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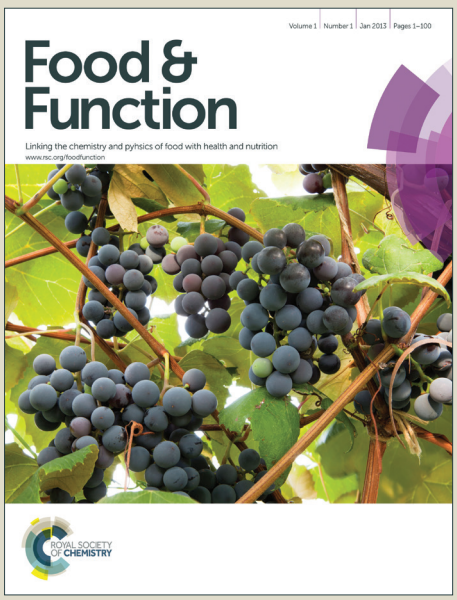

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Quercetin 7-*O*-glucoside suppresses nitrite-induced formation of dinitrosocatechins and its quinones in catechin/nitrite systems under stomach simulating conditions.

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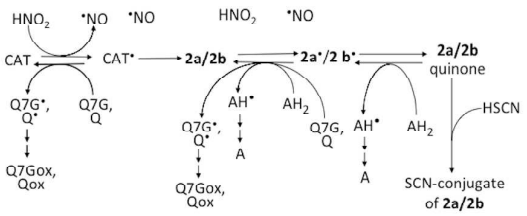
Catechins in foods can be transformed into dinitorosocatechins and the quinones by salivary nitrite in the stomach, and the transformation can be suppressed by flavonols including quercetin and its 7-*O*-glucoside.



CAT, catechin; AH_2, ascorbic acid; AH•, monodehydroascorbic acid;A, dehydroascorbic acid; Q and Q7G, quercetin and its 7-glucoside, Qox and Q7Gox, oxidation products; •, radicals

Abstract

Foods of plant origin contain flavonoids. In adzuki bean, (+)-catechin, quercetin 3-*O*-rutinoside (rutin), and quercetin 7-*O*-β-D-glucopyranoside (Q7G) are the major flavonoids. During mastication of foods prepared from adzuki bean, the flavonoids are mixed with saliva, and swallowed in the stomach. Here we investigated the interactions between Q7G and (+)-catechin at pH 2, which may proceed in the stomach after the ingestion of foods prepared from adzuki bean. Q7G reacted with nitrous acid producing nitric oxide (•NO) and a glucoside of 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(*2H*)-benzofuranone. (+)-Catechin reacted with nitrous acid producing •NO and 6,8-dinitrosocatechin. The production of the dinitrosocatechin was partly suppressed by Q7G, and the suppression resulted in the enhancement of Q7G oxidation. 6,8-Dinitrosocatechin reacted further with nitrous acid generating the *o*-quinone, and the quinone formation was effectively suppressed by Q7G. In the flavonoids investigated, the suppressive effect decreased in the order Q7G ≈ quercetin > kaempferol > quercetin 4'-*O*-glucoside > rutin. Essentially the same results were obtained when (−)-epicatechin was used instead of (+)-catechin. The results indicate that nitrous acid-induced formation of 6,8-dinitrosocatechins and the *o*-quinones can be suppressed by flavonols in the stomach, and that both a hydroxyl group at C3 and *ortho*-hydroxyl groups in B-ring are required for the efficient suppression.

Key words: adzuki bean, catechin radicals, dinitrosocatechins, flavonols, nitric oxide (•NO), nitrous acid.

Abbreviations used: Q7G, quercetin 7-glucoside; Q4'G, quercetin 4'-glucoside; Qox, oxidation product of quercetin; Q7Gox, oxidation product of quercetin 7-*O*-glucoside.

Introduction

Quercetin glycosides and catechins are the common flavonoids found in plants such as adzuki bean, apple, buckwheat, cacao, onion, and tea [1-5]. During the ingestion of foods or beverages prepared from such plants, flavonoids are mixed with saliva in the oral cavity, and then swallowed in the stomach, where the pH is around 2. Under such conditions, nitrite in saliva, which is produced from salivary nitrate by nitrate-reducing bacteria in the oral cavity [6], is transformed into nitrous acid ($pK_a = 3.3$). The concentration of nitrite in mixed whole saliva ranges from 0.05 to 1 mM [7]. Nitrous acid ($E^o = 0.983$ V; 0.865 V at pH 2.0, calculated value) can generate reactive nitrogen species such as NO^+, $^{\bullet}NO_2$ and N_2O_3 by self-decomposition, and can react with (+)-catechin ($E^{o\prime} = 0.49$ V, pH 2.0) (**1a** and **1b**), quercetin ($E^{o\prime} = 0.45$ V, pH 2.0) (**3**) [8,9] (Fig. 1) and other polyphenols producing nitric oxide ($^{\bullet}NO$) [10-13]. The major reaction products of catechins and quercetin have been reported to be 6,8-dinitrosocatechins (**2a** and **2b**) [14] and 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone (**5**) [15,16], respectively. The differences in the oxidation products are supposed to be due to the differences in the reactivity of $^{\bullet}NO$ with semiquinone radicals of (+)-catechin and quercetin generated in (+)-castechin/nitrous acid and quercetin/nitrous acid systems, respectively [17]. The functions of $^{\bullet}NO$ produced by the above reactions in the stomach is reviewed [18], and the functions include antimicrobial activity [19,20], inhibition of stress-induced gastric mucosal injury [21], increase in gastric blood flow, mucus formation [22-24], and inhibition of lipid peroxidation by scavenging the peroxyl and and alkoxyl radicals of unsaturated fatty acids [25]. On the other hand, nitrous acid can produce carcinogenic nitrosoamines through the *N*-nitrosation of secondary amines or amides [26-29]. In flavonoids, catechins are effective inhibitors of the *N*-nitrosoamine formation [14, 30-32].

Recently, it has been reported that (+)-catechin in the methanol extract of adzuki bean and (−)-epicatechin in the methanol extract of apple are transformed into **2a** and **2b**, respectively,

after their incubation in mixed whole saliva under acidic conditions [33,34], and that **2a** and **2b** are oxidized by nitrous acid [17,33]. The oxidation products are postulated to be *o*-quinones of **2a** and **2b** from the results that ascorbic acid reduces the oxidation products to **2a** and **2b** and that thiocyanate reacts with the oxidation products producing 6'-thiocyanate-6,8-dinitrosocatechin and 6'-thiocyanate-6,8-dinitrosoepicatechin, respectively [33]. In addition, quercetin effectively suppresses the oxidation of **2a** and **2b** [17]. Quercetin 7-*O*-glucoside (Q7G) (**4**) is a major flavonoid in adzuki bean in addition to (+)-catechin [4,5], and Q7G can be postulated to be as reactive as quercetin because of the presence of a free hydroxyl group at C3 and *ortho*-hydroxyl groups in the B ring. The presence of both Q7G and (+)-catechin in adzuki bean prompted us to investigate the effects of Q7G on the nitrous acid-induced formation of **2a** and **2b** from catechins and on the formation of *o*-quinones from **2a** and **2b**.

The main aim of this study is to explore the possible interactions of Q7G with catechins and **2a** and **2b** during their oxidation by nitrous acid under stomach simulating conditions. In addition to the above interactions, the interactions of (+)-catechin and **2a** and **2b** with quercetin 3-rutinoside (rutin), quercetin 4'-glucoside (Q4'G), and kaemoferol were also studied in the presence of nitrous acid. Taking the results obtained in this study into account, the importance of interactions of flavonols with catechins and **2a** and **2b** during their reactions with nitrous acid in the stomach is discussed from the point of prevention of formation of **2a** and **2b** and the quinones.

Results and Discussion

Reaction of Q7G with nitrous acid

Figure 2A shows nitrite-induced changes in absorption spectra of Q7G in 50 mM KCl-HCl (pH 2.0). After the addition of sodium nitrite, absorption peaks of Q7G at 254 and 366 nm decreased increasing the absorbance at 288 nm. Such spectral changes have been reported

during nitrous acid-induced oxidation of quercetin, and the oxidation product (Qox) is determined to be **5** [15].

The reaction product of Q7G (Q7Gox) was separated as a single peak by HPLC at the retention time of 2.9 min (Fig. 2B). In Fig. 2B, Qox (retention time, 3.4 min) was co-chromatographed. The UV/visible absorption spectrum of the product had a peak at 289 nm with a shoulder around 320 nm, and the absorption spectrum was similar to Qox (Fig. 2C). To understand the relation between Q7Gox and Qox, Q7Gox was incubated with β-glucosidase. During the incubation of Q7Gox for 40 min, its concentration decreased irrespective of the presence or absence of β-glucosidase in the similar way with a half time of approximately 20 min, but the decrease in the presence of the glucosidase accompanied the formation of a compound that was identified to be Qox from the retention time and UV/visible absorption spectrum. Therefore, we estimated that Q7Gox was a glucoside of **5**.

Figure 3 shows the effects of nitrite concentration on the consumption of Q7G and quercetin and the formation of Q7Gox and Qox. The concentrations of both quercetin (○) and Q7G (●) decreased by nitrite without significant differences. The result suggests that Q7G was oxidized by nitrous acid as effectively as quercetin. During the decrease in the concentrations of Q7G and quercetin, their oxidation products, Q7Gox (■) and Qox (□), were produced. Their production roughly correlated with the consumption of their mother compounds.

Rate constants of the consumption of Q7G and quercetin were estimated under anaerobic conditions by postulating the following reaction;

$$\text{Quercetin/Q7G} + HNO_2 \rightarrow \text{Quercetin/Q7G radical} + {}^{\bullet}NO + H_2O \qquad (1)$$

The values were $(12.5 \pm 1.2) \times 10^2\ M^{-1}\ min^{-1}$ (n = 4) for Q7G and $(13.5 \pm 1.1) \times 10^2\ M^{-1}\ min^{-1}$ (n = 4) for quercetin. The oxidation of Q7G and quercetin by nitrous acid was faster under aerobic than anaerobic conditions, suggesting the contribution of ${}^{\bullet}NO_2$ produced by the

autoxidation of •NO to the oxidation of Q7G under aerobic conditions. It has been reported that the suppressive effect of Q7G is about 50% of that of quercetin for the lipid peroxidation induced by 2,2′-azobis(2-amidopropyl) dihydrochloride in egg yolk phosphatidylcholine large unilamellar vesicles [35]. The smaller effect can be attributed to the difference in the solubility to lipid bilayer between Q7G and quercetin.

Characterization of reaction products of (+)-catechin

Figure 4 (**I** and **II**) shows HPLC of (+)-catechin and products generated in (+)-catechin/nitrous acid systems, and Fig. 4 (**III**) shows their absorption spectra. Accompanying the reaction of (+)-catechin with nitrite under acidic conditions for 10 min, **2a** was formed (trace **I**-2). The retention time (7.5 min) and absorption spectrum (peak at 274 nm with a shoulder around 320 nm) were identical with those of 6,8-dinitrosocatechin, which had been prepared in this study (see Experimental section). As the mechanism of **2a** formation, following reactions have been proposed [17,33].

(+)-Catechin + HNO_2 → (+)-Catechin radical + •NO + H_2O (2)

(+)-Catechin radical + •NO → Mononitrosocatechin (3)

Mononitrosocatechin + HNO_2 → Mononitrosocatechin radical + •NO + H_2O (4)

Mononitrosocatechin radical + •NO → **2a** (5)

During further incubation of the reaction mixture, the concentration of (+)-catechin decreased without increasing the concentration of **2a**, and a new component (P1) was produced (trace **I**-3). From the retention time (7.0 min) and absorption spectrum (peak at 269 nm with a shoulder around 320 nm) and from the ascorbic acid-induced disappearance of P1 increasing the concentration of **2a** by 33% (**II**-3), P1 was postulated to be a quinone of **2a**. The postulation is supported by not only its reduction to **2a** by ascorbic acid but also its transformation into 6'-thiocyanate-6,8- by thiocyanate at pH 2 [33]. *o*-Quinones of caffeic acid, chlorogenic acid, and rutin also react with thiocyanate at pH 2 producing the thiocyanate

conjugates, which are hydrolyzed to oxathiolone derivatives and NH_3 [36,37]. Following reactions may be possible for the formation of *o*-quinone from **2a**.

2a + HNO_2 → **2a** radical + $^{\bullet}NO$ + H_2O (6)

2 × (**2a** radical) → **2a** + **2a** quinone (7)

Effects of Q7G on the formation of **2a** and P1 were studied. Under the experimental conditions of trace **II**-2, both P1 and **2a** were produced in a (+)-catechin/nitrous acid system during the incubation for 10 min. The addition of Q7G to the above system and the incubation for 10 min resulted in the formation of Q7Gox that was estimated from the retention time and the absorption spectrum. The formation of Q7Gox accompanied the inhibition of P1 formation by about 80% and enhancement of **2a** production by about 30%, suppressing slightly the decrease in the concentration of (+)-catechin (trace **II**-3). The result suggests that Q7G might have interacted with (+)-catechin and **2a** during their reactions with nitrous acid. Therefore, we studied the interactions of Q7G with (+)-catechin and **2a** under stomach simulating conditions.

Interactions of Q7G with catechins

Figure 5A shows interaction of Q7G with (+)-catechin during their reaction with nitrous acid. The formation of **2a** was enhanced by about 20% by 5 μM Q7G, and suppressed by about 20% by 50 μM Q7G (■). The enhancement may be explained by the increase in $^{\bullet}NO$ production by Q7G/nitrous acid systems (see below). The consumption of (+)-catechin was suppressed by about 35% by 5 and 15 μM Q7G and by about 65% by 50 μM Q7G (□). According to reactions 2−5, we can deduce that the suppression of (+)-catechin consumption may be due to Q7G-dependent reduction of semiquinone radical of (+)-catechin, and that the suppression of **2a** formation may be due to the reduction of semiquinone radicals of both (+)-

183 catechin and mononitrosocatechin. Nitrous acid-induced formation of semiquinone radical of
184 (+)-catechin has been reported [11].

185 Figure 5A also shows effects of (+)-catechin on the nitrous acid-induced consumption of
186 Q7G and the formation of Q7Gox. The consumption of Q7G during 1 min incubation
187 increased nearly linearly with the increase in Q7G concentration (●), and 50 μM (+)-
188 catechin enhanced the consumption of 5 μM Q7G by about 40% and the consumption of 15
189 and 50 μM Q7G by about 20% (○). The enhancement of Q7G consumption by (+)-catechin
190 accompanied the enhancement of Q7Gox formation (compare ▲ and △). These results can
191 be explained by the oxidation of Q7G by semiquinone radicals generated during the reactions
192 of (+)-catechin with nitrous acid. Such interaction between (+)-catechin and quercetin has
193 been reported to proceed in the presence of nitrite under acidic conditions [17].

194 6,8-Dinitrosoepicatechin (**2b**) was formed in the reaction mixture that contained 50 μM
195 (−)-epicatechin and 0.5 mM sodium nitrite in 50 mM KCl-HCl (pH 2.0), and it was
196 postulated that **2b** might be formed by reactions 2−5, in which (+)-catechin was replaced by
197 (−)-epicatechin. The formation of **2b** was suppressed by Q7G, and the suppression increased
198 with the increase in the concentration of Q7G (■) (Fig. 5B). The consumption of (−)-
199 epicatechin was also suppressed, and degree of the suppression increased with the increase in
200 concentration of Q7G (□). Furthermore, (−)-epicatechin (50 μM) enhanced the nitrous acid-
201 induced consumption of Q7G as (+)-catechin (compare ● and ○), and the enhancement was
202 accompanied by the increase in Q7Gox formation (compare ▲ and △). The result in Fig. 5B
203 suggests that Q7G can also react with semiquinone radicals generated in (−)-
204 epicatechin/nitrous acid systems.

205

206 **Interactions of Q7G with 2a/2b**

6,8-Dinitrosocatechin (**2a**) isolated by preparative HPLC in this study was partly transformed into **2b** during its concentration and lyophilization. It has been reported that **2a** and **2b** are mutually transformed [14]. Then, we studied the effects of Q7G on nitrous acid-induced oxidation of **2a** and **2b** using the isolated dinitrosocatechin that was a mixture of **2a** and **2b** (Fig. 6A). Accompanying the oxidation of **2a**/**2b** by nitrous acid, P1 and P2 were produced as reported previously [17,33]. Their formation was completely suppressed by 30 and 100 μM Q7G (■). The concentration of Q7G required for 50% inhibition was estimated to be 7 μM from the figure. It has been reported that 50% inhibition of the formation of (P1 + P2) from **2a**/**2b** is observed at about 10 μM quercetin under the conditions similar to Fig. 6A [17]. The suppression of (P1 + P2) formation accompanied the inhibition of the consumption of **2a**/**2b** (□). Furthermore, the inhibition of the formation of (P1 + P2) by Q7G accompanied the enhancement of Q7G consumption (compare ● and ○) and Q7Gox formation (compare ▲ and △) (Fig. 6B). These results suggest that Q7G can inhibit the formation of P1 and P2 by scavenging semiquinone radicals produced in **2a**/**2b**/nitrous acid systems by reaction 6, if P1 and P2 are *o*-quinones derived from **2a** and **2b**, respectively, as described in Fig. 4.

•NO production.

During the reactions of nitrite with Q7G, catechin, or **2a**/**2b** under acidic conditions, •NO should be produced by reactions 1, 2, 4, and 6. Then, nitrite-induced •NO production was studied in the presence of the above flavonoids in 50 mM KCl-HCl (pH 2.0) (Table 1). The rate of •NO production in the nitrous acid/Q7G system increased with the increase in Q7G concentration. The rate constant of •NO production by Q7G was calculated to be $(3.6 \pm 0.6) \times 10^3\ M^{-1}\ min^{-1}$ using the data in Table 1, postulating that •NO was produced by reaction 1. The rate constant in the presence of quercetin was calculated to be $(3.9 \pm 0.3) \times 10^3\ M^{-1}\ min^{-1}$. These values were about 2.8-folds of the rate constants for the decreases in concentrations of

Q7G [(12.5 ± 1.2) × 10^2 $M^{-1}min^{-1}$] and quercetin [(13.5 ± 1.1) × 10^2 M^{-1} min^{-1}]. One of the reasons for the larger rate constants for •NO production is the rapid reduction of nitrous acid to •NO by semiquinone radicals of not only Q7G but also quercetin and/or rapid disproportionation of the semiquinone radicals to the mother compounds and quinones that are transformed rapidly into **5** from quercetin and a glucoside of **5** from Q7G [38,39]. The rate constants of •NO production were calculated from the initial slope of •NO production. This may also be a reason for the larger rate constants of •NO production than those of the consumption of Q7G and quercetin.

Table 1 also shows the rates of •NO production in the presence of (+)-catechin, (−)-epicatechin, and isolated **2a**/**2b**. Catechin-induced •NO production under stomach simulating conditions has been reported [11,13]. Nitrous acid-induced •NO production in the presence of 50 μM (+)-catechin or (−)-epicatechin was less than 30%, and the •NO production in the presence of 50 μM **2a**/**2b** was about 45% of that in the presence of 50 μM Q7G. The slower •NO production by **2a**/**2b** than Q7G can be explained by the difference in the rate constants of nitrous acid-induced oxidation of **2a**/**2b** [(6.5 ± 2.5) × 10^2 M^{-1} min^{-1}] [17] from that of Q7G [(12.5 ± 1.2) × 10^2 M^{-1} min^{-1}] (see above). Although the rate constants of nitrous acid-induced oxidation of (+)-catechin [(6.7 ± 1.0) × 10^2 M^{-1} min^{-1}] and (−)-epicatechin [(5.8 ± 0.2) × 10^2 M^{-1} min^{-1}] were similar to that of nitrous acid-induced oxidation of **2a**/**2b** [17,34], •NO production in the presence of (+)-catechin or (−)-epicatechin was significantly slower than that in the presence of **2a**/**2b**. The difference can be explained by •NO consumption by semiquinone radicals derived from (+)-catechin and (−)-epicatechin but not **2a**/**2b** by reactions 3 and 5. The addition of 50 μM **2a**/**2b** to Q7G/nitrous acid systems increased the rate of •NO production; the rates in the presence of both **2a**/**2b** and Q7G were larger than the sums of the rate in the presence of **2a**/**2b** alone and the rates in the presence of Q7G alone at any concentrations of Q7G studied, supporting the insignificant reaction of •NO with semiquinone radical of **2a**/**2b**. The addition of 50 μM (+)-catechin or (−)-epicatechin to

Q7G/nitrous acid systems increased the rate of •NO production, but the rates in the presence of both catechin and Q7G were smaller than the sums of the rate in the presence of (+)-catechin or (−)-epicatechin alone and the rates in the presence of Q7G alone, especially when the concentrations of Q7G were 5 and 15 μM. These data can be explained by the consumption of •NO by semiquinone radicals of (+)-catechin and (−)-epicatechin, both of which were produced in catechin/Q7G/nitrous acid systems.

Interactions of (+)-catechin and 2a/2b with other flavonols.

Rutin, Q4'G, and kaempferol were consumed by nitrous acid (Table 2, column 1), and the rate constants of the consumption were calculated from the data. The values were (1.3 ± 1.0), (2.8 ± 0.8), and (6.2 ± 1.2) × 10^2 M^{-1} min^{-1} (n = 9) for rutin, Q4'G, and kaempferol, respectively, and the values were smaller than those of Q7G [(12.5 ± 1.2) × 10^2 M^{-1} min^{-1}] and quercetin [(13.5 ± 1.1) × 10^2 M^{-1} min^{-1}]. These results are in accordance with a previous report on nitrite-induced oxidation of 50 μM quercetin, Q4'G, rutin, and kaempferol in 50 mM KCl-HCl (pH 2.0) (6.2, 0.9, 0.2, and 1.0 μM/min, respectively) [10].

The effects of 100 μM (+)-catechin and **2a**/**2b** on nitrous acid-induced consumption of rutin, Q4'G, and kaempferol are also shown in Table 2 (columns 2 and 3). Both (+)-catechin and **2a**/**2b** enhanced the consumption of above flavonols, and the enhancement of the consumption seemed to increase in the order rutin < Q4'G < kaempferol. Furthermore, Table 2 shows the effects of flavonols on nitrous acid-induced formation of **2a** from (+)-catechin (column 4) and (P1 + P2) from **2a**/**2b** (column 5). The formation of **2a** and (P1 + P2) was inhibited by Q4'G and kaempferol but not rutin, and degrees of the inhibition were smaller than those of the inhibition by Q7G (Figs. 5 and 6). The data in Table 2 and Figs. 5 and 6 indicate that both a free hydroxyl group at C3 and *ortho*-hydroxyl groups in the B ring are essential for the effective inhibition of the nitrous acid-induced transformation of (+)-catechin

into **2a** and **2a**/**2b** to P1/P2. It has been reported that the hydroxyl group at C3 is a target for the oxidation of flavonols [40-42].

Importance of interactions of catechins with flavonols

Flavonoids mixed with saliva in the oral cavity reach the gastric lumen where they can react with nitrous acid derived from nitrite, which is produced by nitrate-reducing bacteria in the oral cavity. The results of this study show that **2a** and **2b** are possibly produced in the stomach after the ingestion of (+)-catechin- and (−)-epicatechin-containing foods, beverages, or dietary supplements (Fig. 7). The possibility is supported by the reports about the production of **2a** and **2b** in the mixtures of acidified whole saliva and the methanol extracts of adzuki bean [33] and apple [34], respectively. Recently, however, we observed nitrite-induced formation of **2b** by acidification of the juice obtained by mastication of apple fruit, but could not observe the formation of **2a** by acidification of the juice obtained by mastication of boiled adzuki bean (unpublished results). From these results together with the results of in the present study and ref. 33 and 34, we can deduce that (i) the detectable amounts of nitrosocatechins are not always produced in the stomach after the ingestion of catechin-rich foods or beverages, and that (ii) the formation of **2a**/**2b** are dependent on the concentration of the components, which can interfere the reactions of catechins with nitrous acid and can scavenge catechin semiquinone radicals. The formation of **2a**/**2b** in the stomach may be efficient because of the lower O_2 concentration in gastric juice. It has been reported on the increase in the efficiency of **2a**/**2b** formation with the decrease in O_2 concentration [33].

The methanol extract of adzuki bean contained 636 ± 45, 165 ± 18, 41 ± 3, and 29 ± 2 nmol/g of (+)-catechin, Q7G, quercetin, and vignacyanidins, respectively (means with SDs, n = 3), whereas that of boiled aduzki bean used to obtain the juice by mastication contained 138 ± 41, 58 ± 15, 20 ± 4, and 18 ± 2 nmol/g of Q7G, quercetin, and vignacyanidins, respectively (means with SDs, n = 3), as phenolic components that could be readily oxidized by nitrous

acid. Vignacyanidins are cyanidin-catechin adducts present in adzuki bean and can be oxidized by nitrous acid [43]. The molar ratios of (+)-catechin to the sum of the other components were calculated to be about 2.7 and 1.4 in the former and the latter, respectively. The difference in the ratio may contribute to the **2a** formation in the mixture of acidified whole saliva and the failure of its formation in the acidified juice, which was obtained by mastication of boiled adzuki bean. The oxidation products of quercetin and Q7G, namely, **5** and its glucoside, may decompose to more stable components. It is known that **5** decomposes to 2,4,6-trihydroxyphenyl glyoxylic acid and 3,4-dihydroxybenzoic acid, which are derived from the A- and B-ring of **5**, respectively [44]. Taking the report into account, we can deduce that the glucoside of **5** decomposes to a glucoside of 2,4,6-trihydroxyphenyl glyoxylic acid and 3,4-dihydroxybenzoic acid. The oxidation products of viganacyanidins are supposed to be the polymers [43].

The major antioxidative polyphenols of apple juice are chlorogenic acid, (−)-epicatechin, and quercetin 3-glycosides such as arabinoside, xyloside, galactoside, glucoside, and rhamnoside [45,46]. The molar ratio of (−)-epicatechin to chlorogenic acid is about 0.5 [34] and that of (−)-epicatechin to quercetin glycosides is calculated to range from about 1.6 to 4 using the data in ref. 47. In the apple polyphenols, (−)-epicatechin can react more rapidly with nitrous acid than chlorogenic acid and quercetin 3-glycosides. This is deduced by comparing the rate constant of the reaction of nitrous acid with (−)-epicatechin [$(5.8 \pm 0.2) \times 10^2$ M^{-1} min^{-1}] with those of reactions of nitrous acid with chlorogenic acid [$(1.6 \pm 0.3) \times 10^2$ M^{-1} min^{-1}] [34] and a quercetin 3-glycoside, rutin [$(1.3 \pm 1.0) \times 10^2$ M^{-1} min^{-1}] (see above). Then, the observation of nitrite-induced **2b** formation in the acidified apple juice can be explained by the more efficient reaction of nitrous acid with (−)-epicatechin than with the other polyphenols. According to the above discussion, the ingestion of a dietary supplement of catechins is supposed to result in the production of **2a**/**2b** in the stomach because of the absence of polyphenols that suppress the nitrosation. It has also been reported about the

nitrous acid-induced formation of 6,8-dinitirsoepigallocatechin gallate and dinitrisoprocyanidin B2 from epigallocatechin gallate and procyanidin B2, respectively [14,30].

If **2a** and/or **2b** are produced in the gastric lumen after the ingestion of catechin-rich foods, beverages, or dietary supplements, **2a**/**2b** can be oxidized to the *o*-quinones. The *o*-quinones may be absorbed in the body from the stomach and the intestine to give oxidative stresses to cells. The stresses may be due to the increased reactivity of *o*-quinones under neutral conditions than acidic conditions. The increased reactivity may contribute to the cytotoxic and carcinogenic effects through generation of reactive oxygen species, formation of polymers and glutathione-conjugate, binding to DNA, and alkylation of essential macromolecules such as DNA and proteins [48-51]. Fortunately, the formation of *o*-quinones from **2a**/**2b** may not be so efficient in the stomach, because ascorbic acid [52] and thiocyanate [53] derived from gastric juice and saliva, respectively, are present in gastric juice. The former can reduce both semiquinone radicals and *o*-quinones to **2a**/**2b**, and the latter can react with *o*-quinones generating thiocyanate conjugates (Fig. 7). Furthermore, flavonols such as quercetin and Q7G can effectively suppress the formation of *o*-quinone by reducing the semiquinone radicals (Fig. 7).

It has been reported that daily consumption of an aqueous green tea extract does not impair human health [54], but high doses of catechins and other flavonoids as dietary supplement have adverse effects for human health [55-57]. The adverse effects of catechins are discussed to be due to the production of undesired products from catechins in the intestine and the liver [56,57]. The present study suggests the nitrosation of catechins and the oxidation of **2a**/**2b** in the stomach may also contribute to the adverse effects of high doses of catechins, if the **2a**/**2b** and the quinones are absorbed to the body from the gastrointestinal tract. It has been reported that, in addition to quinones, **2a**/**2b** are toxic for Caco cells [14].

Experimental

361 **Reagents**

362 (+)-Catechin (**1a**), (−)-epicatechin (**1b**), quercetin (**3**), kaempferol, and rutin were obtained
363 from Sigma-Aldrich Japan (Tokyo). 1-Hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-
364 methyl-1-triazene (NOC 7) (purity > 90%) was obtained from Dojindo (Kumamoto,
365 Japan). β-Glucosidase from sweet almond was obtained from Oriental Yeast Co., Ltd.
366 (Osaka, Japan). Quercetin 4'-glucoside (Q4'G) was isolated from onion bulbs as reported
367 previously with some modifications [58].

368

369 **Apparatus**

370 UV/visible absorption spectra were recorded using an UV-2450 spectrophotometer
371 (Shimadzu, Kyoto, Japan). Analytical and preparative HPLC was carried out using a
372 Shimadzu LC-10AS pump combined with a SPD M10Avp photodiode array detector
373 (Shimadzu). The columns used for analytical HPLC were a Shim-pack CLC-ODS (15 cm x 6
374 mm i.d.) and a Shim-pack VP-ODS (15 cm x 4.6 mm i.d.) (Shimadzu), and the column used
375 for preparative HPLC was Shim-pack CLC-ODS) (25 cm × 2 cm i.d.) (Shimadzu). The
376 mobile phases were mixtures of methanol and 0.2% formic acid or methanol and 25 mM
377 KH_2PO_4, and their flow rates were 1 and 9 mL/min for analytical and preparative HPLC,
378 respectively. Atmosphere-pressure chemical ionization (APCI) and electrospray ionization
379 (ESI) mass spectra were obtained with a Shimadzu LCMS QP8000α quadrupole mass
380 spectrometer equipped with APCI or ESI ion source. Sample was delivered into the ion
381 source using Ascentis express C18 column (15 cm x 2.1 mm i.d; particle size, 2 μm) (Sigma-
382 Aldrich Japan, Tokyo). The mobile phase was 40% methanol containing 0.2% formic acid
383 and the flow rate was 0.2 mL/min. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra
384 were recorded with ECX-400P FT-NMR spectrometer (JEOL, Tokyo, Japan) with
385 dimethylsulfoxide-d_6 (DMSO-d_6) as the solvent and tetramethylsilane as the internal
386 standard.

387

388 **Isolation of quercetin Q7G**

389 Q7G was isolated by the method developed in this study. Dried adzuki seeds (300 g) were
390 boiled in 700 ml of distilled water for 30 min, and the water extract was filtered under
391 reduced pressure. The pH of the filtrate was adjusted to 2.0 by adding 6 M HCl and then
392 extracted with 200 mL of ethyl acetate. For better separation of the ethyl acetate from water,
393 the mixture was centrifuged at 3000*g* for 5 min. The above ethyl acetate extraction procedure
394 was repeated three times, and ethyl acetate fractions were combined. After removing water in
395 the ethyl acetate fraction by anhydrous sodium sulfate, ethyl acetate was evaporated *in vacuo*.
396 The residue was dissolved in 2 mL of a mixture of methanol and 25 mM KH_2PO_4 (2:3, v/v),
397 and then applied to the preparative HPLC column. After washing the column with a mixture
398 of methanol and 25 mM KH_2PO_4 (1:6, v/v) for 5 min, the concentration of methanol in the
399 mobile phase was increased stepwise using the following mixtures [methanol and 25 mM
400 KH_2PO_4 (1:3, 1:2, and 2:3, v/v)]. Each mobile phase was flowed for 20 min, and Q7G was
401 detected at 360 nm.

402 A compound assigned to be Q7G was eluted at about 7 min after the mobile phase was
403 changed to the mixture of methanol and 25 mM KH_2PO_4 (2:3, v/v). The fraction of Q7G was
404 collected and solvents in the fraction were evaporated *in vacuo*. The residues were dissolved
405 in 2 mL of methanol and purified by repetition of dissolution in methanol and precipitation
406 with water. The purity of Q7G was estimated by analytical HPLC using a Shim-pack VP-
407 ODS column (15 cm × 4.6 mm i. d.). The mobile phase was a mixture of methanol and 25
408 mM KH_2PO_4 (2:3, v/v) and the flow rate was 1.0 mL/min. When no significant contaminants
409 were detected, the precipitate was lyophilized (35 mg from 2.1 kg of adzuki bean). The
410 structure of isolated Q7G was confirmed by spectral data [5,59]: UV-vis (methanol) λ_{max} nm (ε)
411 255 (22,000) and 373 (19,500); negative APCI-MS *m/z* (relative intensity, %) 301, (40, [M –
412 glucose]$^-$) and 463 (100, [M − H]$^-$); negative ESI-MS *m/z* 463 (100, [M − H]$^-$); ^{1}H NMR

(DMSO-d_6) δ 3.20 (m, 1H, H-4"), 3.29 (m, 1H, H-2"), 3.32 (m, 1H, H-3"), 3.47 (m, 1H, H-5"), 3.49 (m, 1H, H-6"a), 3.73 (m, 1H, H-6"b), 5.09 (d, J = 7.3 Hz, 1H, H-1"), 6.43 & 6.44 (d, J = 2.2 Hz, 1H, H-6), 6.78 (d, J = 2.2 Hz, 1H, H-8), 6.91 & 6.92 (d, J = 8.7 Hz, 1H, H-5'), 7.57 & 7.58 (dd, J = 8.2, 2.3 Hz, 1H, H-6'), 7.73 & 7.74 (d, J = 2.2 Hz, 1H, H-2'), 12.52 (s, 1H, O*H*); ^{13}C NMR (DMSO-d_6) δ 61.17 (C-6"), 70.11 (C-4"), 73.67 (C-2"), 76.95 (C-3"), 77.69 (C-5"), 94.80 (C-8), 99.29 (C-6), 100.44 (C-1"), 105.21 (C-4a), 115.90 (C-2'), 116.12 (C-5'), 120.59 (C-6'), 122.34 (C-1'), 136.62 (C-3), 145.61 (C-4'), 148.13 (C-2), 148.48 (C-3'), 156.26 (C-8a), 160.90 (C-5), 163.22 (C-7), 176.55 (C-4).

Isolation of 6,8-dinitrosocatechin

6,8-Dinitrosocatechin was isolated as reported previously [17,33]. In brief, (+)-catechin (29 mg) in 100 mL of 50 mM KCl-HCl (pH 2.0) was incubated with 5 mM nitrite for 5 min under anaerobic conditions, and then the reaction mixture was extracted with 50 mL of ethyl acetate. After removing ethyl acetate, the residue was dissolved in 3 mL of 57% methanol in 0.2% formic acid (v/v) to apply to the preparative HPLC column. The mobile phases were mixtures of methanol and 0.2% formic acid. The concentration of methanol was increased stepwise as follows, 14, 20, 25, and 33% (v/v), and the mobile phases were flowed for 5, 15, 15, and 25 min, respectively. A fraction assigned to be 6,8-dinitrosocatechin, which was eluted around 7 min after changing the mobile phase to 20% methanol, was collected. After removing methanol in the fraction, the residue was lyophilized. The yield was 8−10 mg. LC-MS analysis of the isolated dinitrosocatechin gave two peaks (**2a** and **2b**) with retention times of 2.1 and 3.4 min, respectively, when the mobile phase was 35% of methanol in 0.2% formic acid. The structures were confirmed to be a mixture of 6,8-dinitrosocatechin (**2a**) and 6,8-dinitrosoepicatechin (**2b**) as reported previously [17,33].

Reactions of Q7G, catechin, and 6,8-dinitrosocatechin with nitrous acid

All reactions of flavonoids with nitrite were studied in 1 mL of 50 mM KCl-HCl (pH 2.0) under aerobic or anaerobic conditions. Anaerobic conditions were established by bubbling argon gas through the reaction mixture for 2 min. After initiating the reactions by adding 0.5 mM sodium nitrite, argon gas was blown gently on the surface of the reaction mixture during the reaction period.

The interactions of Q7G with (+)-catechin, (−)-epicatechin, or isolated 6,8-dinitrosocatechins (**2a**/**2b)** were studied in the reaction mixture (1 mL) that contained various concentrations of Q7G and 50 μM (+)-catechin, (−)-epicatechin, or **2a**/**2b** in 50 mM KCl-HCl (pH 2.0). When required, quercetin and other flavonols were used instead of Q7G. Reactions were initiated by adding 0.5 mM sodium nitrite. After incubation for 1 min, 50 μL of the reaction mixture was analyzed by HPLC (see below).

HPLC analysis of reaction products

The reactants and the products of the above reactions were separated and quantified using a Shim-pack CLC-ODS column. The mobile phases used to quantify (+)-catechin, (−)-epicatechin, and their products were mixtures of methanol and 0.2% formic acid (1:4, 1:3, or 1:2, v/v). The products of **2a** and **2b** formed in the presence and absence of Q7G or quercetin were quantified using a mixture of methanol and 0.2% formic acid (2:5, v/v), and the ratios of 1:1 (v/v) was used to quantify rutin, Q7G and the reaction products of Q7G and quercetin. Quercetin, Q4'G, and kaempferol were quantified using the mixture of 2:1 (v/v). The concentrations of reactants and products were estimated from the areas under the peaks: (+)-catechin and (−)-epicatechin at 280 nm; **2a**, **2b**, P1, and P2 at 320 nm; Q7G, quercetin, rutin, and Q4'G at 360 nm; Qox and Q7Gox at 290 nm.

Hydrolysis of reaction product of Q7G

Q7G was oxidized in the reaction mixture (1 mL) that contained 0.1 mM Q7G and 0.2 mM sodium nitrite in 50 mM KCl-HCl (pH 2.0) for 6 min, and then 0.3 mL of 0.1 M Na_2HPO_4 was added to adjust the pH to 6.8. The solution of pH 6.8 was incubated for 10, 20, 30, and 40 min after addition of 20 μL of β-glucosidase (2 mg/mL), and an aliquot (50 μL) of each incubated solution was applied to a Shim-pack VP-ODS column (15 cm x 4.6 mm i.d.). The mobile phase was a mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v).

Measurements of $^{\bullet}$NO production

Nitrite-induced $^{\bullet}$NO production was recorded using a Clark-type electrode (Rank Bothers, Cambridge, UK) at 30°C with a polarization voltage of −0.7 V [25,60]. The reaction mixture (2 mL) contained 5, 15, and 50 μM Q7G in 50 μM KCl-HCl (pH 2.0). When required, 50 μM (+)-catechin, (−)-epicatechin, or **2a**/**2b** was added. After removing air from the reaction mixture by bubbling argon gas, 0.5 mM sodium nitrite was added to initiate $^{\bullet}$NO production. The rate of $^{\bullet}$NO production was estimated from the slope, using an ascorbic acid/nitrous acid system for calibration.

In the ascorbic acid/nitrous acid system, one molecule of ascorbic acid produces two molecules of $^{\bullet}$NO under acidic conditions by the following reaction, if the concentration of nitrous acid is more than two times of that of ascorbic acid

$$\text{Ascorbic acid} + 2HNO_2 \rightarrow \text{Dehydroascorbic acid} + 2^{\bullet}NO + 2H_2O \quad (8)$$

The amount of $^{\bullet}$NO produced by addition of 50 μM ascorbic acid in the presence of 0.5 mM sodium nitrite in 50 mM KCl-HCl (pH 2.0) was essentially the same as the amount produced by 50 μM NOC 7 in the same buffer, one molecule of which produces two molecules of $^{\bullet}$NO, indicating the usefulness of an ascorbic acid/nitrous acid system for calibration of $^{\bullet}$NO production.

Presentation of Data.

Each experiment was repeated more than three times. Typical data or means with SDs are presented in Figures and Tables. The statistical significance of the differences between groups was evaluated by Student's *t*-test.

Conclusion

The ingestion of nitrate-rich leafy vegetables such as lettuce and spinach results in the increase in the concentration of nitrite in mixed whole saliva [7,61]. If catechin-rich foods, beverages, or dietary supplements are taken under such conditions, nitrosation of catechins and oxidation of **2a**/**2b** to the *o*-quinones can proceed in the stomach. The nitrous acid-induced formation of **2a**/**2b** and the *o*-quinones can be suppressed by ascorbic acid in gastric juice cooperating with antioxidative flavonols in foods and beverages. Thus, the results of the present study suggest that the ingestion of catechin-rich dietary supplements accompanied with components, which can reduce the semiquinone radicals of catechins and **2a**/**2b**, may decrease the adverse effects of catechins.

Acknowledgement.

Part of this study was supported by Grants-in-Aid for Scientific Research (22500790 and 23500986) from the Ministry of Education and Science in Japan. S. Veljovic-Jovanovic and F. Morina acknowledge the partial support from Ministry of Education, Science and Technological Development of Republic Serbia (III43010).

References

1. C. Ölschläger, I. Regos, F.J. Zeller and D. Treutter, *Phytochemistry*, 2008, 69, 1389-1397.

2. S. Bhagwat, D.B. Haytowitz and J.M. Holden, USDA database for the flavonoid content of selected foods. Release 3.1; 2013, Available from http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Data/Flav/Flav3-1.pdf

3. A.-M. Danila, A. Kotani, H. Hakamata and F. Kusu, *J. Agric. Food Chem*. 20007, 55, 1139-1143.

4. K. Yoshida, T. Kondo, M. Ito and T. Kondo, *ITE Lett. New Tech. Med*. 2005, 6, 226-231.

5. S.-I. Tebayashi, S. Matsuyama, T. Suzuki, Y. Kuwahara, T. Nemoto and K. Fujii, *J. Pesticide Sci*. 1995, 20, 299-305.

6. J.J. Doel, N. Benjamin, M.P. Hector, M. Rogers and R.P. Allaker, *Eur. J. Oral Sci.* 2005, 113, 14-19.

7. A.S. Pannala, A.R. Mani, J.P.E. Specer, Y. Skinner, K.R. Bruckdorfer, K.P. Moore and C.A. Rice-Evans, F*ree Radic. Biol. Med*. 2003, 34, 576-584.

8. P. Janeiro and A.M.O. Brett, *Anal. Chim. Acta* 2004, 518, 109-115.

9. A.M.O. Brett and M.-E. Ghica, *Electoroanalysis* 2003, 15, 1745-1750.

10. U. Takahama, T. Oniki and S. Hirota, *J. Agric. Food Chem*. 2002, 50, 4317-4322.

11. L. Peri, D. Pietraforte, G. Scorza, A. Napolitano, V. Fogliano and M. Minetti, *Free Radic. Biol. Med*. 2005, 39, 668–681.

12. B. Gago, J.O. Lundberg, R.M. Barbosa and J. Laranjinha, *Free Radic. Biol. Med*. 2007, 43, 1233-1242.

13. B.S. Rocha, B. Gago, R.M. Barbosa and J. Laranjinha, *Toxicology* 2009, 265, 41-48.

14. S.Y.H. Lee, B. Muerol, S. Pollard, K.A. Youdim, A.S. Pannala, G.G.C. Kuhnle, E.S. Debnam, C. Rice-Evans and J.P.E. Spencer, *Free Radic. Biol. Med*. 2006, 40, 323-334.

15. S. Hirota, U. Takahama, T.N. Ly and R. Yamauchi, *J. Agric. Food Chem*. 2005, 53, 3265-3272.

16. T.N. Ly, C. Hazama, M. Shimoyamada, H. Ando, K. Kato and R. Yamauchi, *J. Agric. Food Chem*. 2005, 53, 8183-8189.

17. S. Veljovic-Jovanovic, F. Morina, R. Yamauchi, S. Hirota and U. Takahama, *J. Agric. Food Chem*. 2014, 62, 4951-4959.

18. B.S. Rocha, C. Nunes, C. Pereira, R.M. Barbosa and J. Laranjinha, *Food Funct.* 2014, 5, 1646-1652.

19. N. Benjamin, F. O'Driscoll, H. Dougall, C. Duncan, L. Smith, M. Golden and H. McKenzie, *Nature* 1994, 368, 502.

20. J. Xu, X. Xu and W. Verstraete, *J. Appl. Microbiol*. 2001, 90, 523-529.

21. M. Miyoshi, E. Kasahara, A.M. Park, K. Hiramoto, Y. Minamiyama, S. Takemura, E.F. Sato and M. Inoue, *Free Radic. Res*. 2003, 37, 85-90.

22. H.H. Björne, J. Petersson, M. Phillipson, E. Weizberg, L. Holm and J.O. Lundberg, *J. Clin. Invest.* 2004, 113, 106-114.

23. J. Petersson, M. Phillipson, E.A. Jansson, A. Patzak, J.O. Lundberg, *Am. J. Physiol. Gastrointest. Liver Physiol.* 2007, 292, G718-G724.

24. J.O. Lundberg, E. Weitzberg and M. T. Gladwin, *Nat. Rev. Drug Discov*. 2008, 7, 156-167.

25. J. Volk, S. Gorelik, R. Granit, R. Kohen and J. Kanner, *Free Radic. Biol. Med*. 2009, 47, 496-502.

26. M. Eichholzer and F. Gutzwiller, F. *Nutr. Rev*. 1998, 56, 95-105.

27. K. Ohsawa, S.Y. Nakagawa, M. Kimura, C. Shimada, S. Tsuda, K. Kabasawa, S. Kawaguchi and Y.F. Sasaki, *Mutat. Res*. 2003, 539, 65–76.

28. M. De Lucia, L. Panzella, A. Pezzella, A. Napolitano and M. D'Ischia, *Chem. Res. Toxicol*. 2008, 21, 2407–2413.

29. B.S. Rocha, B. Gago, C. Pereira, R.M. Barbosa, S. Bartesaghi, J.O. Lundberg, R. Radi and J. Laranjinha, *Curr. Drug Targets*, 2011, 12, 1351-1363.

30. L. Panzella, P. Manini, A. Napolitano and M. d'Ischia, *Chem. Res. Toxicol*. 2005, 18, 722-729.

31. K. Tanaka, T. Hayatsu, T. Negishi and H. Hayatsu, *Mutat. Res*. 1998, 12, 91-98.

32. S. Masuda, S. Uchida, Y. Terashima, H. Kuramoto, M. Serozawa, Y. Deguchi, K. Yanai, C. Sugiyama, I. Oguni and N. Kinae, *J. Health Sci*. 2006, 52, 211-220.

33. U. Takahama, R. Yamauchi and S. Hirota, *Free Radic. Res*. 2014, 48, 956-966.

34. S. Hirota and U. Takahama, *Food Sci. Technol. Res.* 2014, 20, 439-447.

35. J. Terao and M.K. Piskula, in Flavonoids in Health and Disease, ed. C.A. Rice-Evans and L. Packer, Marcel Dekker, New York, 1998, pp. 277-293.

36. U. Takahama, M.Tanaka, T. Oniki, S. Hirota and R. Yamauchi, *J. Agric. Food Chem.* 2007, 55, 4169-4176.

37. U. Takahama, M. Tanaka and S. Hirota, *Free Rardic. Res.* 2010, 44: 293-303.

38. A.K. Timbola, C.D. de Souza, C. Giacomelli and A. Spinelli, *J. Braz. Chem. Soc.* 2006, 17, 139-148.

39. A.M. Bondžić, T.D. Lazarević-Pašti, B.P. Bondžić, M.B. Čolović, M.B. Jadranin and V.M. Vasić, *New J. Chem.* 2013, 37, 901-908.

40. L.V. Jørgensen, C. Claus, J. Ulla, L.H. Skibsted and L.O. Dragsted, *Free Radic. Res*. 1998, 29, 339-350.

41. I.G. Zenkevich, A.Y. Eshchenko, S.V. Makarova, A.G. Vitenberg, Y.G. Dobryakov and V.A. Utsal, *Molecules* 2007, 12, 654-672.

42. H. Ohashi, T. Kyogoku, T. Ishikawa, S Kawase and S. Kawai, *J. Wood Sci*. 1999, 45, 53-63.

43. U. Takahama, R. Yamauchi and S. Hirota, *Food Chem.* 2013, 141, 2600-2606.

44. B.M. Fahlman and E.S. Krol, *J. Photochem. Photobiol B: Biology* 2009, 97, 7123-131.

45. Y. Lu and L.Y. Foo, *Food Chem.* 1997, 59, 187-194.

46. J.-S. Xiao, L. Liu, H. Wu, B.-J. Xie, E.-N. Yang and Z.-D. Sun, *J. Agric. Food Chem.* 2008, 56, 2096-2101.

47. C. Manach, A. Scalbert, C. Morand, C. Remesy and L. Jimenez, *Am. J. Clinic. Nutr.* 2004, 79, 727-747.

48. J.L. Bolton, M.A. Trush, T.M. Penning, G. Dryhurst and T.J. Monks, *Chem. Res. Toxicol.* 2000, 13, 135-40.

49. A.G. Siraki, T.S. Chan and P.J. O'Brien, *Toxicol. Sci.* 2004, 81, 148–159.

50. P.J. O'Brien, *Chem. Biol. Interact.* 1991, 80, 1–41.

51. E.L. Cavalieri, K.-M. Li, N. Balu, M. Saeed, P. Devanesan, S. Higginbotham, J. Zhao, M.L. Gross and E.G. Rogan, *Carcinogenesis* 2002, 23, 1071-1077.

52. G.M. Sobala, C.J. Schorah, M. Sanderson, M.F. Dixon, D.S. Tompkins, P. Godwin and A.T. Axon, *Gastroenterology* 1989, 97, 357-363.

53. C.P. Schultz, M.K. Ahmed, C. Dawes and H.H. Mantsch, *Anal Biochem*. 1996, 240, 7-12.

54. J. Frank, T.W. George, J.K. Lodge, A.M. Rodriguez-Mateos, J.P.E. Spencer, A.M. Minihane and G. Rimbach, *J. Nutr*. 2009, 139, 58-62.

55. S. Egert and G. Rimbach, *Adv. Nutr*. 2011, 2, 8-14.

56. A.H. Schonthal, *Mol. Nutr. Food Res.* 2011, 55, 874-875.

57. J.D. Lambert, S. Sang and C.S. Yang, *Mol. Pharmaceutics* 2007, 4, 819-825.

58. S. Hirota, T. Shimoda and U. Takahama, *J. Agric. Food Chem*. 1998, 46, 3497-3502.

59. L. Jurd, in The Chemistry of Flavonopid Compounds, ed. T.A. Geissman, Macmillian Co., New York, 1962, pp. 107-155.

60. U. Takahama and S. Hirota, *Chem. Res. Toxicol*. 2012, 25, 207-215.

61. C.P. Bondonno, L.A. Downey, K.D. Croft, A. Scholey, C. Stough, X. Yang, M.J. Considine, N.C. Ward, I.B. Puddey, E. Swinny, A. Mubarak and J.M. Hodgson, *Food Fuct*. 2014, 5, 849-858.

Fig. 1. Compounds concerned in this study. **1a**, (+)-catechin; **1b**, (−)-epicatechin; **2a**, 6,8-dinitrosocatechin; **2b**, 6,8-dinitrosoepicatechin; **3**, quercetin; **4**, quercetin 7-*O*-β-D-glucopyranoside; **5**, 2-(3,4-dihydroxybenzoyl)- 2,4,6-trihydroxy-3(2*H*)-benzofuranone.

Fig. 2. Nitrous acid-induced oxidation of Q7G. (A) Nitrite-induced changes in absorption spectra. The reaction mixture (1 mL) contained 25 μM Q7G in 50 mM KCl-HCl (pH 2.0). Sodium nitrite (0.2 mM) was added to spectrum 1, and scanning was repeated 10 times at 1 min intervals. Light path of the measuring beam was 4 mm. (B) HPLC of the oxidation product of Q7G (Q7Gox). B-1, before incubation of 50 μM Q7G; B-2, after incubation of 50 μM Q7G with 0.25 mM $NaNO_2$ for 2 min in 50 mM KCl-HCl (pH 2.0). An oxidation product of quercetin (Qox) was co-chromatographed for comparison. Mobile phase, methanol and 0.2% formic acid (1: 1, v/v). (C) Absorption spectra of Q7G, Q7Gox, and Qox in the mobile phase.

Fig. 3. Nitrite-induced oxidation of Q7G and quercetin. The reaction mixture (1 mL) contained 50 μM Q7G or quercetin in 50 mM KCl-HCl (pH 2.0). The reactions were initiated by adding various concentrations of $NaNO_2$ under anaerobic conditions. After 1 min of incubation, the concentrations of the reactants and products were determined by HPLC as described in Materials and Methods. (○), quercetin remained; (●), Q7G remained; (□), Qox formed; (■), Q7Gox formed. Formation of Qox and Q7Gox was estimated from the peak area of HPLC at 290 nm. Each data point represents mean with SD (n = 3-4).

Fig. 4. Characterization of reaction products of (+)-catechin. (**I**) HPLC. The reaction mixture contained 0.1 mM (+)-catechin and 0.2 mM $NaNO_2$ in 50 mM KCl-HCl (pH 2.0). (**I**-1)

before addition nitrite; (**I**-2) 10 min after addition of nitrite; (**I**-3) 25 min after the addition of nitrite; (**I**-4) 15 min after addition of 1 mM ascorbic acid to (**I**-3). (**II**) HPLC with Q7G. The reaction mixture contained 0.1 mM (+)-catechin and 0.5 mM $NaNO_2$ in 50 mM KCl-HCl (pH 2.0). (**II**-1) before addition of nitrite; (**II**-2) 10 min after addition of nitrite; (**II**-3) 10 min after addition of nitrite in the presence of 0.1 mM Q7G. HPLC was performed using a Shim-pack CLC-ODS and the mobile phase was a mixture of methanol and 0.2% formic acid (1:3, v/v). Numbers in parenthesis, peak areas of **2a**. (**III**) Absorption spectra of **2a** and P1in the mobile phase. AH_2, ascorbic acid; Cat., (+)-catechin.

Fig. 5. Interactions of Q7G with (+)-catechin (A) and (−)-epicatechin (B). The reaction mixture contained various concentrations of Q7G with and without 50 μM (+)-catechin or (−)-epicatechin in 50 mM KCl-HCl (pH 2.0). One min after the addition of 0.5 mM $NaNO_2$ under anaerobic conditions, the reaction mixture was analyzed by HPLC. Mobile phases were mixtures of methanol and 0.2% formic acid. (A) 1:4 (v/v); (B) 1: 3 (v/v). Each data point represents mean with SD (n = 3-4). (● and ○) consumption of Q7G in the absence and presence of catechins, respectively; (▲ and △) formation of Q7Gox in the absence and presence of catechins, respectively; (■) formation of **2a** and **2b** in the presence of (+)-catechin and (−)-epicatechin, respectively; (□) consumption of (+)-catechin or (−)-epicatechin.

Fig. 6. Interactions of Q7G with **2a**/**2b**. The reaction mixture contained 50 μM **2a**/**2b** and various concentrations of Q7G in 50 mM KCl-HCl (pH 2.0). One min after the addition of 0.5 mM $NaNO_2$ under anaerobic conditions, each component was quantified by HPLC. (A) Q7G-dependent inhibition of **2a**/**2b** oxidation and (P1 + P2) formation. (B) **2a**/**2b**-dependent enhancement of Q7G oxidation. The mobile phase was a mixture of methanol and 0.2%

666 formic acid (2:5, v/v). (□) **2a**/**2b** remained in the reaction mixture; (■) formation of P1 +
667 P2; (● and ○) oxidation of Q7G in the absence and presence of **2a**/**2b**, respectively; (▲ and
668 △) formation of Q7Gox in the absence and presence of **2a**/**2b**, respectively. Each data point
669 represents mean with SD (n = 3-4). In (A), error bars are within the squares.

670

671 Fig. 7. Possible reactions among nitrous acid, catechins, and **2a**/**2b**. AH_2, ascorbic acid; AH,
672 monodehydroascobic acid; A, dehydroascorbic acid; CAT, catechins; Q, quercetin; Qox,
673 oxidized quercetin; Q7G, quercetin 7-*O*-glucoside; Q7Gox, oxidized Q7G; ˙, symbol for
674 radical. References 17 and 33 were referred to prepare this figure.

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676

677 Table 1. •NO production in flavonoid/nitrous acid systems.

Q7G (μM)	0	5	15	50
		•NO produced (μM/min)		
No addition	0	10.3 ± 2.6	29.6 ± 5.5	73.8 ± 12.3
50 μM (+)-catechin	20.9 ± 3.8	28.9 ± 2.6 (93%)	41.8 ± 5.2 (83%)	98.4 ± 19.2 (104%)
50 μM (−)-epicatechin	21.5 ± 4.1	30.9 ± 2.4 (97%)	47.7 ± 4.3 (93%)	100.4 ± 14.4 (105%)
50 μM **2a**/**2b**	33.5 ± 4.0*	50.9 ± 7.1 (116%))	81.2 ± 9.9 (129%)	120.7 ± 18.9 (112%)

678 Reactions were initiated by addition of 0.5 mM sodium nitrite under anaerobic conditions.
679 Each data represents mean with SD (n = 3−4). *, significant differences in •NO production
680 compared with 50 μM (+)-catechin or 50 μM (−)-epicatechin ($P < 0.05$). A number in
681 parenthesis is the ratio of NO production rate in presence of Q7G with (+)-catechin, (−)-
682 epicatechin, or **2a**/**2b** to the sum of NO production rate in the presence of Q7G alone and that
683 in the presence of (+)-catechin, (−)-epicatechin, or **2a**/**2b** alone.

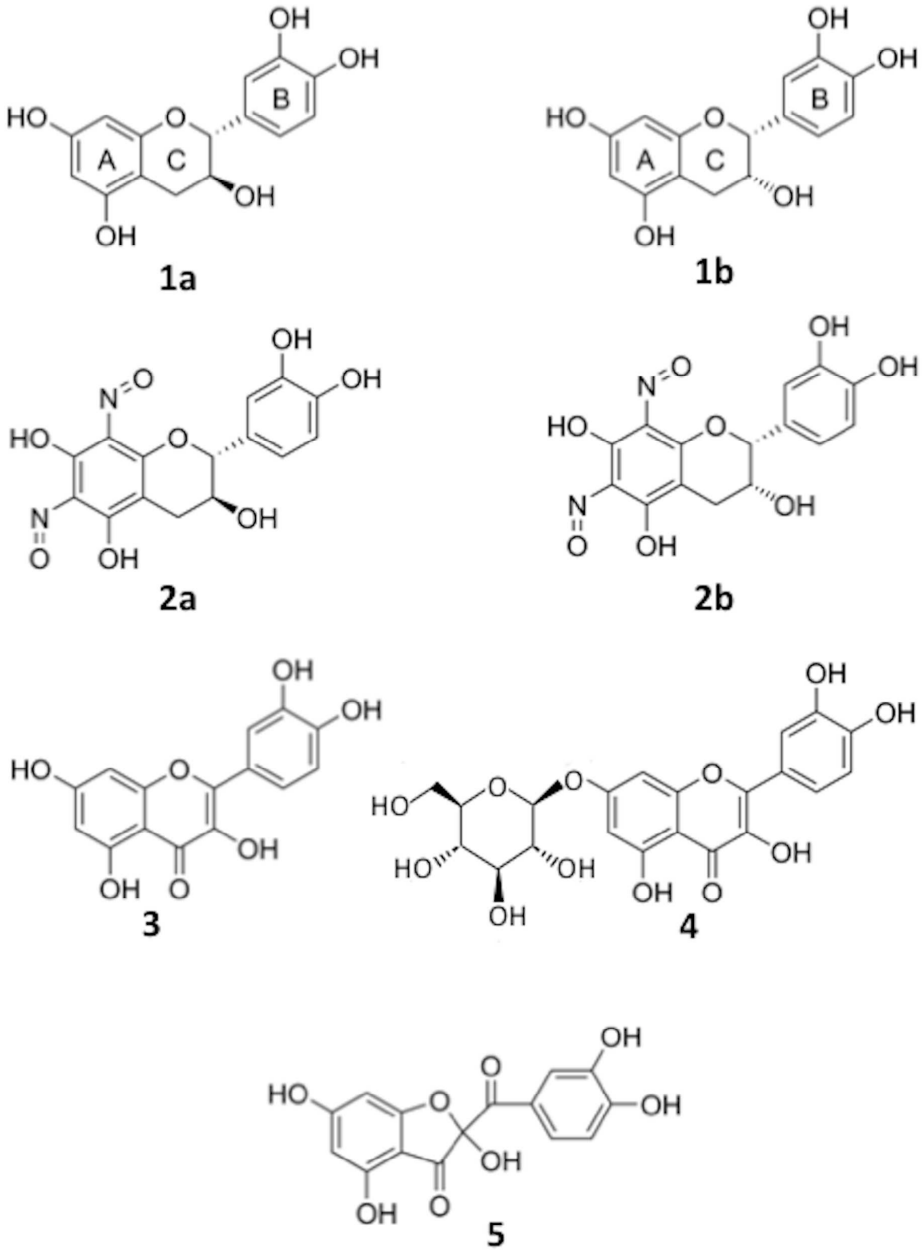
684

Table 2. Interactions between flavonols and (+)-catechin or **2a**/**2b**.

		Consumption of flavonols (μM/min)			Formation of **2a** (per min) (arbitrary unit)	Formation of (P1+P2) (per min) (arbitrary unit)
Addition (100 μM)		(1) No addition	(2) (+)-catechin	(3) **2a**/**2b**	(4) (+)-catechin	(5) **2a**/**2b**
Without flavonols					21.2 ± 0.2	21.4 ± 2.5
Rutin (μM)	10	0.1 ± 0.1	0.2 ± 0.1	0.7 ± 0.1[b]	n.s.d.	n.s.d.
	30	2.5 ± 0.4	3.7 ± 0.3[a]	3.8 ± 0.3[b]	n.s.d.	n.s.d.
	100	10.7 ± 1.1	11.4 ± 4.5	12.8 ± 2.7	n.s.d.	n.s.d.
Q4'G (μM)	10	1.0 ± 0.1	1.1 ± 0.2	3.7 ± 0.5[b]	21.1 ± 0.1	19.4 ± 1.1
	30	5.3 ± 0.4	6.9 ± 0.1[a]	9.5 ± 0.2[b]	20.8 ± 0.2	14.6 ± 0.5[d]
	100	13.9 ± 1.1	20.4 ± 0.5[a]	23.5 ± 0.4[b]	19.7 ± 0.8[c]	9.7 ± 0.2[d]
Kaempferol(μM)	10	3.3 ± 0.6	5.2 ± 0.6[a]	4.6 ± 1.0	20.0 ± 0.6[c]	13.0 ± 1.2[d]
	30	10.7 ± 0.2	12.3 ± 1.0[a]	12.6 ± 2.4	18.9 ± 0.8[c]	7.5 ± 0.6[d]
	100	23.8 ± 1.1	32.5 ± 3.2[a]	41.8 ± 3.0[b]	18.0 ± 0.2[c]	6.5 ± 0.2[d]

All reactions were performed in 50 mM KCl-HCl (pH 2.0). One min after the addition of 0.5 mM $NaNO_2$, the reaction mixtures were analyzed by HPLC. Each data represents mean ± SD (n = 3). [a and b], significant differences between columns 1 and 2 and between columns 1 and 3 ($P < 0.05$); [c and d], significant inhibition of formation of **2a** (column 4) and (P1 + P2) (column 5), respectively, by rutin, Q'4G and kaempferol ($P < 0.05$). n.s.d., no significant difference between the values without rutin.

693 Fig. 1



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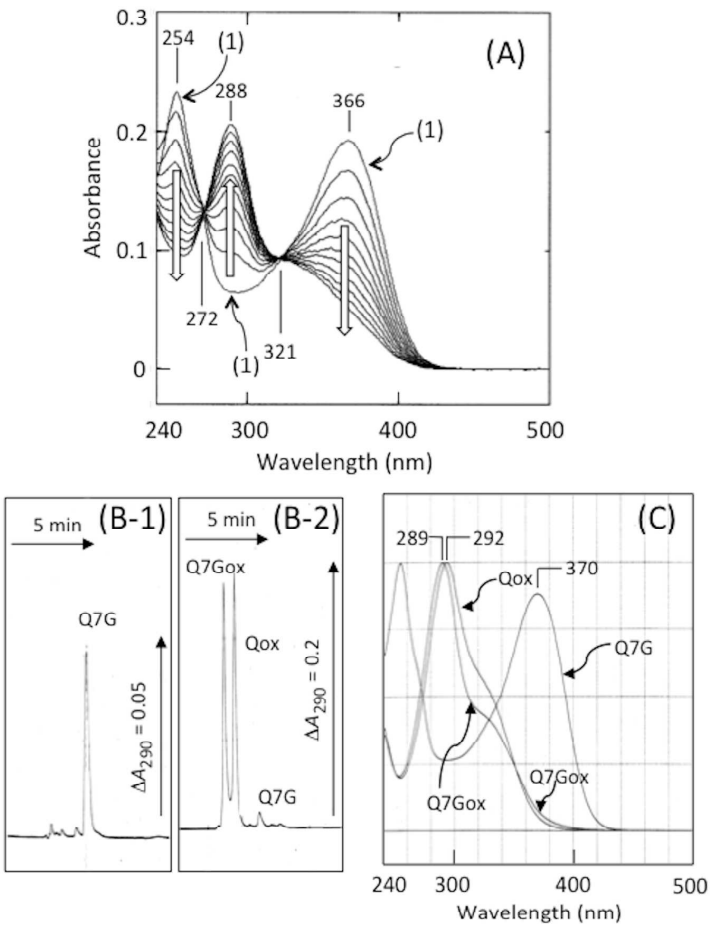
699 Fig. 2



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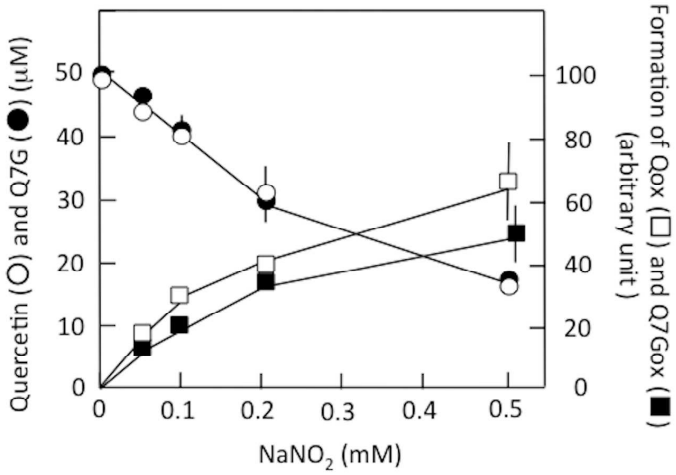
701 Fig. 3



702

Fig. 4

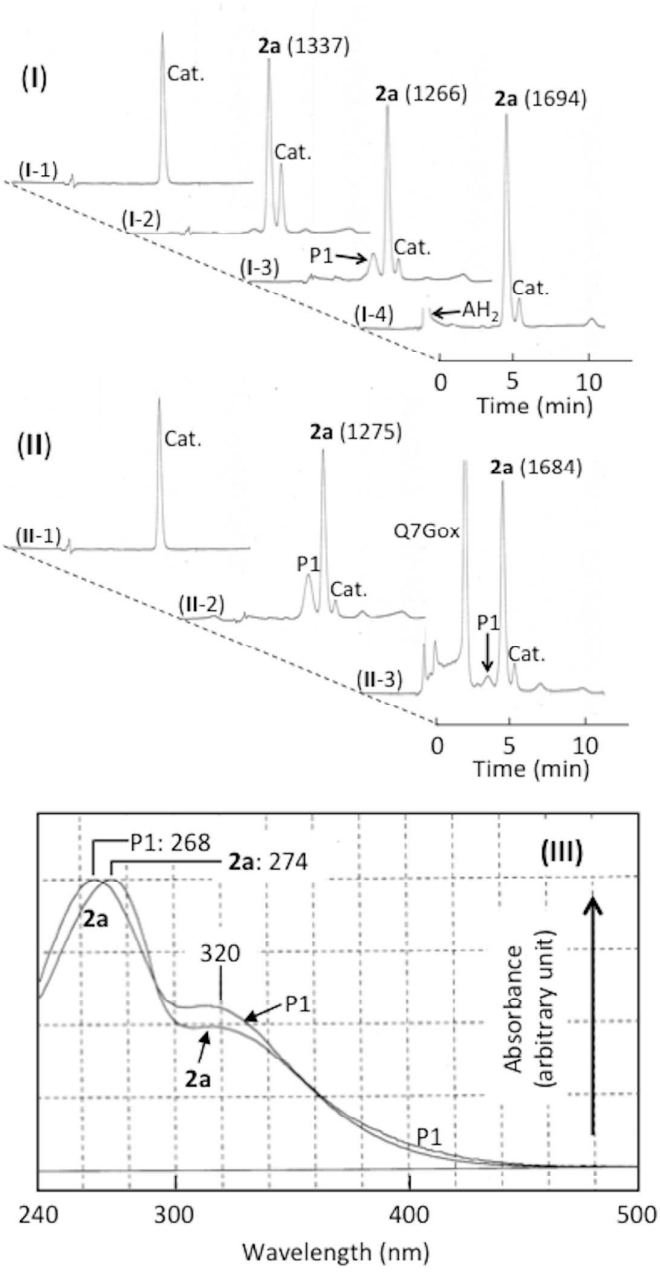


Fig. 5

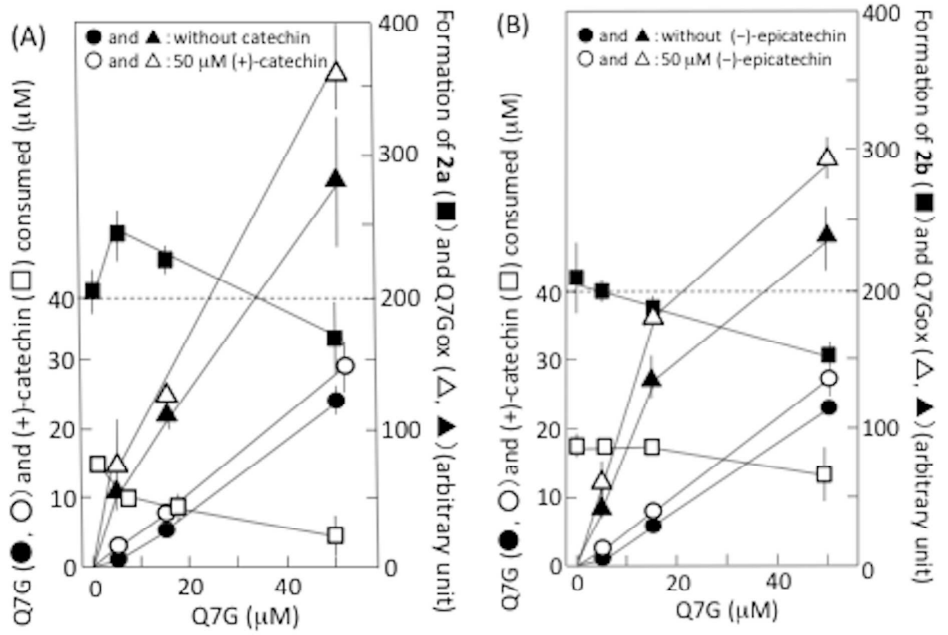
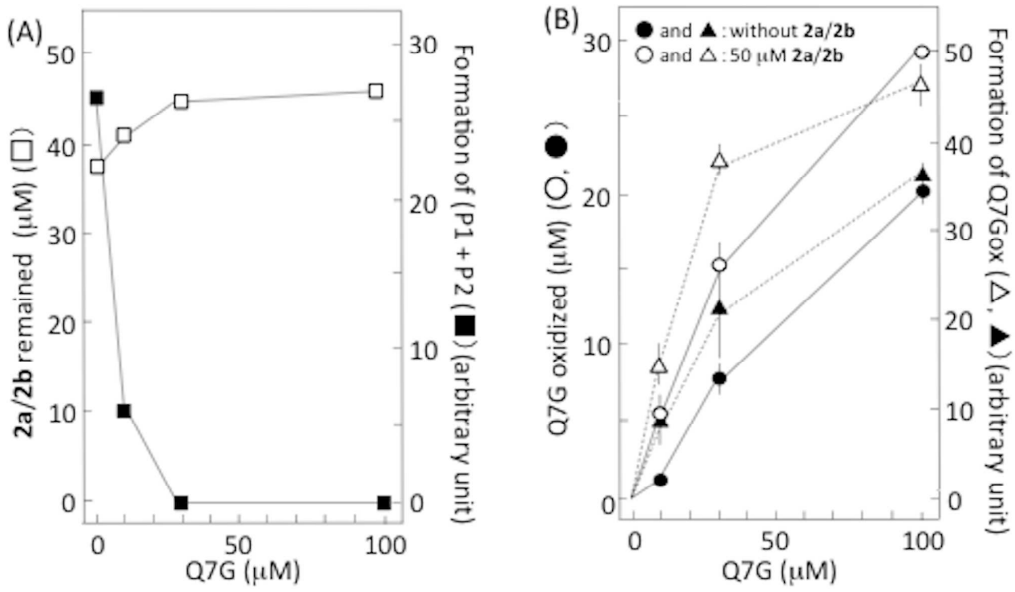
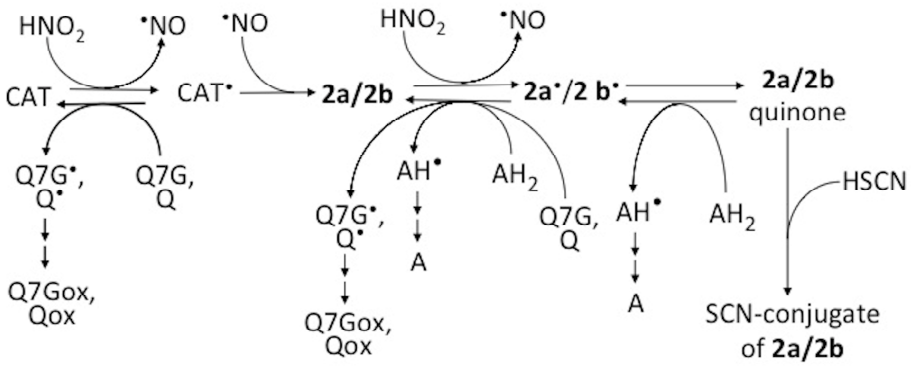


Fig. 6



716 Fig. 7



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