# Antioxidative Activity in the Pericarp and Seed of Japanese Pepper (*Xanthoxylum piperitum* DC)

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The antioxidative activity of the dried pericarp and seed of Japanese pepper was studied. The ethyl acetate extract from the pericarp and the methanol extract from the seed showed strong antioxidative activity against linoleic acid by the ferric thiocyanate and thiobarbituric acid (TBA) methods. Japanese pepper contained 3.9 and 2.9 mg/100 g of dry weight (dw) of tocopherols in the pericarp and seed, respectively, α-Toc in the former constituting 82% of total tocopherol and γ-Toc in the latter constituting 96%. Arbutin and magnoflorine were isolated as antioxidants and their chemical structures determined by instrumental analyses. The contents of arbutin evaluated as the trifluoroacetate derivative by GC-MS were 35 and 3.0 mg/100 g of dw in the pericarp and seed, respectively. Magnoflorine was present only in the seed, and not in the pericarp. Both arbutin and magnoflorine exhibited antioxidative activity against linoleic acid and radical-scavenging activity against the DPPH radical.

**Keywords:** *Japanese pepper; antioxidative activity; DPPH radical-scavenging; arbutin; magnoflorine; Xanthoxylum piperitum DC*

## INTRODUCTION

The young leaves and fruits of Japanese pepper (*Xanthoxylum piperitum* DC) present a pleasant odor and a strong pungent taste and are commonly used as a spice in Japan. Particularly after the fruits have ripened and dried, the pericarps are separated from the seeds and ground. This ground pericarp is widely used as the spice "sansho powder" and as an ingredient of some spice mixes. Sakai et al. (1968, 1970) reported that the essential oil of the ripe fruit was composed mainly of alcohols, aldehydes, ketones, and esters and that citronellal was most important to the characteristic aroma. Sanshool I and II are also known as pungent compounds. Although spices are known to have many biological effects such as antimicrobial, antioxidative, and antiplatelet activities, only the report of Morimitsu et al. (1995) describes the strong inhibitory effect of the methanol extract of Japanese pepper on the formation of advanced glycation end products.

The objectives of this study were (1) to determine the antioxidative activity of the dried pericarp and seeds of Japanese pepper, (2) to isolate and characterize the antioxidants with respect to their structures and radical-scavenging activity, and (3) to compare the antioxidative activity of seeds with that of pericarp to find a practical use for the seeds, which at present are discarded.

## MATERIALS AND METHODS

**Materials.** Ripe and dried pericarp complete with seeds of Japanese pepper collected in Gifu prefecture were purchased from Yasuma Co. (Shizuoka, Japan).

Arbutin was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Magnoflorine iodide was kindly provided by Dr. T. Ishikawa (Chiba University, Chiba, Japan).

**Preparation of Extracts.** The crushed pericarp (600 g) or seed (1 kg) was extracted with hexane (1 L) three times, and the combined filtrate was concentrated. The residue was successively extracted with (3 × 1 L) diethyl ether, ethyl acetate, and 80% methanol, each solvent being evaporated in vacuo to obtain the extract.

**Measurement of the Antioxidative Activity.** *(a) TLC Method.* The antioxidative activity of the separated compound by TLC was checked according to the method of Anet (1971) and Seino et al. (1971). The antioxidants were separated by TLC with suitable solvents. The TLC plate was air-dried, sprayed with 5% linoleic acid/hexane, and then allowed to stand under a UV lamp for 10 min. The antioxidants were revealed as white spots on a pink background by spraying the plate with 1% *N*,*N*-dimethyl-*p*-phenylenediamine HCl/ethanol.

*(b) Ferric Thiocyanate and Thiobarbituric Acid (TBA) Methods.* The antioxidative activity against linoleic acid was measured by using the ferric thiocyanate and TBA methods of Inatani et al. (1983) and Osawa and Namiki (1981). Butylated hydroxytoluene (BHT) was used as a standard.

*(c) Measurement of the DPPH Radical-Scavenging Activity.* The method of Yamaguchi et al. (1998) was adapted for assessment of the radical-scavenging activity. A test sample was dissolved in ethanol, only magnoflorine being dissolved in a Tris-HCl buffer (100 mM, pH 7.4). An aliquot of the antioxidant solution (400 μL) was mixed with the 100 mM Tris-HCl buffer (pH 7.4, 1.6 mL) and then added to 2 mL of 500 μM DPPH in ethanol (a final concentration of 250 μM). The mixture was shaken vigorously and then kept in the dark for 20 min at room temperature. The absorbance of the sample was measured by a spectrophotometer (Shimadzu 240, Kyoto, Japan) at 517 nm; all tests were performed in duplicate and the results averaged.

L-(+)-Ascorbic acid (AsA), BHT, and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used as standards. The absorbance of each sample was expressed relative to that of the control sample without the test sample taken as 100.

**Quantitative Analysis of Tocopherols (Toc).** NaCl (1%, 1 mL), 6% pyrogallol in ethanol (10 mL), ethanol (1 mL)

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incorporating 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC; 10 mg), and 60% KOH (1.5 mL) were added to the crushed pericarp (0.5 g) or seed (1 g). The mixture was kept at 70 °C for 30 min. After cooling in an ice−water mixture, the unsaponified compound was extracted three times with 1% NaCl (15 mL) and 1:9 ethyl acetate/hexane (15 mL). The organic solvent layer was combined and evaporated to dryness under vacuum (*Shin-Shokuhinbunsekiho,* 1996).

The residue was dissolved in hexane (1 mL) to remove interfering impurities and pigments and filtered through a Sep-Pak Plus silica cartridge (Waters Corp.). After a wash with hexane (3 mL), Tocs were eluted with ethyl acetate/hexane (3:7, 2 mL). The resulting fraction was evaporated and dissolved in hexane (1 mL). Tocs (α−γ) were separated into four peaks by HPLC, and the content of each was determined using a standard curve. The conditions for the HPLC analysis were as follows: instrument, JASCO 880-PU; column, Senshu Pak $NH_2$-1251-N (4.6 mm × 250 mm; Senshu Scientific Co., Tokyo, Japan); eluent, *n*-hexane/2-propanol (98:2); flow rate, 0.8 mL/min; UV detector, JASCO UV-970, 295 nm; internal standard, PMC.

**Isolation of the Antioxidants.** The crushed seed (1 kg) was extracted three times with 80% methanol, and the combined filtrate was concentrated in vacuo. The residual aqueous solution was partitioned three times between diethyl ether and ethyl acetate.

*(a) Isolation of the Antioxidant from the Ethyl Acetate Extract.* The ethyl acetate extract was concentrated and fractionated by ODS flash column chromatography (ODS-SS-1020T, 30 mm i.d. × 200 mm; Senshu Scientific Co., Tokyo, Japan). Elution was successively performed by 10, 20, 50, and 100% methanol/$H_2O$ (1 L each). Solvent of each eluate was evaporated, and its antioxidative activity was detected by the TLC method.

The 10% methanol fraction, which showed strong antioxidative activity, was fractionated by HPLC in a PEGASIL-ODS column (20 mm i.d. × 250 mm, Senshu Scientific Co.). The eluent (methanol/$H_2O$) was changed linearly from 0 to 40% methanol in 80 min. The flow rate was set at 7 mL/min, and the eluate was monitored with UV detection at 280 nm.

*(b) Isolation of the Antioxidant from the Aqueous Extract (Water Layer).* The water layer was evaporated and subjected to adsorption column chromatography in an AXT-204 column (5.5 cm i.d. × 85 cm; Organo Corp., Tokyo, Japan). After a wash with water (∼6L), the residual substances were eluted with methanol (3 L). The eluate was concentrated and fractionated by ODS flash column chromatography as described above. Elution was successively performed by 5% (1.5 L), 10% (1.8 L), 20% (2.3 L), 50% (1.1 L), and 100% (0.7 L) methanol/$H_2O$, and the eluates were concentrated and their antioxidative activity was detected by the TLC method.

The 20% methanol fraction was further fractionated by HPLC in a PEGASIL-ODS column (20 mm i.d. × 250 mm; Senshu Scientific Co.). The eluent (methanol/$H_2O$) was changed linearly from 10 to 60% methanol in 80 min. The flow rate was set at 8 mL/min, and UV detection was at 280 nm. Rechromatography used an eluent (methanol/$H_2O$) that was linearly changed from 25 to 30% in 25 min, and UV detection was at 365 nm.

**Instrumental Analyses.** *(a) Mass Spectrometry (MS).* HRFAB mass spectra were measured with a JEOL MStation JMS-700. Glycerin was used as the matrix.

*(b) Nuclear Magnetic Resonance (NMR) Spectrometry.* $^1H$ and $^{13}C$ NMR spectra were recorded by a JEOL JNM-GX 270 or 400 FT NMR spectrometer in a $D_2O$ solution with 3-(trimethylsilyl)propionic acid, sodium salt (TSP), as the internal standard or in a $CD_3OD$ solution with tetramethylsilane (TMS).

*Arbutin:* $^1H$ NMR ($CD_3OD$, TMS) δ 3.37−3.42 (m, H-2′−5′), 3.67−3.71 (br d, H-6′b), 3.88 (d, H-6′a, $J$ = 12.2), 4.73 (d, H-1′, $J$ = 7.6), 6.69 (d, H-3,5, $J$ = 8.9), 6.96 (d, H-2,6, $J$ = 9.2); $^{13}C$ NMR ($CD_3OD$, TMS) δ 62.5 (C-6′), 71.4 (C-4′), 75.0 (C-2′), 78.0 (C-3′,5′), 103.6 (C-1′), 116.6 (C-3,5), 119.4 (C-2,6), 152.4 (C-4), 153.8 (C-1).

*(c) Gas Chromatography−Mass Spectrometry (GC-MS) Analysis.* A Hewlett-Packard 5890 series II gas chromatograph coupled to a Hewlett-Packard 5972 series mass spectrometer was used with a DB-5 column (60 m × 0.25 mm i.d., J&W Scientific). The carrier gas was He at a flow rate of 1 mL/min, and the oven temperature was programmed from 130 °C (2-min hold) to 280 °C at 2 °C/min. The injection temperature was 280 °C, and the split ratio was 30:1. An HP ChemStation System was used to treat the data.

**Quantitative Analysis of Arbutin.** The crushed pericarp or seed (10 g) was extracted with methanol (50 mL). Phenyl-β-D-glucoside dissolved in methanol (3.06 mg for the pericarp and 0.15 mg for the seed) was added as an internal standard before filtration. The residue was extracted twice with 80% methanol, the filtrates being combined and concentrated. Water was added to the concentrate, and the aqueous solution (∼30 mL) was partitioned three times between hexane and diethyl ether. The water layer was concentrated and subjected to adsorption chromatography in an XAD-2 column (3.5 cm i.d. × 45 cm; Organo Corp.). After a wash with water (2 L), the remaining compounds were eluted with methanol (1 L) and concentrated to obtain the crude glycosidic fraction.

Dry pyridine (20 μL) and *N*-methylbis(trifluoroacetamide) (MBTFA, 20 μL) were added to 10 mg of the crude glycosidic fraction, and the mixture was kept at 60 °C for 30 min to obtain the TFA derivative of the glycosidic fraction. The content of the TFA derivative was investigated by comparing the total ion current of the peak with that of phenyl-β-D-glucoside by GC-MS.

## RESULTS AND DISCUSSION

**Antioxidative Activity of the Pericarp and Seed.** The antioxidative activity of the four solvent extracts (hexane, diethyl ether, ethyl acetate, and methanol) that had been prepared from the dried pericarp and seed was studied. The absorbance value of each extract by both the ferric thiocyanate and TBA methods when that of the control was sufficiently high (it was 1.2) is shown in Figure 1A, and the TBA value on the next day is shown in Figure 1B. Each value is relative to a control value of 100. The ethyl acetate extract from the pericarp and the methanol extract from the seed showed strong antioxidative activity, similar to that of BHT against linoleic acid by both methods; however, the methanol extract from the pericarp was pretty weak, and the ethyl acetate extract from the seed showed no activity.

**Content of Tocopherols.** Tocopherols in the pericarp and seed were quantitatively analyzed by HPLC, and the results are summarized in Table 1. The content of total was 3.9 mg/100 g of dry weight (dw) in the pericarp and 2.9 mg/100 g of dw in the seed. The pericarp contained all of the tocopherol isomers (α, β, γ, and δ), with α-Toc constituting 82% of the total. The seed contained only α-Toc and γ-Toc, with γ-Toc constituting 96%. Although the concentration of Tocs in Japanese pepper was not high, the distributions of Toc between the pericarp and seed were quite different. It is known that γ-Toc is effective for stabilizing plant oils under general storage conditions and that α-Toc deactivates $^1O_2$ to $^3O_2$ more effectively than γ-Toc, so different antioxidative effects could be expected from the pericarp and seed of Japanese pepper.

**Isolation and Structural Determination of Further Compounds with Antioxidative Activity.** An aqueous solution of the 80% methanol extract from the seed was partitioned between diethyl ether and ethyl acetate. The ethyl acetate and aqueous fractions that showed antioxidative activity were successively subjected to flash column chromatography with ODS resin and preparative HPLC. Compound **1** was isolated from

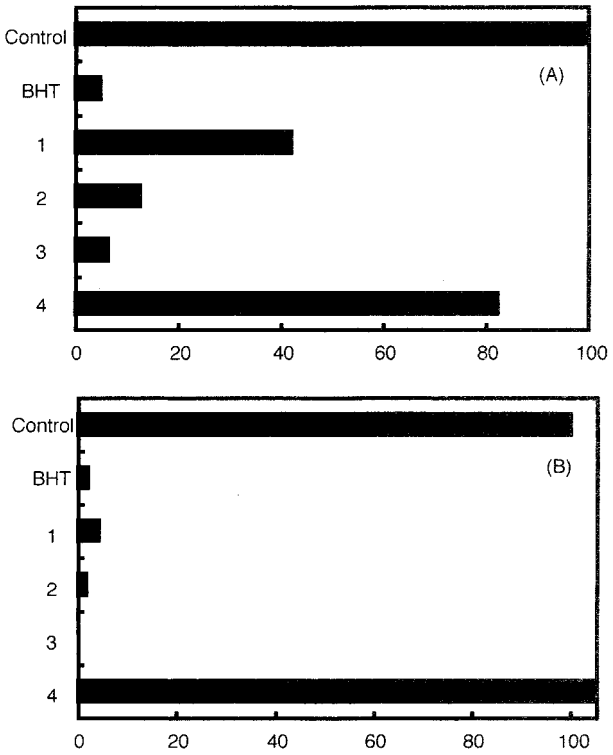


**Figure 1.** Antioxidative activity of the extracts prepared from the Japanese pepper pericarp and seed as measured by the ferric thiocyanate method (A) and the TBA method (B): 1, methanol (pericarp); 2, ethyl acetate (pericarp); 3, methanol (seed); 4, ethyl acetate (seed).

**Table 1. Tocopherol Composition of Japanese Pepper**

| | pericarp (mg/100 g of dw) | seed (mg/100 g of dw) |
|---|---|---|
| α-Toc | 3.2 | 0.12 |
| β-Toc | 0.12 | |
| γ-Toc | 0.35 | 2.8 |
| δ-Toc | 0.27 | |
| total Toc | 3.9 | 2.9 |

**Table 2. $^1$H and $^{13}$C NMR Chemical Shifts of Compound 1 [δ, ppm (J, Hz)][a]**

| | $^1$H NMR | | $^{13}$C NMR |
|---|---|---|---|
| $H_{2,6}$ | 6.96 d ($J$ = 8.9) | $C_1$ | 153.8 |
| $H_{3,5}$ | 6.68 d ($J$ = 9.2) | $C_{2,6}$ | 119.4 |
| $H_{1'}$ | 4.72 d ($J$ = 7.6) | $C_{3,5}$ | 116.6 |
| $H_{2'-5'}$ | 3.37−3.41 m | $C_4$ | 152.4 |
| $H_{6'a}$ | 3.88 d ($J$ = 11.9) | $C_{1'}$ | 103.6 |
| $H_{6'b}$ | 3.67−3.70 br d | $C_{2'}$ | 75.0 |
| | | $C_{3'}$ | 78.0 |
| | | $C_{4'}$ | 71.4 |
| | | $C_{5'}$ | 78.0 |
| | | $C_{6'}$ | 62.5 |

[a] Solvent, $CD_3OD$/TMS. d, doublet; m, multiplet; br, broad.

the ethyl acetate fraction as a colorless crystal. HRFAB-MS spectroscopy indicated its molecular formula as $C_{12}H_{16}O_7$ through the appearance of a negative ion peak at $m/z$ 271.0780 (error mmu = −3.8). The $^1$H NMR signals (270 MHz, $CD_3OD$, TMS) at 3.37−3.88 ppm and the $^{13}$C NMR signals (100 MHz, $CD_3OD$, TMS) at 103.6, 75.0, 78.0, 71.4, 78.0, and 62.5 ppm indicate the existence of glucose as shown in Table 2 and Figure 2. An anomeric proton appeared as a doublet at 4.72 ppm ($J$ = 7.6 Hz), indicative of a sugar β-configuration. The $^1$H NMR signals of two doublets at 6.96 and 6.68 ppm and the $^{13}$C NMR signals at 153.8, 119.4, 116.6, and 152.4 ppm showed the presence of a benzene ring substituted

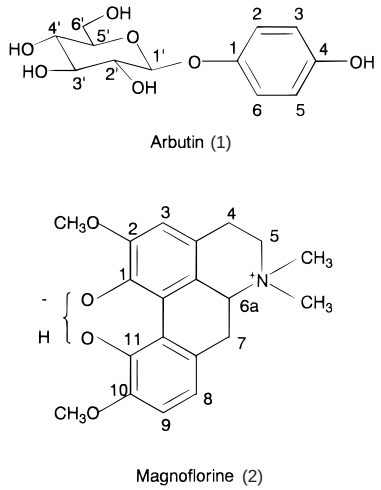


**Figure 2.** Structures of arbutin, compound **1**, and magnoflorine, compound **2**.

at the 1- and 4-positions. Therefore, compound **1** was provisionally assigned as 4-hydroxyphenyl-β-D-glucopyranoside, known as arbutin. The $^1$H and $^{13}$C NMR data of commercial arbutin matched those of compound **1**, confirming its identity as arbutin. Arbutin has been mainly found in leaves of Ericaceae, for example, blueberry, cranberry, and bearberry. The whitening effect of arbutin is also known, and it is used for making a lotion or milky lotion (Kuroda et al., 1985; Fujinuma et al., 1986; Kinomura and Sakakibara, 1987; Senoo and Uehara, 1988; Yokoyama, 1991). In this study, arbutin was identified in Japanese pepper for the first time.

The aqueous fraction was subjected to XAD column chromatography and then to ODS flash column chromatography. The 20% methanol fraction from ODS flash column chromatography was further fractionated by HPLC in a PEGASIL-ODS column, and one main peak was collected. Repetitive rechromatography by HPLC at 365 nm yielded a pale yellow amorphous compound (compound **2**). HRFAB-MS spectroscopy indicated the molecular formula of the compound as $C_{20}H_{23}O_4N$ through the appearance of a positive ion peak at $m/z$ 342.1713 (error mmu = +0.7). It had absorption maxima at 228.9, 276.1, and 318.9 nm in the UV spectrum. The NMR spectra are summarized in Table 3. The $^1$H NMR signals at 3.77 and 3.86 ppm and the $^{13}$C NMR signals at 58.3 and 58.5 ppm showed the existence of two methoxyl groups (the solvent was $D_2O$/TSP). The $^1$H NMR signals at 6.52−6.77 ppm and the $^{13}$C NMR signals at a magnetic field lower than 110 ppm showed the existence of a benzene ring. Because these $^1$H and $^{13}$C NMR data and the UV absorption maxima agree very closely with published data (Ishii et al., 1994), compound **2** was provisionally identified as 5,6,6a,7-tetrahydro-1,11-dihydroxy-2,10-dimethoxy-6,6-dimethyl-4*H*-dibenzo[de,g]quinolinium, known as magnoflorine (Figure 2). Compound **2** was finally characterized as the iodide derivative, which was a colorless crystal, mp (dec) 256 °C. This mp value corresponds with that for magnoflorine iodide given by Chiba University. Compound **2** was thus unequivocally identified as magnoflorine. Magnoflorine belongs to the aporphine alkaloids and has been found in many plants of the Xanthoxylum, Papaveraceae, and Magnoliaceae (Ishii et al., 1994; Harborne and Baxter, 1993). Magnoflorine has also been found in the root and stem of Japanese pepper (Tomita and Ishii, 1957; Abe et al., 1973), but it was identified in the seed for the first time in this study.

**Table 3. $^1$H and $^{13}$C NMR Chemical Shifts of Compound 2 [$\delta$, ppm ($J$, Hz)]**[a]

| | $^1$H NMR | $^{13}$C NMR |
|---|---|---|
| 1 | | 151.0 |
| 2 | | 153.9 |
| 3 | 6.59 (s) | 112.0 |
| 4 | 2.55 (dif d, $J$ = 17.7) | 25.9 |
| | 3.04 (dif d, $J$ = 15.0) | |
| 5 | 2.74 (dif d, $J$ = 12.8) | 63.7 |
| | 3.40 (dd, $J$ = 12.0, 5.0) | |
| 6a | 3.56 (dif d, $J$ = 13.7) | 71.9 |
| 7 | 2.28 (t, $J$ = 13.0) | 32.6 |
| | 2.87 (dif d, $J$ = 12.5) | |
| 8 | 6.52 (d, $J$ = 7.9) | 119.3 |
| 9 | 6.77 (d, $J$ = 7.9) | 113.3 |
| 10 | | 152.4 |
| 11 | | 149.9 |
| OMe | 3.77 (s) | 58.3 |
| OMe | 3.86 (s) | 58.5 |
| $N^+$Me | 2.70 (s) | 45.4 |
| $N^+$Me | 3.14 (s) | 56.1 |
| 3a | | 118.7 |
| 6b | | 122.7 |
| 7a | | 127.9 |
| 11a | | 124.2 |
| 11b | | 124.5 |

[a] Solvent, $D_2O$; internal standard, TSP. s, singlet; d, doublet; t, triplet; dd, double doublet; dif, diffused splitting pattern.

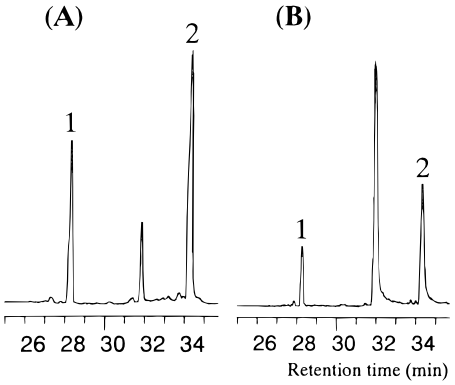


**Figure 3.** TIC chromatograms of the TFA derivative of the glycosidic fraction from the pericarp (A) and from the seed (B) of Japanese pepper: 1, peak of the TFA derivative of the internal standard, phenyl-$\beta$-D-glucoside; 2, peak of the TFA derivative of arbutin.

**Quantitative Analysis of Arbutin.** The contents of arbutin in Japanese pepper were compared between the pericarp and seed. As arbutin is a glycosidic compound, a crude glycosidic fraction was prepared from the pericarp or seed. TFA derivatives of the fraction were synthesized as described above and quantitatively analyzed by using GC-MS. An internal standard, phenyl-$\beta$-D-glucoside, having a structure similar to that of arbutin, was used. The total ion current (TIC) chromatograms of the TFA derivative of the crude glycosidic fraction from both the pericarp and seed are shown in Figure 3. A comparison of the retention time and MS spectrum of the TFA derivative of an authentic arbutin sample enabled its peak to be detected on the TIC chromatogram. The concentration of arbutin in the pericarp was 35 mg/100 g of dw and ~10-fold less that in the seed, 3.0 mg/100 g of dw.

**Antioxidative Activity and DPPH Radical-Scavenging Activity of the Isolated Compounds.** The antioxidative activity of both arbutin and magnoflorine was investigated by the TBA and/or ferric thiocyanate methods, and the results are summarized in Figure 4.

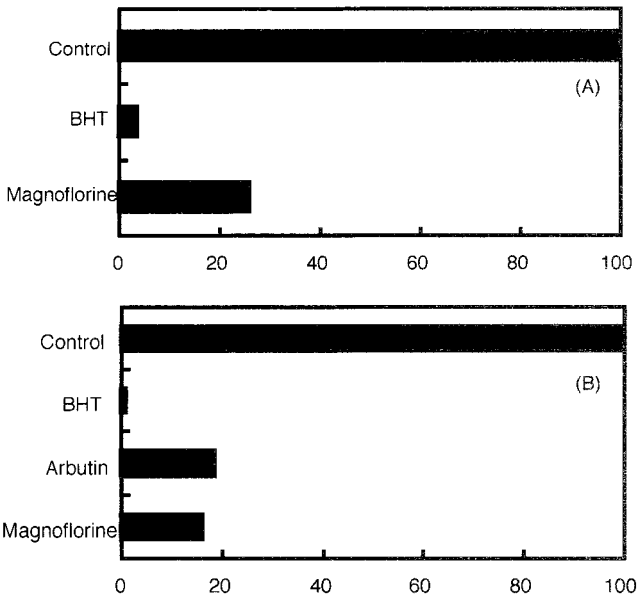


**Figure 4.** Antioxidative activity of arbutin and magnoflorine against linoleic acid by the ferric thiocyanate method (A) and the TBA method (B).

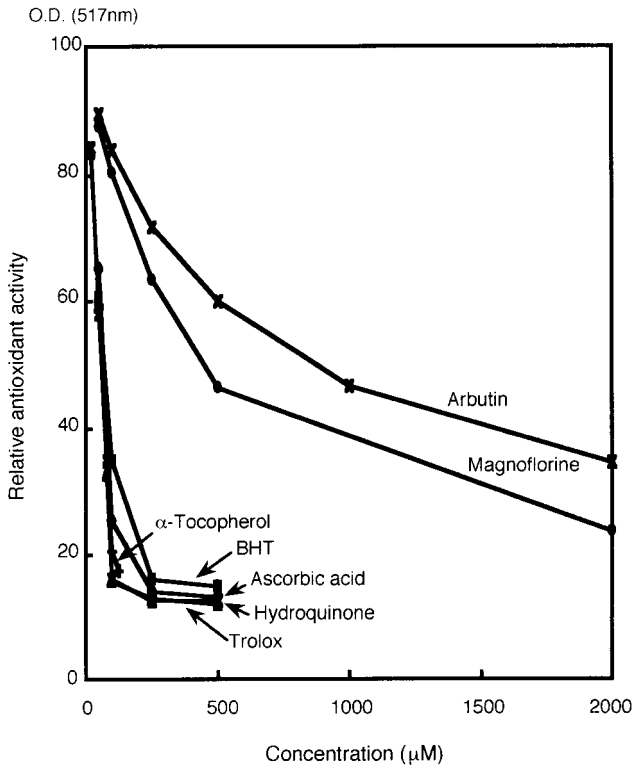


**Figure 5.** DPPH radical-scavenging activity (colorimetry). L-(+)-Ascorbic acid (AsA), BHT, and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used as standards, and the absorbance of each sample is with respect to that of the control set to 100.

Ioku et al. (1992) reported that arbutin showed antioxidative activity by the ferric thiocyanate method, although it was not strong. We also observed relatively weak activity for arbutin by the TBA method. According to Misik et al. (1995), magnoflorine inhibited lipoxygenase activity and lipid peroxidation and had lower antioxidative activity than corytuberine, a structure similar to magnoflorine apart from only one methyl group being attached to the nitrogen atom. Apparently the presence of two OH groups did not contribute to this

antioxidative effect, but that the presence of a free electron pair on the nitrogen atom seemed to be essential (Misik et al., 1995).

The DPPH radical-scavenging activity of arbutin, magnoflorine, α-Toc, and γ-Toc was determined by colorimetry. In addition, hydroquinone, which is the aglycon of arbutin, ascorbic acid, BHT, and Trolox, all known antioxidants, were evaluated. As shown in Figure 5, Trolox and hydroquinone had the highest radical-scavenging activity, followed by α-Toc, AsA, and BHT. The activity of γ-Toc could not be measured because it was insoluble in the solvent used. The radical-scavenging activity of arbutin and magnoflorine was very low, and no synergistic effect of arbutin and α-Toc or of arbutin and magnoflorine was apparent. However, Ioku et al. (1998) suggested that dietary flavonoid glucosides were primarily hydrolyzed and liberated aglycons in the jejunum of rat. A similar hydrolysis of arbutin could be expected to liberate hydroquinone within the human body. Besides, Williams and Francis (1996) reported that the release of aroma volatiles occurred during a period of cellar storage for slow acid-catalyzed hydrolysis from glycosides, which are major contributors to aroma. From these results, it must be said that arbutin is a possible antioxidant in food because it might be hydrolyzed during cooking or digestion.

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