Antioxidizing Component, Musizin, in *Rumex japonicus* Houtt.

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A substance with antioxidant properties was obtained from the hexane extract of roots of *Rumex japonicus* Houtt. The active component of the hexane extract was isolated and characterized as 2-acetyl-1,8-dihydroxy-3-methyl naphthalene, trivially named musizin (MUS). The antioxidant activities of MUS in six types of fats and oils were higher than that of butyl hydroxyanisole (BHA) and δ-tocopherol (δ-TOC). Together, TOC and MUS have a synergistic effect, because comparable amounts of either had lower antioxidant activity than various combinations of the two antioxidants. When we studied the antioxidant properties of a mixture of MUS and δ-TOC with methyl linoleate (MeLH), we found that the rates of destruction of the two antioxidants were comparable, but that their destruction occurred sequentially, with MUS first followed by δ-TOC, after which the oxidation of MeLH quickly occurred. Comparison of the antioxidant activity of MUS and its analogs suggests that only one of the two hydroxyl groups in MUS is involved in its antioxidant activity. Intermolecular hydrogen bonding may be involved.

KEY WORDS: Antioxidant, methyl linoleate, musizin, naphthalenediol derivatives, *Rumex japonicus* Houtt., synergistic effect, tocopherol.

Butyl hydroxyanisole (BHA), butyl hydroxytoluene (BHT), tocopherol (TOC) and L-ascorbic acid (AA) have been used widely as antioxidants for foods. Recently, it has been reported that synthetic antioxidants such as BHA and BHT are carcinogenic (1,2), so these antioxidants have been used less frequently although they have high antioxidant activity. At present, natural antioxidants such as TOC and AA are widely used because they are considered safer and have been linked with fewer adverse reactions.

The antioxidant activities of TOC and AA are, however, lower than those of synthetic antioxidants such as BHA and BHT. Hence, much research has been conducted to find safe antioxidants with high antioxidant activity from natural sources (3-8). As a result, essence of spice and preparations of natural gallic acid are sold commercially. Uses of these products are limited because they compromise the flavor and color of foods.

The authors have recently found that an herb, *Rumex japonicus* Houtt. of the family Polygonaceae, contains a component with high antioxidant activity. Several papers report on the plant components of the *Rumex* genus. Ogweno *et al.* (9) investigated plants of the *Rumex* genus in Kenya, and Bauch *et al.* (10) investigated the species *R. alpinas* L. Odani *et al.* (11) described antibacterial substances in *R. japonicus* Houtt.

This paper presents the results of the isolation and identification of antioxidants in *R. japonicus* Houtt., the antioxidant activity of musizin (MUS), the synergistic effect of TOC with MUS, and the antioxidant activities of the analogs of MUS.

MATERIALS AND METHODS

Roots of *R. japonicus* Houtt. collected in the Wakayama Prefecture in Japan were lyophilized for 24 hr in preparation for the experiment. The dried roots were pulverized in a household mixer. The pulverized root was then fractionated by the method shown in Figure 1 with the solvents water, n-butanol (nBA), chloroform (CF), ethyl acetate (EA) and n-hexane (HEX).

Standard reference material used for comparison of antioxidant activities were α-, γ- and δ-TOC (99% pure, Eisai Co., Ltd., Tokyo, Japan). Also used were BHA, L-ascorbic acid, 1-naphthol, 1,5-naphthalenediol, naphthoic acid and 1-hydroxy naphthoic acid (obtained from Tokyo Kasei Kogyo Co., Tokyo, Japan).

Refined corn oil, rapeseed oil, palm oil, soybean oil, beef tallow and lard (Nippon Oil and Fats Co., Ltd., Tokyo, Japan) were used as oxidation substrates. Methyl linoleate was used after purification through a column packed with silica gel (12). The peroxide value (PV) of purified methyl linoleate, determined according to AOCS methods (13), was 0.1 meq/kg or less. Panaseat 810 (Nippon oil and Fat Co.) was used as the medium chainlength triglyceride (MCT).

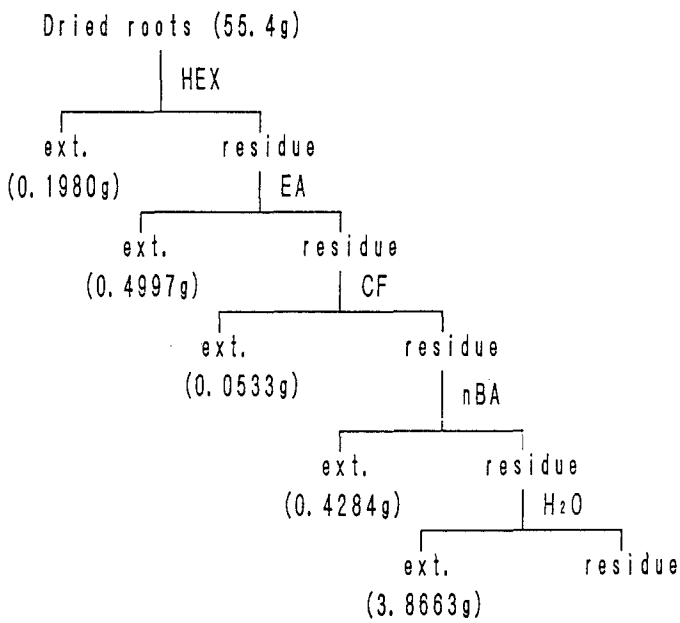

FIG. 1. Fractionation scheme for extracts of the root of *Rumex japonicus* Houtt. Water, n-butanol (nBA), chloroform (CF), ethyl acetate (EA) and n-hexane (HEX) were used as solvents.

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A Gilson Model 303 with 10SC pump head was used. The columns used were: 25 cm × 20 mm Deverosil 60-10; 25 cm × 20 mm Deverosil ODS10; and 25 cm × 4.6 mm Develosil ODS5 (Nomura Chemical Co., Ltd., Aichi, Japan). Columns (a) and (b) were used for the purification of the HEX extract. Column (c) was used for the simultaneous analyses of TOC and MUS. Chloroform, an 80:20 mixture of acetonitrile and water, and methanol were used as the mobile phases for columns (a), (b) and (c), respectively. A Model SPD-M6A detector (Shimadzu Works, Tokyo, Japan) was used at 273 nm for columns (a) and (b). For column (c), TOC and MUS were detected at 299 and 273 nm, respectively. The flow rate was 6 mL/min for columns (a) and (b), and 1 mL/min for column (c).

Other instruments used for the identification of the antioxidizing material were as follows: UV absorption spectrophotometer, Model UV 260 (Shimadzu Works); FT-IR 8300 (Japan Spectroscopic Co. Ltd., Tokyo, Japan); NMR, Model JNM-FX 200 [Japan Electron and Optics Laboratory (JEOL) Co. Ltd., Tokyo, Japan]; mass spectrometer, Model JX-303 (JEOL).

Measuring antioxidant activity. Specified quantities of the antioxidant solution (for example, 0.2 mL of a solution of MUS containing 1 mg per mL in n-hexane) were placed in small-mouthed bottles 3-cm in diameter. The solvent was carefully removed with a stream of nitrogen. The bottles were then weighed, and to each was added 1.0000 ± 0.005 g of the substrate (*e.g.*, MeLH) from a dropper. The contents of bottles were mixed sufficiently to ensure uniform distribution of the antioxidants in the oil. Each bottle was reweighed and placed in a constant-temperature oven (60°C). They were then covered with watch glasses. At daily intervals the bottles were removed from the oven, allowed to cool at room temperature for 30 min and weighed (to ± 0.0001 g). The induction periods up to the sharp increase in weight of the solutions were then compared with each other (14).

RESULTS AND DISCUSSION

The weight increase of MeLH after the addition of 200 ppm of various root extractions prepared by the method of Figure 1 was measured at 60°C and the induction periods were compared with each other. As defined by the induction periods, antioxidant activities of the HE, EA, CF, nBA and water fraction are shown in Table 1. The water and nBA extracts showed no activity, while the HEX extract showed high activity and the EA and CF extracts produced intermediate activities.

The HEX extract was fractionated by HPLC into two fractions as shown in Figure 2. The antioxidant activities of these fractions, (1) and (2), are also shown in Figure 2. The antioxidant activity of fraction (1) is low, while that of fraction (2) is high.

Fraction (2) collected by high-performance liquid chromatography (HPLC) was fractionated further into two fractions, 2-1 and 2-2, by HPLC as shown in Figure 2. The antioxidant activities of fractions 2-1 and 2-2 are also shown in Figure 2. Fraction 2-1 is the major antioxidizing component of *Rumex japonicus* Houtt. The chemical structure of fraction 2-1 was then determined by instrumental analysis as follows.

The result of mass spectrometry indicates that the molecular weight of fraction 2-1 is 216. Other fragmentation ions of 201, 155, 127, 91, 75, 60, 43 were also observed. The signals of δ2.45[3H, s (Φ-CH_3)], δ2.55[3 H, s (Φ-(C=O) -CH_3)], δ6.84[H, dd (Φ-H7)], δ6.89[1H, s (Φ-H4)] δ7.05[1H, dd (Φ-H5)], δ7.23[1H, t (Φ-H5)], δ10.22[1H, s (Φ-OH8)] and δ17.33[1H, s (Φ-OH1)] were observed by ^{1}HNMR. Infrared absorption bands (KBr) were observed at 3449.14 (-OH), 1586.64 (C=0), 1168.04 (C-O) and 857.46 cm^{-1} (aromatic C-H). Ultraviolet absorption bands were observed at 224.4, 261.6, 320.6, 336.0 and 394.0 nm (MeOH).

TABLE 1

Antioxidant Activity of Various Root Extracts of *Rumex japonicus* Houtt.[a]

Fraction	Induction period (days)
HEX	3.0
EA	2.0
CF	2.0
nBA	0.8
H_2O	0.7
Control	0.7

[a]Concentrations of the fractions were 200 ppm in methyl linoleate (MeLH). Induction period was determined by measurement of weight increase of MeLH at 60°C.

Comparison of these results with the data of Ogweno *et al.* (9) indicates that fraction 2-1 is 2-acetyl-1,8-dihydroxy-3-methyl naphthalene, trivially named musizin (MUS).

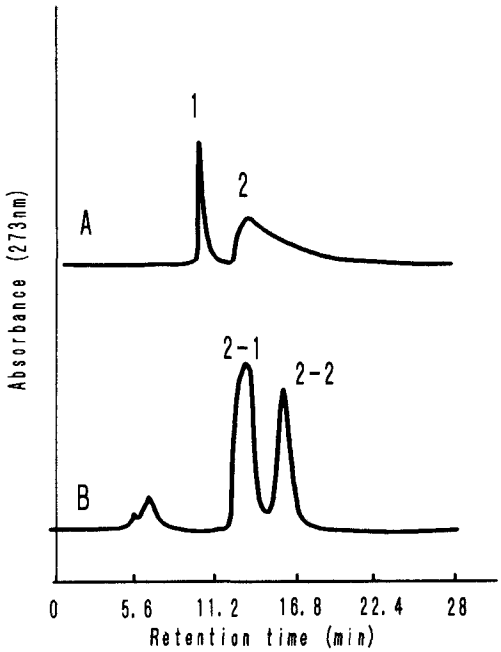
Antioxidant activity of MUS in edible fats and oils. The antioxidant activities of MUS on various edible fats and oils are shown in Table 2. Typical synthetic and natural antioxidants, BHA and δ-TOC, respectively, did little to reduce the oxidation of corn, rapeseed, palm and soybean oils. Perhaps this is because these vegetable oils already contained tocopherol and tocotrienol (15,16), antioxidizing substances derived from the raw materials, and thus the addition of TOC and BHA did not improve their oxidative stabilities any further.

The oxidative stabilities of those four vegetable oils were significantly improved by adding MUS. It was found, therefore, that MUS is a more efficient antioxidant of vegetable fats and oils than conventional antioxidants.

The addition of TOC, BHA and MUS to beef tallow lengthened the induction periods by approximately 4, 2 and 4.5 times, respectively. In the case of lard, TOC, BHA and MUS lengthen the induction period by approximately 3, 3.5 and 5.5 times, respectively. These results indicate that BHA and TOC are less effective for vegetable oils, while these compounds show enough antioxidant activity for beef tallow and lard substances hardly expected to contain TOCs (17). It can be presumed that BHA and TOC are less effective for oils already containing TOCs.

Meanwhile, the addition of 200 ppm of MUS to corn, rapeseed, palm and soybean oils lengthens the induction periods by 1.29–1.67 times. In the case of beef tallow and lard, MUS lengthens the induction periods by 4.5 and 7.5 times, respectively. These results indicate that MUS shows antioxidant activity regardless of the TOC content. The antioxidant activity of MUS and synergistic effects of MUS with TOC were examined next.

Concentration vs. *antioxidant activity of MUS.* The



Hexane Fraction No.	Induction Period, Days
1	1.4
2	3.0
2-1	3.0
2-2	1.0
Control	0.7

FIG. 2. HPLC chromatograms and antioxidant activity of fractions of the hexane extract of *Rumex japonicus* roots. Concentrations of the extracts were 200 pm. Induction periods were determined at 60°C with methyl linoleate (MeLH) as the substrate. A, initial hexane extract; B, fraction 2 of initial hexane extract.

TABLE 2

Comparison of Antioxidant Effect of MUS with that of BHA on the Oxidation of Various Oils[a]

Oil	Induction period (days)[b]			
	Control	δ-TOC	BHA	MUS
Corn	9.0	9.6	9.9	15.9
Rapeseed	9.3	11.4	10.2	15.9
Palm	35.4	39.6	34.5	48.6
Soybean	6.3	6.3	6.3	8.1
Beef tallow	12.0	52.5	24.0	54.0
Lard	5.4	15.6	19.2	30.6

[a]Concentrations of the antioxidants were 200 ppm. Induction period was determined at 60°C.

[b]δTOC, δ-tocopherol; BHA, butyl hydroxyanosole; and MUS, musizin, 2-acethyl-1, 8-dihydroxy-3-methyl naphthalene.

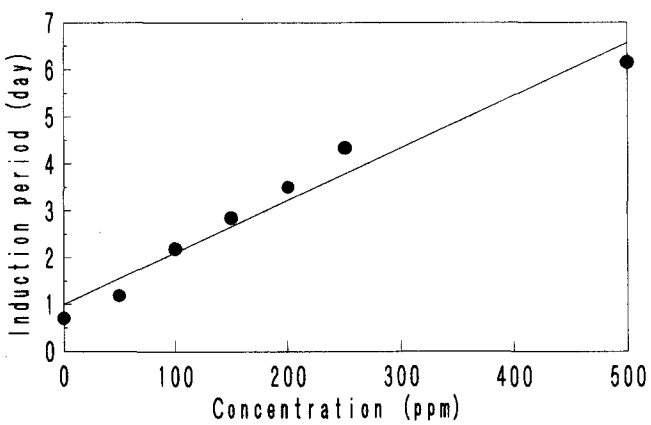


FIG. 3. Induction period as a function of concentration of musizin (MUS) in the oxidation of methyl linoleate (MeLH) at 60°C.

antioxidant activity of MUS was investigated in further detail with methyl linoleate (MeLH). The weight increase of MeLH after the addition of MUS, ranging in concentration from 50 to 500 ppm, was periodically measured. The concentration dependence of the antioxidant activity of MUS is shown in Figure 3. The Figure reveals that when MeLH is the substrate, the induction period is proportional to the concentration of MUS up to 500 ppm.

Synergistic effects. We thought that the relatively high antioxidant activity of MUS exhibited in fats and oils naturally containing TOCs might be attributed to the presumed synergistic effect of MUS with TOC (15,16). Hence, the presumed synergistic effect was further investigated. A total quantity of 200 ppm of a mixture of MUS and either α-, γ- or δ-TOC was added to MeLH at TOC/MUS ratios of 200:0 ppm, 150:50, 100:100, 50:150 and 0:200 ppm, respectively. The induction period was gravimetrically measured to determine the oxidative stability of the substrate (MeLH). The results are shown in Figure 4. The synergistic effect of MUS is greatest with γ-TOC, the induction period being approximately double (1.7) that of MUS alone when the γ-TOC/MUS ratio is 100:100 ppm. This synergistic effect is reflected by the fact that, when the total antioxidant concentration is 200 ppm and the MUS concentration is greater than 50 ppm, the antioxidant activity of all combinations of TOC and MUS exceeds that of either TOC or MUS alone.

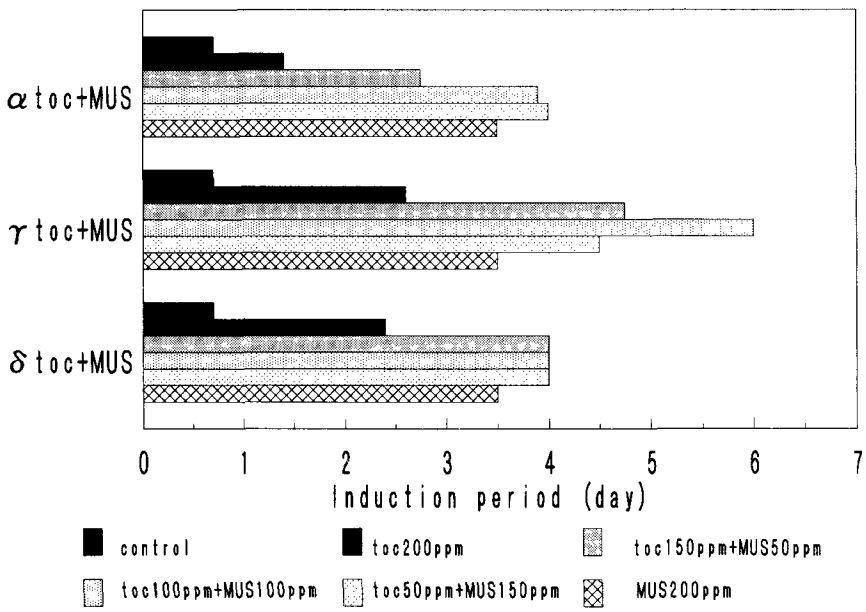


FIG. 4. Synergistic effects observed with several concentration ratios of tocopherol (TOC) and musizin (MUS). Induction periods were determined at 60°C with methyl linoleate (MeLH) as substrate.

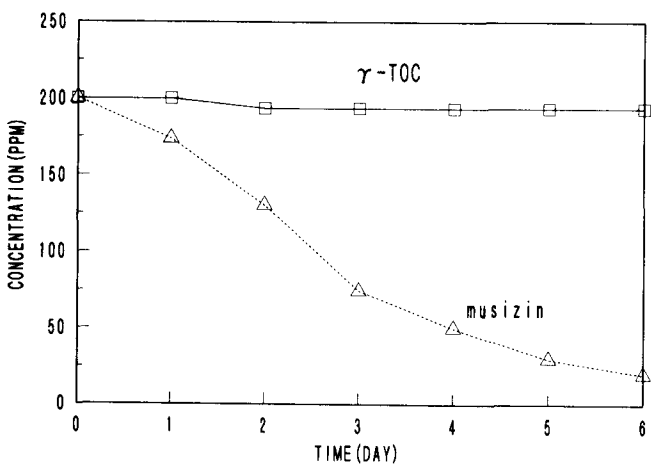


FIG. 5. Relative stabilities of γ-tocopherol (γ-TOC) and musizin (MUS) in medium chainlength triglyceride (MCT) at 60°C.

These results suggest that MUS and naturally occurring TOC interact synergistically to confer greater oxidative stability to a number of fats and oils, such as corn and soybean oils.

The antioxidant activity of L-ascorbic acid (AA) with MUS was also investigated. The antioxidant activity of AA (200 ppm), as measured by the induction period, was the same as the control (induction period = 0.7 days) without the antioxidant. When AA was replaced with increasing amounts of MUS (from 50 to 200 ppm), the induction period increased as expected for those MUS concentrations (see Fig. 4), and therefore no synergism was observed for the combination of AA and MUS.

Stability of MUS. Both γ-TOC and MUS (200:200 ppm) were added to a medium chainlength triglyceride (MCT), a highly stable oil. The change in concentration of these antioxidants with time was measured at 60°C. The results are shown in Figure 5, which reveals that MUS decreases to <5% with time, while γ-TOC changed little over a six-day period. These results indicate that MUS is relatively unstable.

Since MUS is consumed faster, or is less stable, than TOC the rate of disappearance of the two antioxidants under oxidizing conditions and in the presence of MeLH was investigated. The results plotted in Figure 6 show that the concentration of MUS decreases rapidly during the initial induction period, while only about 5% of γ-TOC is lost. However, when the MUS concentration reaches 0 ppm, then γ-TOC decreases rapidly at the same rate observed for MUS. Only after MUS is completely gone, and the γ-TOC concentration has decreased to <2.0% of its initial concentration does MeLH begin to oxidize. MeLH oxidation occurs rapidly when both MUS and γ-TOC have been totally depleted.

Comparison of antioxidant activities of analogs of MUS. The correlation of the chemical structure with antioxidant activity was determined for MUS and four analogs of MUS. The results are shown in Table 3. Naphthoic acid with a carboxyl group and no hydroxyl had no antioxidant activity. The antioxidant activity of naphthol with one hydroxyl group is equal to that of MUS, while naphthalene 1,5-diol has two oxidizible hydroxyl groups perfectly positioned to double the antioxidant activity of naphthol and MUS. The antioxidant activity of MUS can only involve one of the two hydroxyl groups and therefore is similar to naphthol in antioxidant activity. Therefore, intramolecular hydrogen bonding by one hydroxyl in MUS

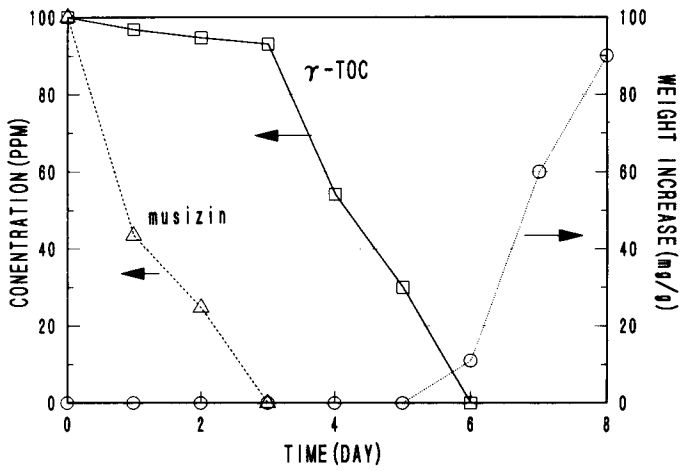


FIG. 6. Change in concentration of γ-tocopherol (γ-TOC) and musizin (MUS), and the weight increase due to oxidation of methyl linoeate (MeLH) at 60°C.

TABLE 3

Comparison of Antioxidant Effect of Musizin Analogs on the Oxidation of Methyl Linoleate (MeLH)[a]

Sample	Induction period (days)
1,5-Naphthalenediol	7.9
1-Naphthol	4.0
Musizin	3.5
Naphthoic acid	0.7
1-Hydroxynaphthoic acid	0.7
Control	0.7

[a]Concentrations of the antioxidants were 200 ppm. Induction period was determined at 60°C.

is expected to have little effect on the antioxidant activity of MUS. However, hydrogen bonding of the single hydroxyl group in 1-hydroxy naphthoic acid may be responsible for its lack of antioxidant activity.

An active antioxidizing substance in *Rumex japonicus* Houtt. has been identified as MUS, which has higher antioxidant activity than BHA and δ-TOC in six types of fats and oils, especially those that may contain residual natural TOC levels. TOC and MUS have a synergistic relationship, and MUS is preferentially destroyed before TOC when oxidized in the presence of MeLH. TOC then disappears at a similar rate oxidation of the substrate (MeLH) occurs. It is estimated from comparison of the antioxidant activity of MUS with that of several of its analogs that only one hydroxyl group of MUS is involved in its antioxidant activity. Intramolecular bonding may occur.

This is the first report of high antioxidant activity in extracts of *Rumex japonicus* Houtt., and of the isolation and characterization of musizin as the active component. MUS, like gossipol, is an uncommon type of natural antioxidant, both having a naphthalene skeleton. Since the antioxidant activity of MUS is higher than that of either BHA or δ-TOC, MUS is of interest for use in foods and cosmetics as well as in fats and oils. The heat stability of MUS, and its toxicity and safety for use in foods and personal care products will continue to be of interest.

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