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# Antioxidant activity of Japanese pepper (*Zanthoxylum piperitum* DC.) fruit

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**Abstract**

Antioxidants were extracted from Japanese pepper (*Zanthoxylum piperitum* DC.) fruit and characterized. The antioxidant activity of the methanol extract from Japanese pepper fruit was found to be equal to that of α-tocopherol and stable under heat treatment. The main compounds that gave a significant antioxidant activity from the methanol extract were identified to be hyperoside (quercetin-3-*O*-galactoside) and quercitrin (quercetin-3-*O*-rhamnoside) as determined by HPLC, mass spectrometry, UV/Vis spectroscopy, and TLC. Radical-scavenging activities of hyperoside and quercitrin from Japanese pepper fruit were evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. As a result, hyperoside and quercitrin scavenged DPPH radical strongly with $IC_{50}$ values of 16 and 18 μM, respectively. These observations show the presence of strong antioxidants, namely hyperoside and quercitrin in Japanese pepper fruit.


*Keywords:* Japanese pepper; Antioxidant; 1,1-Diphenyl-2-picrylhydrazyl; Hyperoside; Quercitrin

## 1. Introduction

Lipid peroxidation and free radicals are well known to be among the main causes for compromising the quality of food during processing and storage. Therefore, it is obvious that the prevention of lipid peroxidation in the food is effective in not only the stability of the nutritional content but also the extension at the best-before date. In living systems, biomembranes are composed of lipids including unsaturated fatty acids that react easily to form lipid peroxide and free radicals. Accumulation of lipid peroxides in living systems induces functional abnormalities and pathological changes (Culter, 1984; Harman, 1982, chap. 8; Yagi, 1987). Therefore, much attention has been paid to the antioxidants, which are expected to work effectively to prevent food and living systems from peroxidative damage. Though butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are artificial strong antioxidants, have been much used in the food industry, they are suspected to be toxic in the lung and carcinogenic (Mizutani, Nomura, Nakanishi, & Fujita, 1987; Williams, Maeura, & Weisburger, 1983). Hence, the importance of research for finding natural antioxidants has been greatly increased in recent years (Katsube, Iwashita, Tsushida, Yamaki, & Kobori, 2003; Noda, Kaneyuki, Mori, & Packer, 2002; Schaffer, Schmitt-Schillig, Müller, & Eckert, 2005; Tsuda, Ohshima, Kawakishi, & Osawa, 1994).

Japanese pepper (*Zanthoxylum piperitum* DC.) that belongs to the Rutaceae family is a deciduous and shrubbery tree and is distributed in the Japanese islands, China, and the Korean peninsula. It is well known that Japanese pepper has a special aroma over the whole plant and its pericarp is commonly used as a spice in Japan. Hence, Japanese pepper has been investigated for its characteristic aroma and stimulant effect. For example, citranellal and sanshool are known to be the most important compounds involved (Sakai, Yoshihara, & Hirose, 1968,

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1970). The strong antioxidant activity of Japanese pepper leaves has also been reported for leaves (Cho et al., 2003; Hur, Park, & Hwang, 2001) and fruits (Hisatomi, Matsui, Kobayashi, & Kubota, 2000; Yamazaki & Inagaki, 2004). In addition, antioxidants have been purified and identified to be magnoflorine (5,6,6a,7-tetrahydro-1,11-dihydroxy-2,10-dimethoxy-6,6-dimethyl-4*H*-dibenzo[de,g]quinolium) in the pericarp and arbutin (4-hydroxyphenyl-*b*-D-glucopyranoside) from seed (Hisatomi et al., 2000). However, the antioxidant activities and DPPH radical-scavenging activities of magnoflorine and arbutin were not as strong as they were expected.

The aims in this work were to isolate, identify, and characterize antioxidant(s), other than magnoflorine and arbutin, in Japanese pepper fruit and to examine their effects in in vitro models.

## 2. Materials and methods

### 2.1. Materials

Japanese pepper fruit, which was collected at Higashi Kishuu area in Japan, was a kind gift from Isefunzai Co. (Mie, Japan).

### 2.2. Chemicals

Quercetin-3-*O*-galactoside (hyperoside) and quercetin-3-*O*-rhamnoside (quercitrin) were obtained from Funakoshi Co. (Tokyo, Japan). Unless otherwise indicated, all chemicals and solvents used were of analytical grade.

### 2.3. Preparation of the methanol extract from Japanese pepper

Fresh Japanese pepper fruit (100 g) was ground and extracted with methanol (500 ml) at room temperature overnight. The extract was filtrated through 0.45 μm filter paper and then water was added to the filtrates to afford 80% methanol solution. The solution was defatted five times by adding equal volumes of *n*-hexane. The resultant aqueous phase was collected and evaporated in vacuo.

### 2.4. Determination of total polyphenol

The concentration of polyphenol in the methanol extract of Japanese pepper fruit was determined using the Folin–Ciocalteu method (Singleton & Rossi, 1965) and results were expressed as quercitrin equivalent. The methanol extract was diluted in 80% methanol beforehand. Twenty microlitres of sample were added to 125 μl of $Na_2CO_3$ in 0.1 M NaOH and incubated at 37 °C for 10 min. Then 25 μl of previously twofold-diluted Folin–Ciocalteu reagent solution were added. After standing for 20 min at 37 °C, the absorbance was measured at 750 nm using a Model 550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.5. Measurement of the antioxidant activity

Measurement of the antioxidant activity was carried out by the ethanol–linoleic acid autoxidation system (Osawa & Namiki, 1981). Antioxidant (100 μg) was added to a solution mixture, which contained linoleic acid (0.13 ml), ethanol (10 ml), 50 mM phosphate buffer (pH 6.8, 10 ml), and water (5 ml). The solution was mixed in a conical flask and incubated at 40 °C. The oxidation was monitored at intervals during incubation by the thiocyanate method (Osawa & Namiki, 1981) by reading the absorbance at 500 nm after colour development using $FeCl_2$ and thiocyanate; the results were expressed as 100% lipid peroxidation ratio of the control with no antioxidant added.

### 2.6. Stability of the methanol extract by heat treatment at different pH

Stability of the methanol extract of Japanese pepper fruit under heat treatment at different pH was carried out using the method of Miyake, Yamamoto, and Osawa (1997). The buffer solutions were prepared at pH 3.5 and 5.0 with 50 mM sodium acetate, and pH 7.0 with 50 mM sodium phosphate. The final concentration of 1.0 mg/ml of the methanol extract was added to each buffer and then heated at 80 °C for 30 min or at 120 °C for 20 min. After the treatment, the solutions were subjected to the ethanol–linoleic acid autoxidation system measured by the thiocyanate method. The data were reported as mean ± SD values for three independent experiments and stabilities were estimated against unheated samples.

### 2.7. Purification and identification of the antioxidant from the methanol extract of Japanese pepper fruit

The methanol extract of Japanese pepper fruit was dissolved in methanol and fractionated using preparative HPLC (650E Advanced Protein Purification System, Waters, Milford, MA, USA) equipped with a column of μ Bondasphere-15 μC18 300A (19.0 mm i.d. × 300 mm, Waters, Milford, MA, USA) with a 996 photodiode array detector (Waters, Milford, MA, USA). The elution was first under isocratic condition using 30% methanol for 10 min and then with a linear gradient ranging from 30% to 100% methanol for 130 min. The flow rate was kept constant at 2 ml/min. The separated fractions were assayed using the ethanol–linoleic acid autoxidation system. The antioxidative fractions were collected and purified again under the same condition using preparative HPLC, and the purified fractions were concentrated in vacuo. Each purified fraction was confirmed for giving a single peak by HPLC with a Waters 2697 Alliance (Waters, Milford, MA, USA) and 996-photodiode array detector using a semi-micro column (Inertsil C18, 1.5 mm i.d. × 150 mm, GL Science, Tokyo, Japan). The flow rate was 0.1 ml/min and the elution was first under isocratic condition of 30% methanol for 5 min and then using a linear gradient ranging

from 30% to 100% methanol for 20 min. The purified antioxidants were used as samples for identification purposes and determination of DPPH radical-scavenging activity.

### 2.8. UV/Vis spectroscopy

For identification of the antioxidants, UV/Vis spectra were measured in methanol, sodium methylate, methanolic aluminum chloride, methanolic aluminum chloride–hydrochloric acid, methanolic sodium acetate, and methanolic sodium acetate–boric acid solution by the method of Markaham (1982). All spectra were measured using a V-580 System spectrophotometer (Jasco, Tokyo, Japan).

### 2.9. Acid hydrolysis of the antioxidants to determine the aglycon and sugar

To determine the aglycone and sugar, the antioxidants were subjected to acid hydrolysis with HCl. The antioxidants were suspended in 5% HCl and incubated for 1 h at 100 °C and then cooled and extracted with ether. Each of the ether and the residual aqueous layer were concentrated in vacuo and subjected to TLC (Silica gel 60 $F_{254}$, Merck, Darmastadt, Germany) in an ethyl acetate/*n*-butanol/water (65:22.75:12.25, -v/v/v-). After development, the RF (rate of flow) of the ether layer was measured at 254 nm. On the other hand, the aqueous layer was identified using methanol/*p*-anisaldehyde/sulfuric acid (8.5:0.5:1, v/v/v).

### 2.10. ESI/MS analysis

Mass spectrometry analysis was performed using a Finnigan LCQ (Thermo Electron, San Jose, CA, USA) equipped with an API source, using an electrospray ionization (ESI) probe. The antioxidant was dissolved in methanol and infused into the ESI source by using a syringe pump; the flow rate was 2–10 μl/min. The capillary voltage, the spray voltage, the tube lens offset, and the capillary temperature were at 8 V, 4.5 kV, 55 V, and 200 °C, respectively. When the molecular ion of the antioxidant was detected, its $MS^2$ spectrum was obtained using a relative energy of collision of 12. $MS^3$ of the main fragment ion in the $MS^2$ spectrum was also obtained using a relative energy of collision of 30.

### 2.11. Measurement of the DPPH radical-scavenging activity

The DPPH radical-scavenging activity was assessed by the method of Singh, Murthy, and Jayaprakasha (2002). Different concentrations of the antioxidants dissolved in 25 μl of methanol were taken in different test tubes. Then 1.0 ml of a 0.2 mM DPPH in methanol was added to each the test tube. The reaction mixtures were allowed to stand for 20 min at room temperature. The control was prepared without any antioxidants, and methanol was used for the baseline correction. Changes in the absorbance of the reaction mixtures were measured at 490 nm using a Model 550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). DPPH radical-scavenging activity was expressed as the inhibition percentage and was calculated using the following formula: DPPH radical-scavenging activity (%) = (1 − antioxidant OD/control OD) × 100.

## 3. Results and discussion

### 3.1. Antioxidative activity of the Japanese pepper fruit

The polyphenol content of the methanol extract from Japanese pepper fruit was 6.0% (w/w) quercitrin equivalent using Folin–Ciocalteu reagent. The methanol extract was then diluted to be 100 μg quercitrin equivalent. The antioxidative activity of the methanol extract in the linoleic acid–ethanol system measured by the thiocyanate method is shown in Fig. 1. The methanol extract showed potent antioxidant activity, which was equivalent to the same concentration of α-tocopherol. These data suggested that the some strong antioxidants must be present in the methanol extract of Japanese pepper fruit.

### 3.2. Stability of the methanol extract by heat treatment at different pH

For the methanol extract's use in beverage and/or food, it is important to examine its stability toward sterilization (Miyake et al., 1997). Table 1 shows the stability of the methanol extract from Japanese pepper fruit upon heat treatment at different pH. Under acidic conditions (pH 3.5 and 5.0), the methanol extract was stable upon treatments. Though its stability was slightly weaker and the solution got discoloured under neutral conditions at high temperatures (120 °C, 20 min), the residual amount still exhibited more than 70% activity. In general, flavonoids are stable under acidic conditions, however, in alkaline solutions flavonoids are unstable and discolour because hydroxyl group(s) of flavonoid dissociate(s) and O-1 → C2 linkage in C-ring cleaves. These results suggest that the use of the methanol extract is suitable under neutral pH for beverage and/or food.

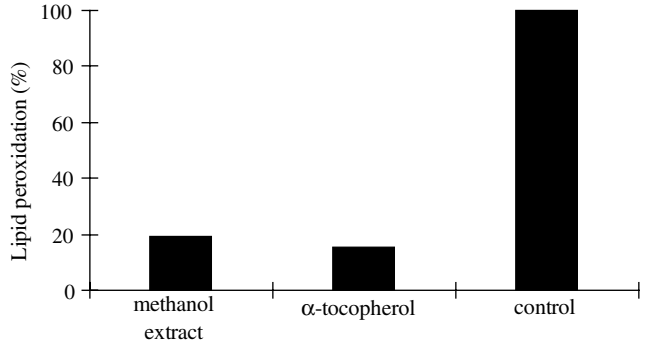



Fig. 1. Antioxidant activity of the methanol extract from the Japanese pepper fruit measured by the thiocyanate method. α-Tocopherol (100 μg) was used for the standard sample. A control with no antioxidant represents 100% lipid peroxidation.

Table 1
Stability of the methanol extract from Japanese pepper fruit by heat treatment at different pH

| pH | Temp. (°C) | Time (min) | Residual ratio (%) |
|---|---|---|---|
| 3.5 | 80 | 30 | 85.9 ± 1.1 |
| | 120 | 20 | 88.7 ± 0.3 |
| 5.0 | 80 | 30 | 85.8 ± 1.9 |
| | 120 | 20 | 89.2 ± 0.2 |
| 7.0 | 80 | 30 | 85.2 ± 0.8 |
| | 120 | 20 | 73.0 ± 3.0 |

The stability of the methanol extract by heat treatment was determined by the residual ratio of the antioxidant activity after heat treatment. Reported values are the mean ± SD ($n = 3$).

### 3.3. Purification and identification of the antioxidants from the methanol extract

The methanol extract of Japanese pepper fruit was successively subjected to preparative HPLC. Two slightly yellow pigments were isolated in 45% and 50% methanol fractions; pigments 1 and 2, respectively. We confirmed that each of the purified pigment was due to a single peak as evidenced by a reversed-phase HPLC using a semi-micro column. The purified pigments were collected and used for identification and estimation of DPPH radical-scavenging activity.

Each pigment was adjusted by dilution with methanol so that the absorbance of the major absorption peaks around 250 nm (band II peak region) and 350 nm (band I peak region) gave reading in the region 0.6–0.8.

The UV/Vis spectrum of pigment 1 showed two peaks at 253 and 358 nm and a shoulder at 269 nm, which indicate that pigment 1 was a flavone or flavonol. The presence of a free hydroxyl-group at the 4′-position in the B-ring was indicated by a bathochromic shift of 50 nm of band I at 358 nm after addition of sodium methoxide. A new peak appeared at 328 nm suggesting the presence of a free hydroxyl-group at the 7′-position. Since addition of sodium methoxide to pigment 1 did not induce immediate disappearance of band I, absence of a free hydroxyl-group at the 3-position in the C-ring was indicated. The addition of anhydrous aluminum chloride produced a bathochromic shift of 81 nm of band I at 358 nm, and a hypochromic shift of 33 nm of band I. Then two new peaks at 300 and 357 nm appeared upon the addition of hydrochloric acid ($AlCl_3$/HCl), indicated that pigment 1 was 5-hydroxy-3-substituted flavonol. The addition of anhydrous sodium acetate produced a bathochromic shift of 19 nm for band II at 255 nm, which indicated the presence of a free hydroxyl-group at the 7-position in the A-ring. A bathochromic shift of 22 nm of band I at 358 nm upon addition of boric acid-sodium acetate ($NaOAc/H_3BO_3$) indicated the presence of a free *O*-dihydroxy group in the B-ring.

The UV/Vis spectrum of pigment 2 showed two peaks at 254 and 348 nm, and a shoulder at 266 nm, indicating that pigment 2 was a flavone or flavonol. The presence of a free hydroxyl-group at the 4′-position in the B-ring was indicated by a bathochromic shift of 47 nm for band I at 348 nm after the addition of sodium methoxide. A new peak appeared at 324 nm suggesting the presence of a free hydroxyl-group at the 7′-position. Since addition of sodium methoxide to pigment 2 did not induce immediate disappearance of band I, absence of free hydroxyl-group at the 3-position in the C-ring was indicated. The addition of anhydrous aluminum chloride produced a bathochromic shift of 83 nm of band I at 348 nm. Then a hypochromic shift of 29 nm for band I and two new weak peaks at 303, 348 nm appeared upon the addition of hydrochloric acid ($AlCl_3$/HCl), indicating that pigment 2 was 5-hydroxy-3-substituted flavonol. Addition of anhydrous sodium acetate produced a bathochromic shift of 14 nm of band I at 255 nm, which indicated the presence of a free hydroxyl-group at the 7-position in the A-ring. A bathochromic shift of 20 nm of band I at 348 nm upon addition of boric acid-sodium acetate ($NaOAc/H_3BO_3$) indicated the presence of a free *O*-dihydroxy group in the B-ring.

To identify the molecular structures, these pigments were subjected to acid hydrolysis and TLC. The hydrolysates were extracted with ether. Each of the ether and residual aqueous layer was subjected to TLC. As a result, both the ether layers of these pigments corresponded with authentic quercetin (3,5,7,3′,4′-pentahydroxyflavone) and the aqueous layers of pigment 1 and 2 corresponded with authentic galactose and rhamnose, respectively. Each intact pigment 1 and 2 was in good agreement with hyperoside (quercetin-3-*O*-galactoside) and quercitrin (quercetin-3-*O*-rhamnoside), respectively.

Pigments 1 and 2, and their ether layer of the hydrolysates were individually analyzed by ESI/MS. The ether layer of pigment 1 produced a $[M - H]^-$ at *m*/*z* 301, which was consistent with an authentic quercetin. Pigment 1 produced a $[M - H]^-$ ion at *m*/*z* 463 and fragment ions of $[M - H - 162]^-$ at *m*/*z* 301 and $[M - H - 302 + H_2O]^-$ at 179. These ion masses were fitted exactly with the structure of hyperoside.

The ether layer of pigment 2 produced a $[M - H]^-$ at *m*/*z* 301, which is consistent with authentic quercetin. Pigment 2 produced a $[M - H]^-$ ion at *m*/*z* 447 and fragment ion $[M - H - 146]^-$ at *m*/*z* 301, which is consistent with the loss of rhamnose residue. These ion masses fitted exactly with the structure of quercitrin.

Based on the UV/Vis spectral characteristics, TLC and mass spectrometry (Table 2), pigments 1 and 2 were identified to be hyperoside and quercitrin, respectively (Fig. 2).

The analysis of phenolic compounds has been mostly been carried out by HPLC coupled with photodiode array system. Recently, electrospray mass spectrometry has proved to be useful in the identification of phenolic compounds (Cardoso et al., 2005; Ye, Yan, & Guo, 2005).

Quercetin glucosides are widely distributed in plants (Müller, Vasconcelos, Coelho, & Biavatti, 2005; Xu, Qi, Wang, & Chen, 2005; Ye et al., 2005) and recently have attracted much attention because of their multiple chemical and biological effects, such as antioxidant (Kirakosyan et al., 2003; Zou, Lu, & Wei, 2004), anticarcinogenic (Kern

Table 2
UV/Vis spectra ($\lambda$ max), TLC, and mass spectrometry data of the pigments of the methanol extract from Japanese pepper fruit

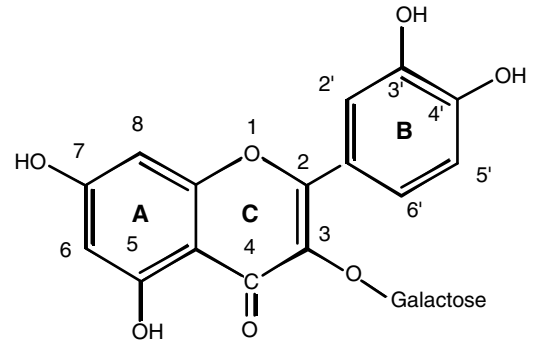
| Substance | $\lambda_{max}$ (nm) | | | | | | *m/z* | TLC |
|---|---|---|---|---|---|---|---|---|
| | MeOH | +NaOMe | +$AlCl_3$ + $AlCl_3$/HCl + NaOAc + NaOAc/$H_3BO_3$ | | | | | |
| Pigment 1 | 253 (II) | 270 (II) | 274 (II) | 268 (II) | 272 (II) | 262 (II) | 463 $[M-H]^-$ | Hyperoside |
| | 269 sh | 328 | 439 (I) | 300 | 318 | 380 (I) | 301 $[M-H-162]^-$ | |
| | 358 (I) | 408 (I)↑ | | 357 | 392 (I) | | 179 $[M-H-301+H_2O]^-$ | |
| | Not immediate disappearance (I) | | | 406 (I) | | | | |
| Hydrolysate (ether layer) | | | | | | | 301 $[M-H]^-$ | Quercetin |
| Hydrolysate (aqueous layer) | | | | | | | | Galactose |
| Pigment 2 | 254 (II) | 271 (II) | 274 (II) | 271 (II) | 268 (II) | 262 (II) | 447 $[M-H]^-$ | Quercitrin |
| | 266 sh | 324 | 431 (I) | 303 | 319 | 368 (I) | 301 $[M-H-146]^-$ | |
| | 348 (I) | 395 (I)↑ | | 348 | 360 (I) | | | |
| | Not immediate disappearance (I) | | | 402 (I) | | | | |
| Hydrolysate (ether layer) | | | | | | | 301 $[M-H]^-$ | Quercetin |
| Hydrolysate (aqueous layer) | | | | | | | | Rhamnose |

I, bandI; II, bandII; sh, shoulder; +, addition of reagents; ↑, increase of the abs. regarding to MeOH sol.

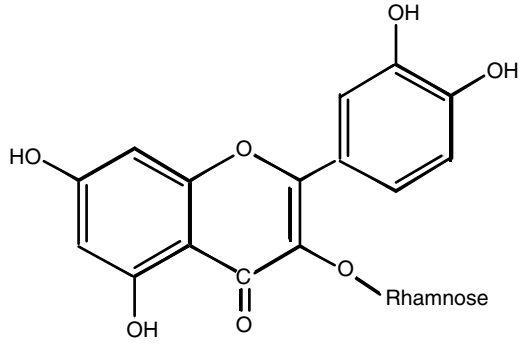
et al., 2005), bacteriostatic (Dall'Agnol et al., 2003; Li et al., 2005), antiinflammatory (Lee et al., 2004), and inhibition of glucose uptake (Cermak, Landgraf, & Wolffram, 2004).

### 3.4. Antioxidant activity and DPPH radical-scavenging activity of hyperoside and quercitrin

Antioxidant activities of hyperoside and quercitrin purified from the methanol extract of Japanese pepper fruit were estimated in the ethanol–linoleic acid autoxidation system by the thiocyanate method as shown in Fig. 3. The antioxidant activities of hyperoside and quercitrin were equivalent at the same concentration of α-tocopherol. The radical-scavenging activities of hyperoside, quercitrin, and α-tocopherol at different concentrations were examined by the DPPH method. A radical scavenger reacts with DPPH radical, which is a stable free radical, and converts it to 1,1-diphenyl-2-picrylhydrazine. The degree of discoloration indicates the scavenging activity of the antioxidant. As a result, hyperoside, quercitrin and α-tocopherol showed DPPH radical-scavenging activity in a dose-dependent manner. $IC_{50}$ values of hyperoside, quercitrin, and α-tocopherol were 16, 18, and 36 μM, respectively (Fig. 4). Both hyperoside and quercitrin showed higher DPPH radical-scavenging activities than α-tocopherol. On the other hand, Hisatomi et al. (2000) reported that $IC_{50}$ values of magnoflorine and arbutin were 480 and 890 μM, respectively, which were over 20 times less than those of hyperoside and quercitrin. In addition, the content of arbutin in Japanese pepper fruit was below 40 mg/100 g (dry weight) while the contents of hyperoside and quercitrin were 439




Hyperoside (qercetin-3-*O*-galactoside)




Quercitrin (quercetin-3-*O*-rhamnoside)

Fig. 2. Chemical structures of hyperoside and quercitrin of the methanol extract from Japanese pepper fruit.

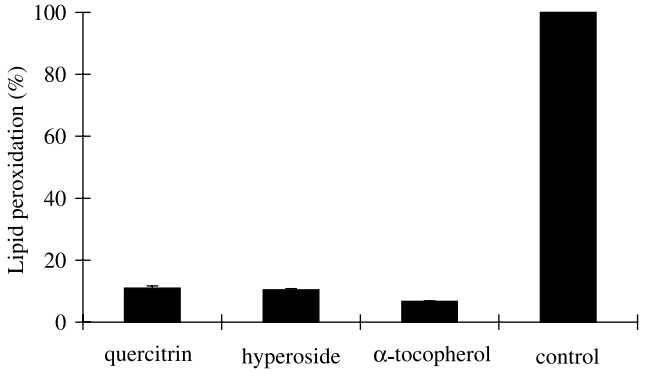



Fig. 3. Antioxidant activity of hyperoside and quercitrin (100 μg) from Japanese pepper fruit measured by the thiocyanate method. α-Tocopherol (100 μg) was used for the standard sample. The values are the mean ± SD ($n = 3$). A control with no antioxidant represents 100% lipid peroxidation.

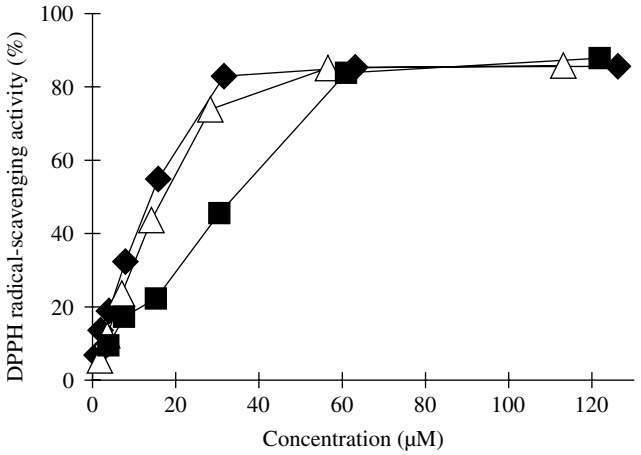



Fig. 4. DPPH radical-scavenging activities of hyperoside and quercitrin from Japanese pepper fruit. α-Tocopherol was used for the standard sample. The values are the mean ± SD ($n = 3$). Symbols: ◆, hyperoside; △, quercitrin; ■, α-tocopherol.

and 39 mg/100 g (fresh weight), respectively (magnogflorine's was not noted). These data suggest that hyperoside and quercitrin are the main contributors for the antioxidant activity of Japanese pepper fruit.

Antioxidant activity and radical-scavenging activity of flavonoids depend highly on their structure, especially a free C-3–OH, a free C-4′–OH, a double bond between C-2 and C-3, and an *O*-dihydroxy grouping in the B-ring are important (Burda & Oleszek, 2001). Both hyperoside and quercitrin were good match for these requirements with the exception of the absence of a free C-3–OH. However, in biological systems both quercetin glycosides might undergo enzymatic hydrolysis, resulting in the formation of quercetin, a highly antioxidative aglycone with a free C-3–OH (Murota & Terao, 2003).

In conclusion, hyperoside and quercitrin from the methanol extract of Japanese pepper fruit inhibited lipid peroxidation effectively and exhibited strong radical-scavenging activity, which would be expected to increase shelf life of foods and protect against peroxidative damage in living systems in relation to aging and carcinogenesis. Thus, Japanese pepper fruit presents a potentially valuable source of natural antioxidant and bioactive material.

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