Antioxidative Components, Xanthone Derivatives, in *Swertia japonica* Makino

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Substances with antioxidative properties were obtained from an ether extract of *Swertia japonica* Makino. Six active components of the extract were isolated and identified as methylbellidifolin, methylswertianin, swertianin, bellidifolin, norswertianin and desmethylbellidifolin. These six xanthone derivatives were shown to possess different antioxidant activities by chemiluminescent assay. The antioxidative activities of bellidifolin, norswertianin and desmethylbellidifolin were higher than those of butylated hydroxytoluene (BHT) and α-tocopherol. On autoxidation of methyl linoleate, bellidifolin had activity similar to that of BHT. On the basis of the data present on antioxidative properties and data reported on the mutagenicities of the xanthones, both activities were shown to give a good correlation.

KEY WORDS: Antioxidant, chemiluminescence, purification, *Swertia japonica* Makino, xanthone.

Antioxidants play important preventive roles not only on undesirable changes in flavor and nutritional quality of foods, but also on tissue damage in various human diseases (1). Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are widely used as food additives to prevent fat rancidity. However, the use of these synthetic antioxidants in foods has recently been reassessed by consumers because of possible toxic or carcinogenic effects (2). On the other hand, tocopherol is widely used as a safe natural antioxidant, but it is less effective than BHA and BHT. Recently, many reports have been published describing antioxidants from natural sources, such as spices (3), leaf wax (4), *Rumex japonicus* (5), peanut hulls (6) and Japanese and Chines folk medicines (7).

In a previous paper, the authors devised a chemiluminescent method for the simple evaluation of antioxidant activity for the purpose of screening large numbers of natural compounds (8). By using this chemiluminescence (CL) assay, the authors have recently reported that a natural Japanese folk medicine for a peptic, named Senburi (*Swertia japonica* Makino), contained some components with antioxidative activity (8).

The present report is concerned with the isolation, identification and characterization of antioxidative substances in *S. japonica*. Antioxidative activities of the isolated components, which are six xanthone derivatives, are compared with α-tocopherol and BHT by the CL method and by a method involving autoxidation of methyl linoleate (ML).

EXPERIMENTAL PROCEDURES

Material. Swertia japonica or Senburi was purchased commercially. Cytochrome c (from horse heart, type VI) was obtained from Sigma Chemical Co., Ltd. (St. Louis, MO). ML, supplied by Nippon Oil & Fats Co., Ltd. (Tokyo, Japan) was further purified by column chromatography with Florisil (Florisin Co., Berkely Springs, WV; 60:100 mesh) before use (9). ML hydroperoxide (MLHPO) was prepared from ML and purified as described elsewhere (8). All other chemicals of analytical grade were purchased from Wako Pure Chemical Ind. (Osaka, Japan).

Measurement of antioxidant activity. The CL assay for the evaluation of antioxidant activity was carried out in a manner similar to that described in the previous report (8). An appropriate amount of antioxidant and MLHPO was diluted in chloroform/methanol (1:9, vol/vol). The concentration of MLHPO was adjusted to 50 μM. After the mixture was shaken, 10 μL of sample aliquot was withdrawn and injected into a flow of chloroform/methanol (1:9, vol/vol) at 0.75 mL/min with a pump [980-PU; Japan Spectroscopic Co., Ltd. (JASCO), Hachioji, Japan]. The flow of chloroform/methanol was mixed with a flow of 50 mM borate buffer (pH 9.3), containing 10 μg/mL cytochrome c and 1 μg/mL luminol, at 0.60 mL/min. The CL, generated by the reaction of MLHPO, cytochrome c and luminol, was monitored with a CL-detector equipped with a flow-cell (825-CL; JASCO) (8).

The antioxidant activity of xanthones on autoxidation of ML, initiated with 2,2′-azo-*bis*(2,4-dimethylvaleronitrile) (AMVN), was examined by the method of Terao (10). An appropriate amount of antioxidant in tetrahydrofuran (5 mM) was added to a mixture of *n*-hexane/isopropanol (1:1, 1.0 mL) containing ML (100 μmol). Oxidation was initiated by adding an *n*-hexane solution of AMVN (10 μmol in 0.1 mL), and the mixture was incubated with continuous shaking at 37°C.

At appropriate intervals, 10-μL sample aliquots were injected into a high-performance liquid chromatography (HPLC) column. The HPLC consisted of a pump (980-PU; JASCO) and an ultraviolet (UV) detector (970-UV; JASCO), monitored at 234 nm, and a TSK gel Silica-60 column (i.d. 4.6 mm, length 250 mm; Tosoh Co., Ltd., Tokyo, Japan). The mobile phase was 1.0% isopropanol in *n*-hexane at 2.0 mL/min.

Extraction and separation of xanthones in S. japonica. Commercially dried *S. japonica* or Senburi was cut and pulverized in a mixer. The pulverized Senburi powder was extracted with methanol. An aqueous suspension of the methanolic extract was fractionated with diethyl ether (Et_2O), ethyl acetate (EA) and *n*-butanol (nBA) saturated with H_2O, as shown in Scheme 1 (where ext. = extract).

Column chromatography and thin-layer chromatography (TLC). The Et_2O fraction was evaporated to dryness and redissolved in EA. Aliquots of 2.5 g of the Et_2O fraction were injected into a Sephadex LH-20 column (i.d. 5 cm, length 50 cm) with EA and separated into four active fractions F-I, F-II, F-III and F-IV. The antioxidant activity was monitored by the CL method. All of the Et_2O fraction was fractionated by this procedure, and testing was repeated five times. Fractions F-I and F-II were passed through a Sep-pak cartridge packed with Silica gel (51900 Waters; Milford, MA) with a solvent system of benzene/Et_2O (20:1) and benzene/EA (9:1), respectively, before TLC.

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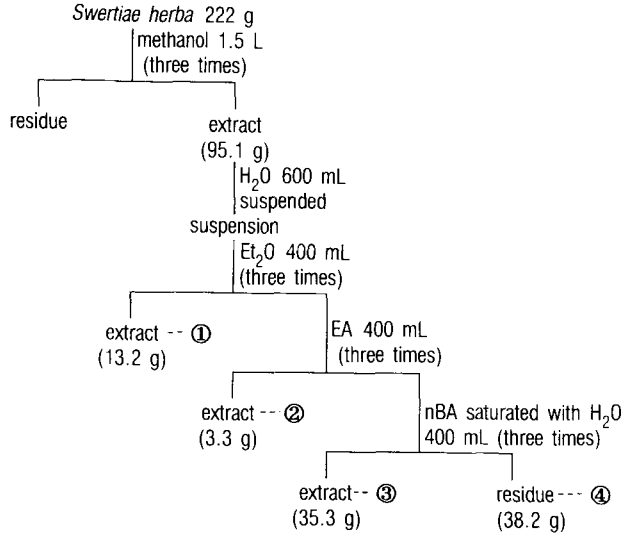


SCHEME 1

TLC plates precoated with Silica gel (5715 Merck; Merck, Darmstadt, Germany) were used to separate the constituents of fractions F-I, F-II and F-IV. The plate was developed with a solvent system of benzene/Et_2O/acetate (40:1:0.2), benzene/EA (85:15) or benzene/EA (1:2). The antioxidant activity of each band on the TLC plates was identified by the CL method. Bands of interest were scraped from the TLC plates, soaked overnight in 20 mL benzene/methanol (1:1), filtered and evaporated to dryness.

Instrumental analysis for identification. Melting points were determined on a micro hot stage and were uncorrected. UV spectra were measured with a spectrophotometer, Model U-3210 (Hitachi Ltd., Tokyo, Japan). Infrared (IR) spectra were measured on KBr discs with an IR spectrophotometer, Model 260-50 (Hitachi). Electron impact mass spectra were measured with Autospec (VG Analytical Ltd., Manchester, United Kingdom).

Statistical analysis of experimental data. All experiments, except the experiments of autoxidation of ML, were run in triplicate, and the presented results are the average of three measurements. The ML autoxidation tests were repeated three times, and typical results are shown from one of the three independent experiments. Significance of treatments was estimated by the Student's *t*-test (11).

RESULTS AND DISCUSSION

Separation and identification of xanthones. The changes of CL intensity with the addition of 400 ppm of the various *S. japonica* extracts, prepared by the method of Scheme 1, were measured. Table 1 shows the antioxidant activities of these extracts, as defined by the rate of decrease of CL intensity. The antioxidant activity was mainly found in the Et_2O fraction (1 in Scheme 1). Therefore, the Et_2O extract was investigated further in the following study.

The Et_2O extract was separated on a Sephadex LH-20 column with EA into four active fractions, F-I, F-II, F-III and F-IV (Fig. 1).

TABLE 1

Antioxidant Activity of *Swertia japonica* Fractions with Various Solvents by the Chemiluminescence (CL) method[a]

Fraction (solvent)	Activity [decrease of CL (%)]
Diethylether	34.1
Ethyl acetate	26.2
n-Butanol	19.9
H_2O	0.0

[a]Concentrations of the fractions were 200 ppm in chloroform-methanol (1:9, vol/vol) containing methyl linoleate hydroperoxide (50 μM).

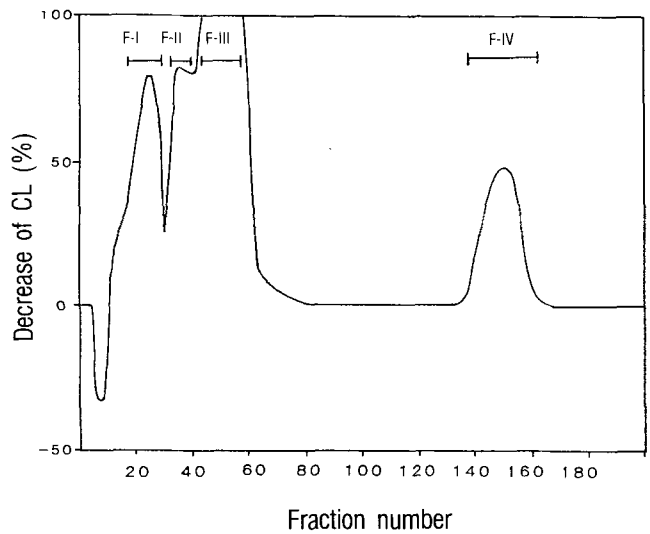


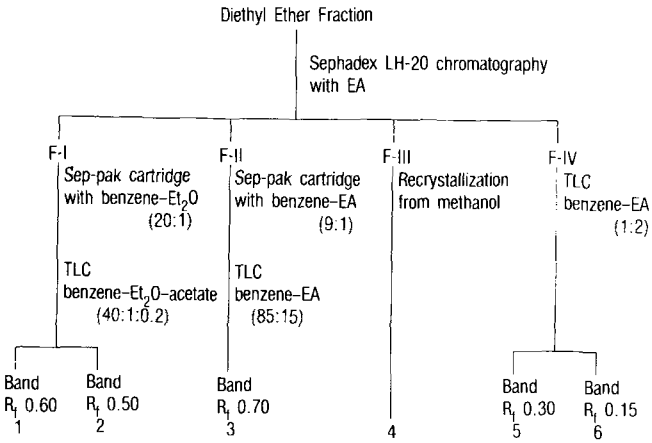
FIG. 1. Sephadex LH-20 column chromatogram of ether extract of *Swertia japonica*: eluant, ethyl acetate; fraction (each tube) = 10 mL; chemiluminescence (CL) assay, concentrations of the fractions were 0.2%.

By using a Sep-pak cartridge with benzene/Et_2O (20:1) and subsequent preparative TLC with benzene/Et_2O/acetate (40:1:0.2), F-I was separated into two yellow pigments No. 1 (R_f 0.60) and No. 2 (R_f 0.50) in yields of 0.02 and 0.01% of the original weight of dried *S. japonica* (40 and 20 mg in 222 g), respectively (Scheme 2). By using a Sep-pak cartridge with benzene/EA (9:1) and subsequent preparative TLC with benzene/EA (85:15), a yellow pigment No. 3 (R_f 0.70) was isolated from F-II in a yield of 0.02% (40 mg) (Scheme 2).

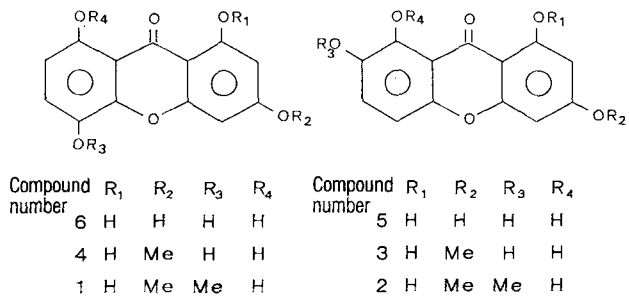
Recrystallization of F-III from methanol gave a yellow pigment No. 4 in a yield of 0.2% (450 mg). Fraction-IV was separated into two yellow pigments, No. 5 (R_f 0.30) and No. 6 (R_f 0.15), on preparative TLC with benzene/EA (1:2) in yields of 0.002 and 0.001% (4 and 2 mg), respectively (Scheme 2).

Table 2 presents the data of the UV, IR and electron impact mass spectra (EI-MS) and the melting points of pigments No. 1-6. Comparison of the results with the data of Komatsu *et al.* (12) and Kanamori *et al.* (13) suggests that No. 1-6 are methylbellidifolin, methylswertianin, swertianin, bellidifolin, norswertianin and desmethylbellidifolin, respectively (Fig. 2).

Antioxidant activities of the xanthones on the CL method. To compare the efficiency of these isolated six



SCHEME 2



Compound number	R_1	R_2	R_3	R_4
6	H	H	H	H
4	H	Me	H	H
1	H	Me	Me	H

Compound number	R_1	R_2	R_3	R_4
5	H	H	H	H
3	H	Me	H	H
2	H	Me	Me	H

FIG. 2. Structures of xanthones isolated from *Swertia japonica*.

xanthone derivatives on CL intensity, the concentration expressed in terms of the half amount of the CL intensity (IC_{50}) was measured. Concentrations corresponding to IC_{50} were 55.0 ± 2.0, 9.1 ± 0.3, 7.1 ± 0.2, 0.49 ± 0.02, 2.0 ± 0.08 and 1.9 ± 0.05 μM for methylbellidifolin, methylswertianin, swertianin, bellidifolin, norswertianin and desmethylbellidifolin, respectively. Because IC_{50} of BHT and α-tocopherol are 6.8 ± 0.02 and 2.5 ± 0.05 μM, respectively (8), bellidifolin, norswertianin and desmethylbellidifolin are stronger antioxidants than both typical commercial antioxidants in the CL assay ($P < 0.001$, between BHT and norswertianin; $P < 0.05$).

Antioxidant activity of the xanthones on ML autoxidation. Antioxidant activities of the isolated xanthones on AMVN-initiated oxidation of ML were investigated. Figure 3 shows the effects of four xanthones, not including norswertianin and desmethylbellidifolin, at 0.77 mM (1.0 mol% relative to ML) on the rate of formation of MLHPO. Norswertianin and desmethylbellidifolin were excluded because their yields were not sufficient for the ML-autoxidation test. In the absence of xanthones, MLHPO accumulated linearly at the rate of 4.83 μM/min. Each xanthone suppressed the oxidation of ML at a definite rate. The rate of the accumulation of MLHPO was 4.15, 4.39, 2.15 and 0.37 μM/min in the presence of methylbellidifolin, methylswertianin, swertianin and bellidifolin, respectively.

The inhibitory effect of swertianin and bellidifolin on the oxidation of ML was compared with that of BHT and α-tocopherol. Under the conditions listed in Figure 3, the rate of the accumulation of MLHPO in the presence of BHT and α-tocopherol was similar to that of bellidifolin. Figure 4 shows the rates of formation of MLHPO with the addition of 0.18 mM (0.2 mol% to ML) of antioxidants to the test solution. The curve of the formation of MLHPO with the addition of bellidifolin was similar to that of BHT. Bellidifolin was shown, therefore, to possess an antioxidative activity equivalent to BHT. α-Tocopherol seemed to inhibit the formation of MLHPO during the first 200 min. However, MLHPO sharply accumulated after 200 min, and the rate of accumulation increased further and faster than that of BHT and bellidifolin after 300 min. Swertianin was least effective among the four antioxidants tested (Fig. 4).

The relationship between mutagenicity and antioxidant activity of the xanthones. For the practical application of new substances, such as xanthone derivatives to foods, large amounts of data and careful considerations on their safety are needed. Because data on the Ames test for xanthone derivatives have been reported by Kanamori *et al.* (13), the relationship between antioxidant activity and

TABLE 2

Data of Instrumental Analysis of Separated Pigments No. 1-6

Compound number	Melting point (°C)	Mass spectra (M^+)	Ultraviolet (MeOH, nm)	Infrared (cm^{-1})	Component identification
1	187	288	202, 253, 277, 333	3450, 1665, 1640, 1610, 1580	Methylbellidifolin
2	190	288	202, 238, 261, 330	3450, 1670, 1605, 1575	Methylswertianin
3	221	274	203, 238, 261, 322	3450, 1660, 1640, 1605, 1580	Swertianin
4	264	274	204, 254, 279, 334	3450, 1660, 1635, 1610, 1595	Bellidifolin
5	335	260	202, 237 266, 330	3340, 1660, 1640, 1610, 1590	Norswertianin
6	317	260	202, 252, 279, 342	3400, 1670, 1645, 1620, 1595	Desmethylbellidifolin

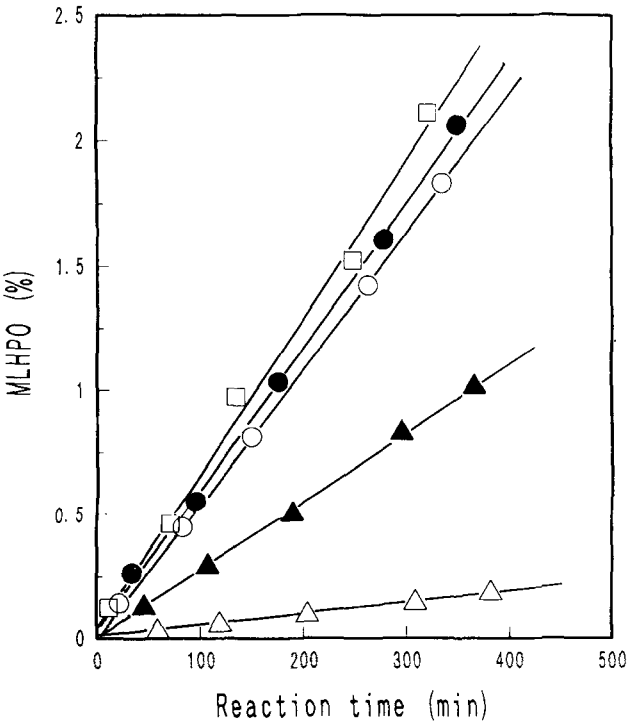


FIG. 3. Effect of xanthones on the oxidation of methyl linoleate. MLHPO—methyl linoleate hydroperoxide. The reaction system consisted of methyl linoleate (77 mM), xanthones (0.77 mM) and 2,2′-azo-*bis*(2,4-dimethylvaleronitrile) (7.7 mM) in a mixture of *n*-hexane/isopropanol/tetrahydrofuran (6:5:2, 1.3 mL); ○, methylbellidifolin, $y = 5.39 \cdot 10^{-3}x + 1.18 \cdot 10^{-2}$, $r^2 = 0.993$; ●, methylswertianin, $y = 5.70 \cdot 10^{-3}x + 3.97 \cdot 10^{-2}$, $r^2 = 0.999$; ▲, swertianin, $y = 2.79 \cdot 10^{-3}x - 1.02 \cdot 10^{-2}$, $r^2 = 0.999$; △, bellidifolin, $y = 4.78 \cdot 10^{-4}x - 3.17 \cdot 10^{-3}$, $r^2 = 0.999$; □, no addition, $y = 6.28 \cdot 10^{-3}x + 4.96 \cdot 10^{-2}$, $r^2 = 0.993$.

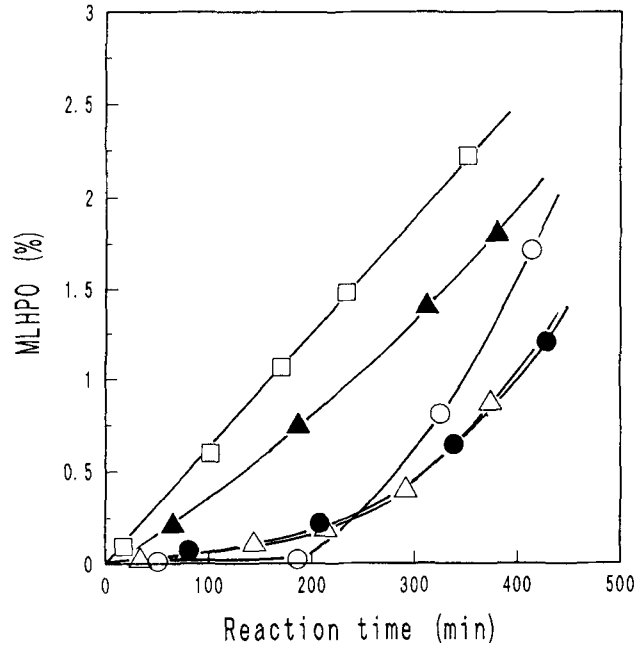


FIG. 4. Comparison of antioxidant activity of swertianin and bellidifolin with butylated hydroxytoluene (BHT) and α-tocopherol on the oxidation of methyl linoleate. The reaction system consisted of methyl linoleate (88 mM), antioxidant (0.18 mM) and 2,2′-azo-*bis*(2,4-dimethylvaleronitrile) (8.8 mM) in a mixture of *n*-hexane/isopropanol/tetrahydrofuran (6:5:0.4, 1.14 mL); ▲, swertianin; △, bellidifolin; ●, BHT; ○, α-tocopherol; □, no addition. See Figure 3 for other abbreviation.

mutagenicity of the xanthones was investigated in the present study as a preliminary safety check.

The antioxidant activities of the xanthones in this work and the data on mutagenicity in the study of Kanamori *et al.* (13) are summarized in Table 3. The order of the antioxidative and mutagenic activities of the xanthones indicates similar trends. These results suggest a relationship between antioxidative activity and mutagenicity.

If these xanthones possess carcinogenicity, they cannot be applied in foods as antioxidants. However, it is well-known that a great number of positive compounds in the Ames test are actually negative to carcinogenicity. The safety of the xanthones must be examined with other evaluation methods. To practically apply the xanthones of the extract of *S. japonica* to foods as antioxidants, we hope to examine the antioxidative effects on various foods as well as the safety of the xanthones.

TABLE 3

Orders of Antioxidant Activities and Mutagenicities of Isolated Xanthones

Method	Order of activity[a] (high)					(low)
Antioxidant activity						
Chemiluminescence method	4 >	6 >	5 >	3 >	2 >	1
(IC50, μM)	(0.49)	(1.9)	(2.0)	(7.1)	(9.1)	(55.0)
Methyl linoleate-autoxidation method	4 >	3 >	1 >	2		
(accumulation of MLHPO, μM/min)	(0.37)	(2.15)	(4.15)	(4.39)		
Mutagenicity						
Ames test[b]	4 >	6 >	5 =	3 >	1 >	2
(His^+ revertant, colonies/μg)	(53.3)	(47.5)	(16.5)	(16.5)	(7.9)	(3.4)

[a]1:methylbellidifolin, 2:methylswertianin, 3:swertianin, 4:bellidifolin, 5:norswertianin, 6:desmethylbellidifolin.
[b]Data by Kanamori *et al.* (13).

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