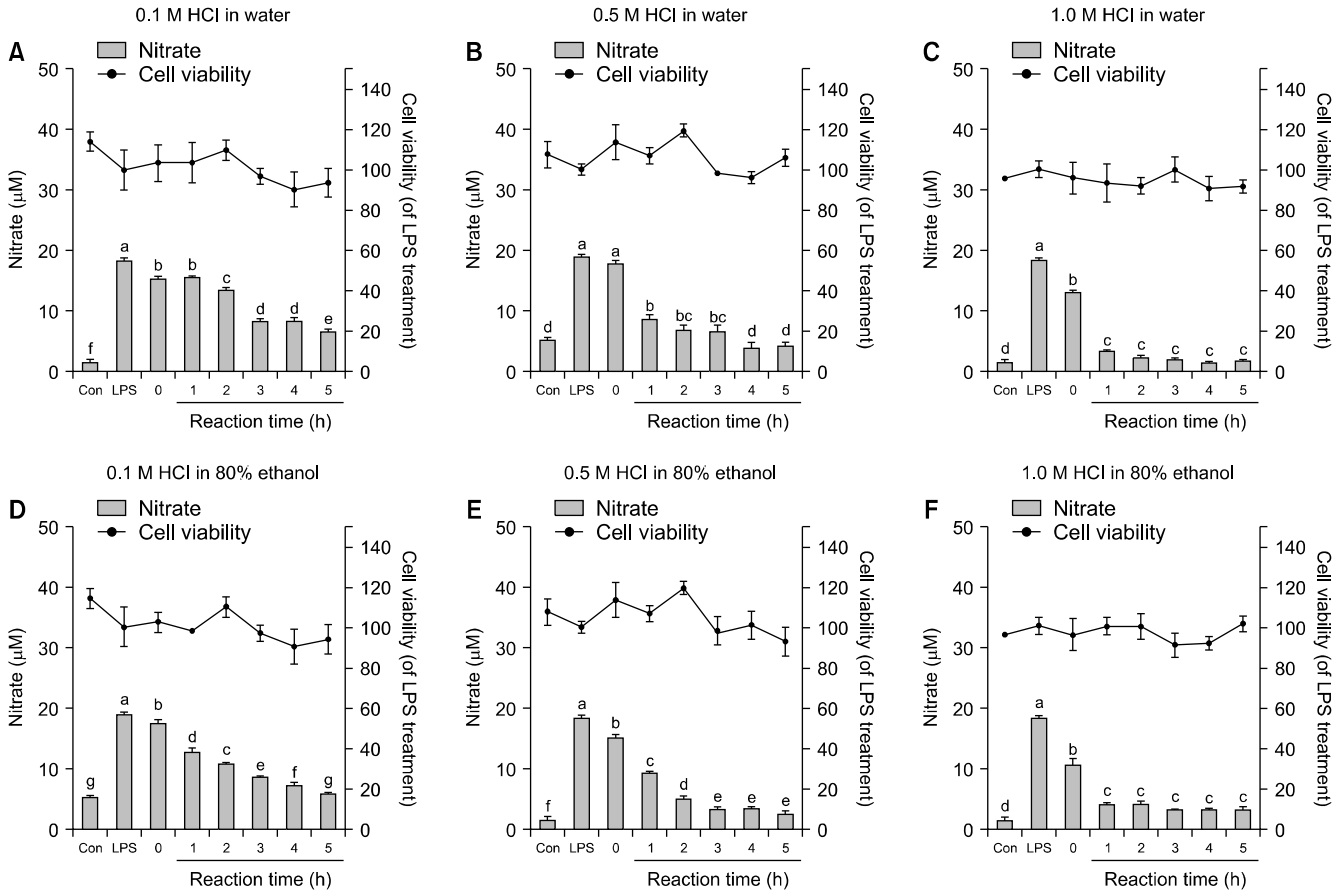

Fig. 4. The inhibitory effect of rutin reacted in water or ethanol with various concentrations of HCl and reaction time on nitrate production induced by lipopolysaccharide (LPS). (A) 0.1 M HCl, (B) 0.5 M HCl, and (C) 1.0 M HCl in water, and (D) 0.1 M HCl, (E) 0.5 M HCl, and (F) 1.0 M HCl in 80% ethanol. The used concentration is 15 μg/mL. NO production was expressed as μM of nitrite, and cell viability was expressed as percent of LPS-treated cells. Each data point represents the mean of triplicates. Error bars indicate standard error (n=3). Mean values with different letters (a-g) are significantly different (P<0.05).

an aglycone flavonoid, has higher biological activities than rutin (Shen et al., 2002; Ahn et al., 2008). Our results show that hydrolyzed rutin (30 μg/mL) does not affect viability of 3T3-L1 adipocytes (Fig. 5). Hydrolysis of rutin for 1 h with 0.5 M HCl in water or 80% ethanol resulted in 86.41% and 76.96% lipid accumulation, respectively. Moreover, the rate of triglyceride accumulation decreased with higher HCl concentration due to increased generation of quercetin by rutin hydrolysis. These results suggest that quercetin, which is produced by rutin acid hydrolysis, has higher anti-adipogenic effects than rutin.

In conclusion, rutin hydrolysis occurred more rapidly in 80% ethanol than in water and was accelerated with increased HCl concentrations. Since pH is an important factor for flavonoid hydrolysis, 0.5 M HCl in water or 80% ethanol resulted in conversion of almost all available rutin to quercetin within approximately 3 h. We also investigated whether this conversion affects the physiological activities of rutin/quercetin. We confirmed that conversion of rutin to quercetin increases its antioxidant, anti-inflammatory, and anti-adipogenic activities.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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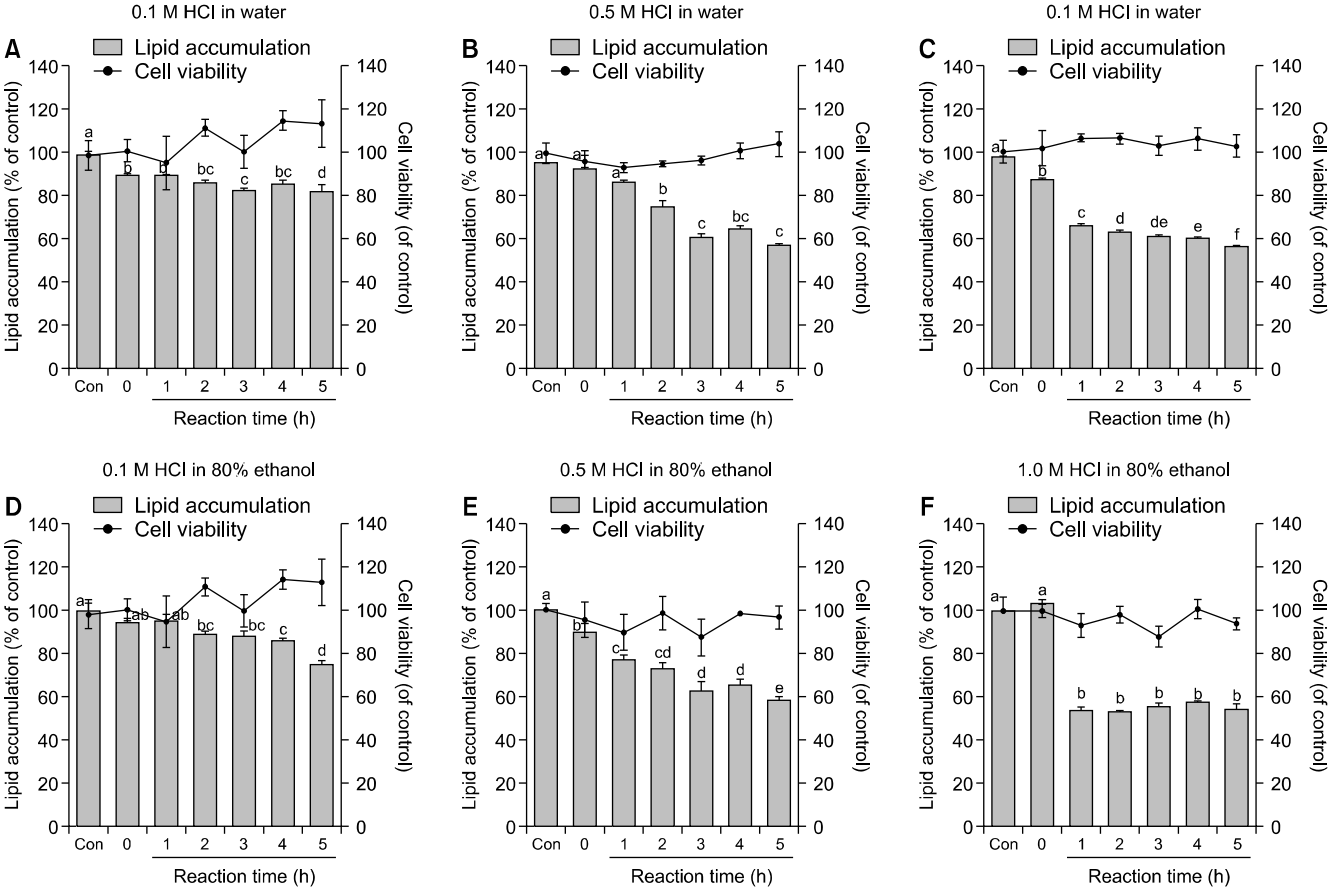

Fig. 5. The inhibitory effect of rutin reacted in water or ethanol with various concentrations of HCl and reaction time on lipid accumulation. (A) 0.1 M HCl, (B) 0.5 M HCl, and (C) 1.0 M HCl in water, and (D) 0.1 M HCl, (E) 0.5 M HCl, and (F) 1.0 M HCl in 80% ethanol. The used concentration is 30 μg/mL. Cell viability and lipid accumulation was expressed as percent of the control group. Each data point represents the mean of triplicates. Error bars indicate standard error (n=3). Mean values with different letters (a–f) are significantly different (P<0.05).

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