Phenylacetic acids were detected in the plasma and urine of rats administered with low-dose mulberry leaf extract

Chooi Yeng Leea,⁎, Si Mui Simb, Hwee Ming Chenga

aDepartment of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia
bDepartment of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia

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Abstract

The use of a high quercetin dose to demonstrate its absorption and bioavailability does not reflect the real dietary situation because quercetin glycosides are usually present in small amounts in the human diet. This study aimed to demonstrate the absorption and bioavailability of quercetin in mulberry leaves that represents a more physiologic dietary situation. Mulberry leaf ethanol extract was prepared similar to tea infusion, which is the way the tea leaves are generally prepared for consumption. Accordingly, rats were fed by oral intubation the mulberry leaf ethanol extract (15 g%/rat per day) or pure rutin (135 μg/rat per day) for 2 weeks. The control group received a similar volume of the vehicle, 10% ethanol. There was a significant increase in total antioxidant activity (TAA) in the urine and feces of the antioxidants-fed rats. Phenylacetic acid, a microbial metabolite of quercetin, was detected in the urine of the test animals, and quercetin was present in the fecal samples. By using an in situ intestinal preparation, 3-hydroxyphenylacetic acid, another microbial metabolite of quercetin, was detected in the plasma when the duodenal segment was instilled with 2 mg of rutin. This microbial metabolite retained 50% of the TAA of quercetin. The results of this study indicate that in a more realistic dietary situation, an increase in TAA in the body after consumption of quercetin-containing foods is contributed mainly by the microbial metabolites.

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1. Introduction

Mulberry (Morus alba L) plant was originally grown in China and has had wide use in both the agricultural and medicinal fields. Its leaves, bark, and branches have been used to treat fever, strengthen the joints, improve eyesight, reduce blood pressure, and the same can be used as a diuretic and liver tonic [1-4]. Mulberry leaves have antihyperglycemic effect because of the presence of an α-glucosidase inhibitor, 1-deoxynojirimycin [5,6], and fagomine, which potentiates glucose-induced insulin secretion [7]. The mulberry leaf extract has neuroprotective effects [8], and it can be used as a skin whitening agent [9] and antiinflammatory compound [10]. Isoquercitrin [11], rutin, and quercetin 3-(6-malonylglucoside) [12,13] in the mulberry leaf ethanol extract are able to inhibit low-density lipoprotein oxidation. Rutin is the major compound in the mulberry leaf ethanol extract. Mulberry leaves are now widely consumed as tea infusion.

Increased popularity in the intake of plant-based food antioxidants has prompted extensive research on their absorption and bioavailability in both human and animals. Studies on the bioavailability of dietary antioxidants, which provide evidence of the efficacy of antioxidants in the body, are more relevant than those that just report on their in vitro antioxidant properties. However, most studies involved the use of high-dose antioxidants, which range from 50 to 250 mg [14-19]. Although plasma concentration of quercetin rarely exceeds 1 μmol/L even with the consumption of 100 mg of quercetin from plant foods [20,21], the use of high-dose antioxidants to demonstrate their bioavailability...
must be justified. Because the daily intake of polyphenols is low [22], about 20 to 35 mg/d for flavonols [23], it is more realistic to also use a dose closer to the one ingested generally when these studies are carried out. Nevertheless, because of high variability in polyphenols intake, mainly because of variations in individual food preferences, and because of difficulty in evaluating polyphenols intake by using dietary questionnaires [21], a simple and direct approach is to adopt a general preparation for a consumable plant extract. Therefore, the mulberry leaf extract that was tested in this study was prepared by soaking the mulberry leaves in solvent used for extraction, a simulated tea preparation. The downside of this design is the limitation of the dose of mulberry leaf extract that can be prepared. Bioavailability of polyphenols is largely influenced by their chemical structures [20]. Clearly, bioavailability of quercetin from different food sources varies, depending on the types of glycosides that are bound to quercetin [23]. Low quercetin bioavailability has always been attributed to its poor absorption because of the presence of a sugar moiety at the C-ring of quercetin. However, bioavailability is a subject that integrates several variables. These include the intestinal efflux of glucuronides into the intestinal lumen, metabolism by microflora, and biliary and urinary excretions. Increased information on these aspects will help to better assess bioavailability of a compound. We hypothesized that with the low intake of polyphenols by the human, any increase of total antioxidant activity (TAA) in the body is contributed by the microbial metabolites.

The main objective of this study was to show oral absorption and bioavailability of quercetin that resembled more closely the actual dietary intake. Mulberry leaf extract, prepared closely to its general dietary intake, was orally administered to rats. Rutin, prepared at a dose equivalent to the amount found in the leaf extract, was also given to rats for comparison purpose. In another series of experiments, rutin was instilled into the intestinal segment using an in situ intestinal model. This study is important because we showed that apart from poor absorption, bioavailability of quercetin glycosides is greatly dependent on their extensive microbial metabolism in the intestine. Quercetin and its hepatic metabolites were not detected in the plasma and urine samples, unlike the observations reported by others [24]. Instead, phenylacetic acids, the major microbial metabolites of quercetin and rutin, were found in these samples. An increase in total antioxidant activity (TAA) in the body from the ingestion of dietary antioxidants is more likely to be contributed by the microbial metabolites.

2. Methods and materials

2.1. Rats

Sprague-Dawley rats of both sexes (n = 53) were housed, 2 per cage, in temperature-controlled rooms (25°C), with a dark period from 6:00 PM to 7:00 AM. The rats were fed with standard pelleted food and tap water. The pelleted food was composed mainly of protein, 21%; fiber, 5%; fat, 3%; moisture, 13%; ash, 8%; and nitrogen-free extract, 49% (Gold Coin Feedmills, Kuala Lumpur, Malaysia). Before the experimental procedure, rats were fasted 12 to 18 hours by placing them individually in metabolism cages. The Animal Care and Use Committee, Faculty of Medicine, University of Malaya (Kuala Lumpur, Malaysia) approved all surgical procedures carried out on the rats.

2.2. Mulberry leaf extract and rutin preparations

Mulberry leaf extract was prepared using a procedure as described previously [25]. In this study, an ethanol extract of mulberry leaves (7.5 g of mulberry leaves in 10 mL of ethanol) and 675 μg/mL of pure rutin in ethanol were prepared. These samples were diluted 10 times with water for the oral dosing. A 10% ethanol (1:9 ethanol-water, vol/vol) solution is often used as a vehicle to dissolve lipophilic compounds for absorption studies in animals [26].

2.3. Experimental design and collection of the biological samples

In the 2-week oral feeding study, rats were randomly divided into 3 groups (n = 6 for each group) as follows: the control, the ethanol extract-treated, and the rutin-treated groups. Predose urine and feces were collected for 24 hours before the start of the dosing regimen, whereas predose plasma (0.5 mL) was collected from the tail vein at about 17 hours before dosing. Mulberry leaf extract (7.5 g%/rat) or rutin (67.5 μg/rat) was given orally (1 mL) to a rat twice daily, 6 hours apart. The control group was given a similar volume of 10% ethanol. Twenty-four-hour urine samples were collected everyday after the start of the regimen, whereas 24-hour fecal samples were collected only on day 14, which was the last day of the dosing regimen. Plasma (0.5 mL) collection from blood of tail veins was performed on days 4, 7, 9, 11, and 14, each time at 7 hours postdosing. All samples were subjected to TAA measurement. High-performance liquid chromatography (HPLC) analysis was also carried out on urine and fecal samples. Hydrochloric acid at 1 mol/L was added into each urine sample at a ratio of 1:2 before storage at −20°C.

In the in situ rat intestinal preparation, rutin (6.75 μg) was instilled into the isolated duodenal, jejunal, and ileal segments (n = 5 for each segment) using a method described previously [25]. Control rats were given 10% ethanol. The experiment was repeated for a higher dose of rutin (2 mg) instilled into the duodenum (n = 5). Plasma samples were subjected to TAA measurement and HPLC analysis. Before HPLC analysis, the plasma sample was acidicified with 10 mmol/L acetic acid before storage at −20°C.

2.4. Sample preparations for TAA measurement

Urine collected for TAA measurement was diluted 60 times with 50 mmol/L of sodium acetate buffer (pH 5.0). Plasma samples were diluted 30 times with purified water for the assay.
For the fecal samples, the following procedure was carried out based on the method used by Manach et al [24] with slight modifications. The feces (0.5 g) of the control, ethanol extract-treated, and rutin-treated rats were defatted in 20 volumes of hexane by heating at 65°C for 2 hours. This process was repeated twice. The hexane extracts were discarded. The feces were then extracted in 10 volumes of absolute ethanol. The extraction process, twice repeated, was carried out at 75°C for 30 minutes. The fecal ethanol extracts were collected and diluted 30 times with water for the antioxidant assay.

2.5. Antioxidant assay using the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt reagent

This reagent was prepared according to the method described by Pellegrini et al [27]. When used for analysis, the stock reagent was diluted 20 times with phosphate buffered saline (5 mmol/L, pH 7.4). All the biological samples were dispensed in triplicates of 20 μL each into a 96-well microplate. 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) solution (200 μL) was added, and the mixtures were incubated at room temperature for 6 minutes. Absorbance was read at 630 nm (Sunrise Absorbance Reader, Tecan, Austria). Water in place of the biological sample was used as the negative control.

Total antioxidant activity was expressed as the percentage of inhibition and calculated as follows:

\[
\text{Percentage inhibition (\%)} = \frac{A_{630 \text{ nm}(\text{negative control})} - A_{630 \text{ nm}(\text{sample})}}{A_{630 \text{ nm}(\text{negative control})}} \times 100
\]

For the urine samples, TAA was first expressed as ascorbic acid equivalent in millimoles per liter concentration, based on the ascorbic acid standard curve. Because samples have been diluted 60 times before the assay, the ascorbic acid equivalent obtained was multiplied by 60. Therefore, ascorbic acid equivalent (mmol) in each of the total urine collected was calculated as follows:

\[
\text{Ascorbic acid equivalent (mmol) = } \frac{\text{Ascorbic acid equivalent (mmol) \times 60 \times \text{total volume of the urine (μL)}}}{1 \times 10^6 \text{μL}}
\]

The urine ascorbic acid equivalent and the plasma TAA were normalized against that of the corresponding predose levels so that 1.0 represented the baseline value. The calculations for percentage inhibition and normalization of the values have been described previously [25].

2.6. Pretreatment of urine, fecal, and plasma samples for HPLC analysis

Each urine sample (250 μL) was added 20 μL of sulfatase (38 U in 0.1 mol/L of sodium acetate buffer, pH 5.0) and 20 μL of acetic acid (0.58 mol/L). The mixture was incubated at 37°C for 60 minutes. This was followed by the addition of 10 μL of 1 mol/L of HCl. The treated samples were twice extracted with ethyl acetate, at 400 and 300 μL, respectively, and vortexed for 30 seconds each time [28-30]. The mixture was centrifuged each time at 5000 revolutions per minute for 10 minutes. An aliquot of 550 μL of the combined supernatant was evaporated using purified nitrogen and then stored at −20°C. For HPLC analysis, the dried samples were redissolved in 100 μL of methanol, and 20 μL of the reconstituted sample was injected onto the HPLC column.

Ethanol extract of the feces was filtered using a GHP Acrodisc 13 syringe filter (Gelman Sciences Inc., Ann Arbor, Mich). The extract was subjected to HPLC analysis without additional preparations of the extract.

Plasma samples from the in situ experiments were pooled, and 300 μL of the pooled plasma samples (n = 2-3) was used for HPLC analysis. Hydrochloric acid at 1 mol/L (24 μL), 24 μL of sulfatase, prepared at 38 U, and 12 μL of 8 μg/mL fisetin in ethanol were added to the plasma. Fisetin was used as the internal standard. The mixture was incubated at 37°C for 60 minutes before another addition of 1 mol/L HCl (12 μL). Quercetin was extracted with 2 volumes of acetone [24] and vortexed for 30 seconds. The mixture was centrifuged at 5000 revolutions per minute for 10 minutes. Supernatant (870 μL) was collected, evaporated under purified nitrogen, and then stored at −20°C. For analysis, the dried samples were redissolved in 65 μL of methanol, and 20 μL of the reconstituted sample was injected into the HPLC system.

2.7. High-performance liquid chromatographic analysis for urine, fecal, and plasma samples

For urine samples, the HPLC system consisted of an LC-10A solvent delivery system (Shimadzu Corp., Kyoto, Japan), a Genesis C18 column (4 μm, 150 × 4.6 mm, Jones Chromatography USA, Inc., Lakewood, Colo), a guard column of the same packing material (Waters Corp., Milford, Mass), and a UV-VIS detector SPD-10A (Shimadzu, Japan) set at 370 nm. The reconstituted urine samples were eluted under isocratic condition with acetonitrile-methanol-water (20:7:73, vol/vol) containing 0.07% phosphoric acid at a flow rate of 1.5 mL/min. The chromatograms were recorded and integrated using a Chromatopac C-R8A data processor (Shimadzu, Japan).

Plasma and fecal samples were analyzed using the same HPLC system as described for urine samples. However, the reconstituted plasma and fecal samples were eluted under isocratic condition with acetonitrile-water (25:75, vol/vol plus 0.07% phosphoric acid) at a flow rate of 1.5 mL/min.

2.8. Statistical analysis

The mean TAA values (±SD of n = 5-6) for the plasma and fecal samples were calculated and analyzed for their significance between the control and the test groups using the unpaired Student’s t test (GraphPad Statistical Software Inc, San Diego, Calif). Differences were considered statistically significant for \( P < .05 \). Mean values of urine ascorbic acid equivalent were not calculated because of high variations in the interday individual profiles.
3. Results

In the oral feeding study, rats given the mulberry leaf extract (15 g%/rat per day) or rutin (135 μg/rat per day) did not show any significant increase in their plasma TAA throughout the 14-day study period when compared to the control (Fig. 1).

The daily excretion of ascorbic acid equivalent in urine for the mulberry leaf extract-treated group was generally higher than the control group. However, each animal’s urine profile showed large day-to-day fluctuations and peaked on different days throughout the 14-day study period. In addition, there were also high interindividual variations in the daily excretion of urinary ascorbic acid equivalent profiles. Consequently, the urine profile of each rat (n = 6 per group) is shown (Fig. 2). Mean values for all of the study groups were not calculated.

When the urine samples were analyzed using HPLC, a peak that corresponded to phenylacetic acid standard, a microbial metabolite of quercetin, was detected (retention time at 7 minutes) in the urine of the mulberry leaf extract-treated and rutin-treated rats on days 7, 13, and 14. This phenylacetic acid peak was not observed in the HPLC chromatograms of the control group. Representative HPLC profiles of the urine samples collected on day 14 for the control rats and rats given the mulberry leaf extract or rutin are shown (Fig. 3).

Fecal samples of the rats fed the mulberry leaf extract or rutin showed significantly higher TAA on day 14 than on day 0 (35%, $P < .001$). There was no significant difference of TAA between day 0 and day 14 in the control fecal samples (Fig. 4). This was evident because quercetin was detected (retention time at 10 minutes) in the fecal samples of the antioxidant-fed rats (Fig. 5).

In the in situ study, when rutin (6.75 μg) was instilled into the duodenum, a significant increase in the TAA level was observed at 180 minutes ($P < .01$). When a higher rutin dose (2 mg) was administered in the duodenum, it increased TAA levels at 150 and 180 minutes ($P < .05$) (Fig. 6). More important, HPLC analysis performed on plasma samples from rats given 2 mg of rutin in situ showed the presence of 3-hydroxyphenylacetic acid (retention time at 2.6 minutes), which is the major microbial metabolite of quercetin and rutin (Fig. 7). This observation has not been reported before.

![Fig. 1. Comparison of the plasma TAA profile of the control rats that were given 10% ethanol with that of the rats fed with mulberry leaf ethanol extract (15 g%/rat per day) or rutin (135 μg/rat per day) throughout the 14-day study period. The TAA of plasma was assayed using ABTS reagent. Values are means ± SD of measurements from 6 rats. Data were analyzed using unpaired t test.](image1)

![Fig. 2. Individual urine ascorbic acid equivalent profiles of the (A) control rats, and rats fed with (B) mulberry leaf ethanol extract (15 g%/rat per day) or (C) rutin (135 μg/rat per day) were obtained from the ascorbic acid standard curve. Mean ascorbic acid equivalent for each group that consists of 6 rats was not calculated because of high variations in the individual urine profile.](image2)
was no increase of plasma TAA from instillation of rutin (6.75 μg) into the jejunal and ileal segments (data not shown).

4. Discussion

Generally, a commercial tea bag contains approximately 2 g of dried tea leaves. Hertog et al [31] reported that tea leaves usually contain about 20 mg/kg of quercetin glycosides. Assuming that the extraction or infusion process is complete, 2 g of tea leaves will yield only 40 μg of dietary quercetin. This amount of quercetin glycosides is about 50 to 100 times less than the high dose used in most in situ and in vivo animal studies [29,32-34]. In this study, a 7.5 g% ethanol extract of mulberry leaves was given twice daily via oral intubation to a rat for 14 consecutive days. Daily consumption of rutin, the main antioxidant in the mulberry leaf ethanol extract, was equivalent to 135 μg. Although the rutin dose was low compared to most other studies, we think it is a more realistic approach because this is closer to the dietary situation. Moreover, ingestion of other antioxidants in the extract was postulated to be sufficient to raise the body TAA. Total antioxidant activity measurement is important in addition to the common practice of identifying quercetin and its metabolites in the plasma or urine as biomarkers of absorption and bioavailability. This is because increased levels of these compounds in the circulation may not necessarily be accompanied by an increase in TAA in the body [35].

Our results showed that rutin, given as a single compound, and a fraction of antioxidants in the mulberry leaves were absorbed. Because of the low doses of antioxidants given to the animals, an increase in plasma TAA, which would have indicated absorption, was not measurable in both the treated groups. This could be the result of dilution of a small quantity in the relatively large blood pool. Indirect evidence indicates that absorption did occur because high antioxidant activity, expressed as ascorbic acid equivalent, was found in the urine of both the mulberry leaf extract-treated and rutin-treated groups. However, the urine profiles showed large interindividual variations where a surge in the excretion of ascorbic acid equivalent occurred on different days for rats of the mulberry leaf extract-treated group. The urine profiles of control animals showed less day-to-day fluctuation.

Fig. 3. Representative HPLC chromatograms of the rat urine at day 14 are as follows: (A) control rats and (B) rats fed with the mulberry leaf ethanol extract (15 g%/rat per day) or (C) rutin (135 μg/rat per day). The chromatograms are representatives of 4 similar analyses on each urine sample. Phenylacetic acid, a microbial metabolite, was detected (arrow) in the urine of the rats treated with the mulberry leaf ethanol extract or rutin.

Fig. 4. Fecal samples of the control rats, and rats treated with the mulberry leaf ethanol extract (15 g%/rat per day) or rutin (135 μg/rat per day) were analyzed to determine the presence of unabsorbed antioxidants in the intestine. Values are means ± SD of measurements from 4 rats. Data were analyzed using unpaired t test. *** denotes P < .001.
Renal excretion is a minor pathway for quercetin [14], accounting for only 0.3 to 1.4% of the ingested dose. This implies that the quercetin glycosides either have not been absorbed from the gut, or have been absorbed and excreted in the bile, or have been metabolized by the colonic microflora before absorption [20]. In any case, these glycosylated or conjugated quercetin, including those that are present in the intestine because of efflux from the enterocytes, will be carried to the colon. High catalytic and hydrolytic activities take place in the colon, and deconjugation reactions readily occur [36]. This may account for the presence of microbial metabolites in the urine.

The presence of hydroxyl groups at the aromatic ring of quercetin aglycone increases the susceptibility of the aglycone to degradation by colonic microflora [37]. Quercetin degradation produces mainly 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 3-hydroxyphenylacetic acid [38]. Phenylacetic acids are the major metabolites of rutin, accounting for about half of the ingested rutin. They are subsequently absorbed from the colon before excretion in the urine [39,40]. In this study, we showed that one of the compounds in the urine, as detected in the HPLC analysis, was

Fig. 5. Representative HPLC chromatograms of the rat fecal samples at day 14 are as follows: (A) control rats and rats given (B) the mulberry leaf ethanol extract (15 g%/rat per day) or (C) rutin (135 μg/rat per day). The profiles are representatives of 4 similar analyses on each fecal sample. Quercetin was detected (arrow) in the feces of the treated rats.

Fig. 6. Absorption and bioavailability of dietary antioxidants measured using an in situ intestinal preparation. Rutin or vehicle was instilled into the duodenal segment, and plasma was collected from the carotid artery. Plasma TAA profiles obtained were compared between the control rats and rats with the duodenal segment instilled with: (A) 6.75 μg of rutin or (B) 2 mg of rutin. Values are means ± SD of measurements from 5 rats. Data were analyzed using unpaired t test. * denotes P < .05, and ** denotes P < .01.
phenylacetic acid. Other metabolites that may have contributed to the increase of antioxidant property in the urine have not been identified. Because urine may contain a wide range of metabolic compounds, it is likely that some of the metabolites in the urine will remain unidentified [20].

N of re qu e r c i t i n was detected in the urine of rats treated with either the mulberry leaves or rutin. Quercetin is unlikely to be detected in the urine if it is present at a low concentration in the diet [41]. Differences or changes in the composition of the microflora could explain the large interindividual variations in bioavailability [42]. This may be the reason for high variations between the individual urine ascorbic acid equivalent profiles, in addition to the uncertainty of the fate of the microbial metabolites after absorption from the intestines.

Feces of both the mulberry leaf extract-treated and rutin-treated rats showed significantly higher TAA on day 14 than on day 0. Quercetin was present in the feces of the rats given the mulberry leaf extract and rutin. Thus, the increased TAA in the feces can be explained by either the unabsorbed quercetin glycosides or quercetin microbial metabolites, or both. Taken together, a significant amount of antioxidative compounds was found in the urine or feces of rats that consumed quercetin glycosides either in the pure form or in an extract.

The in situ rat intestinal model showed that when rutin dissolved in 10% ethanol was instilled into the duodenum for 3 hours, an increase in plasma TAA was found. Despite that, quercetin was not detected in the plasma. Instead, 3-hydroxyphenylacetic acid, a major microbial metabolite of rutin, was detected. The TAA of 3-hydroxyphenylacetic acid was half that of the quercetin aglycone, as assayed using the ABTS reagent (unpublished data). The detection of this metabolite in the blood has not been reported previously. However, Olthof et al [40] suggested that it is possible for the phenolic metabolites to circulate in the blood and act as antioxidants in vivo. Phenylacetic acids retain their antioxidant properties, with potency similar to that of Vitamin E [43,44].

Although absorption of quercetin glycosides, especially rutin, from the duodenum is very uncommon, the duodenum is still possibly involved [18,45]. The vehicle of administration greatly affects the efficiency of absorption [46]. However, it was unlikely that ethanol enhanced the absorption of quercetin glycosides in the duodenum because the ethanol concentration used in this study was less than 30% [47]. Therefore, regardless of where in the intestine the quercetin glycosides, including rutin, are absorbed, and these glycosides will be extensively metabolized before absorption into systemic circulation.

In conclusion, our data indicate that ingested quercetin glycosides are extensively degraded before reaching the systemic circulation. A significant amount of the intestinal microbial metabolites with antioxidant property are absorbed. The contribution of TAA in the body may come predominantly from the microbial metabolites. It is only at high dose that quercetin will play a significant role as an antioxidant in the body, if sufficient amount of the intact quercetin is absorbed. Because antioxidants in the diet are present in low amounts, our data, involving the use of low dose of quercetin glycosides, may reflect more closely the real dietary situation.

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