# Triterpenoids from *Hippophae rhamnoides* L. and Their Nitric Oxide Production-Inhibitory and DPPH Radical-Scavenging Activities[1)]

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**In our investigation on the chemical constituents of *Hippophae rhamnoides* L., the chloroform-soluble fraction of the 80% acetone extract of branch bark was observed to inhibit nitric oxide (NO) production in a lipopolysaccharide and recombinant mouse interferon-$\gamma$-activated murine macrophage-like cell line, RAW 264.7 cells. Two new triterpenoids, 2-*O*-*trans*-*p*-coumaroyl maslinic acid (1) and 2-*O*-caffeoyl maslinic acid (2), and three known triterpenoids, oleanolic acid (3), 3-*O*-*trans*-*p*-coumaroyl oleanolic acid (4), and 3-*O*-caffeoyl oleanolic acid (5), and 6-methoxy-2*H*-1-benzopyran (6) and $\beta$-sitosterol (7) were isolated from the branch bark extract. Their inhibitory activities on the production of NO in RAW 264.7 cells and radical-scavenging activities were examined.**

**Key words** *Hippophae rhamnoides* L.; triterpenoid; nitric oxide; macrophage; radical-scavenging activity

*Hippophae rhamnoides* L. (Elaeagnaceae) is a hardy, deciduous shrub with yellow or orange berries. *H. rhamnoides* contains a series of chemical compounds including flavonols,[2,3)] sterols,[4)] lipids,[5)] triterpenoids,[6)] *etc. H. rhamnoides* has been used as a traditional medicine for the treatment of cough, indigestion, and blood stasis in China.[7)]

Macrophages play major roles in host defense, immunity, and inflammatory responses and once activated they induce the production of proinflammatory cytokines and oxygen and nitrogen species, which recruit activated immune and inflammatory cells to the site of a lesion, thereby amplifying and perpetuating the inflammatory state. The inorganic free radical nitric oxide (NO) has been implicated in physiologic and pathologic processes such as vasodilation, nonspecific host defense, ischemia-reperfusion injury, and chronic or acute inflammation. NO can be expressed in response to proinflammatory agents such as lipopolysaccharide (LPS) in various cell types including macrophages, endothelial cells, and smooth muscle cells. In inflammatory disease such as rheumatoid arthritis, excessive NO production may be of therapeutic benefit in various types of inflammation.[8,9)]

In our investigation of the *in vitro* antiinflammatory effects of *H. rhamnoides* extracts, the chloroform-soluble portion of the 80% acetone extract of branch bark was observed to inhibit NO production in LPS-activated macrophages. Furthermore, the chloroform-soluble portion was purified by column chromatography on silica gel and ODS and also by preparative HPLC. Two new triterpenoids, 2-*O*-*trans*-*p*-coumaroyl maslinic acid (**1**) and 2-*O*-caffeoyl-maslinic acid (**2**), and three known triterpenoids, oleanolic acid (**3**),[10)] 3-*O*-*trans*-*p*-coumaroyl oleanolic acid (**4**),[11)] and 3-*O*-caffeoyl oleanolic acid (**5**),[12)] and 6-methoxy-2*H*-1-benzopyran (**6**)[13)] and $\beta$-sitosterol (**7**)[14)] were isolated from the branch bark extract. We also report on the inhibition of NO production and radical-scavenging activities of these compounds.

Compound **1** was obtained as an amorphous powder. The molecular formula of **1** was established to be $C_{39}H_{54}O_6$ by negative high-resolution FAB-MS (HR-FAB-MS). The UV spectrum exhibited absorption maxima at 225 and 310 nm, suggesting the presence of aromatic rings in the molecule. The IR spectrum contained absorption bands for hydroxyls (3550—3100 $cm^{-1}$), $\alpha,\beta$-unsaturated carbonyl (1696 $cm^{-1}$), and aromatic (1605, 1515 $cm^{-1}$) functionalities. The $^1$H-NMR spectrum of **1** indicated seven tertiary methyls [$\delta$ 0.82, 0.88, 0.90, 0.93, 1.06, 1.09, 1.18 (each 3H, s)], two hydroxymethine protons [$\delta$ 5.04 (1H, ddd, $J$=11.8, 10.0, 4.6 Hz and $\delta$ 3.24 (1H, d, $J$=10.0 Hz)], a trisubstituted olefinic double bond [$\delta$ 5.24 ( 1H, t, $J$=3.7 Hz)], and a 1,4-disubstituted

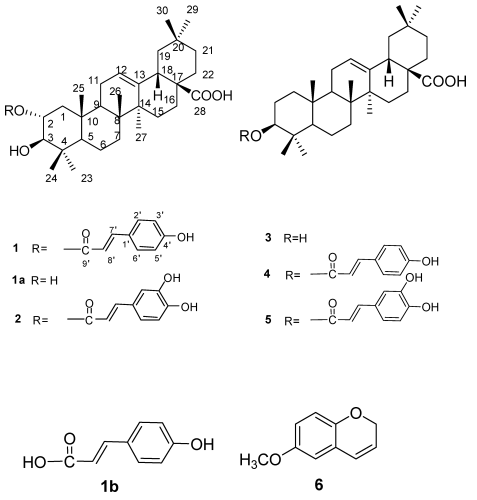


Fig. 1. Structures of Compounds **1**—**6**

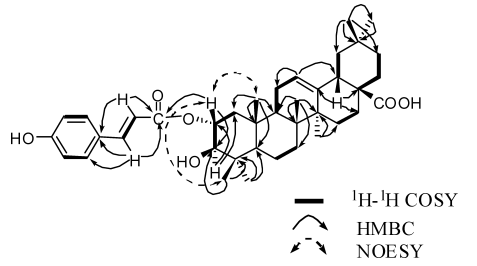


Fig. 2. $^1$H–$^1$H COSY, Key HMBC and NOE Correlations of **1**

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benzene ring [$\delta$ 7.45 (2H, d, $J$=8.6 Hz) and $\delta$ 6.80 (2H, d, $J$=8.6 Hz)]. In addition, the coupling constant of the proton signals at $\delta$ 7.62 and $\delta$ 6.34 were 16.0 Hz, indicating the presence of two *trans-p*-coumaroyl protons. Thus it was presumed that there was an *O-trans-p*-coumaroyl group in **1**.

The $^{13}$C-NMR spectrum of **1** showed 39 carbon atoms that were classified as seven methyls, nine methylenes, 12 methines, and 11 quaternary carbon atoms using distortionless enhancement by polarization transfer (DEPT) spectrum analysis. In addition, there were one ester carbonyl carbon at $\delta$ 169.3 (C-9′), one carboxylic acid carbon at $\delta$ 182.0 (C-28), a pair of olefinic carbons at $\delta$ 123.4 (C-12) and $\delta$ 145.4 (C-13), four double-bond carbons at $\delta$ 116.0 (C-8′), 116.8 (C-3′, 5′), 127.3 (C-1′), 131.1 (C-2′, 6′), 146.3 (C-7′), and 161.2 (C-4′), and two hydroxylated carbons at $\delta$ 73.9 (C-2) and $\delta$ 81.1 (C-3). The negative HR-FAB-MS give the molecular formula of $C_{39}H_{54}O_6$, in agreement with the NMR spectral data. Thus **1** has an *O-trans-p*-coumaroyl group and an oleanane-type skeleton containing 30 carbons. The framework of this compound was deduced from the carbon number and the presence of seven tertiary-linked methyls and double bond and two hydroxymethines. To confirm the location of the *O-trans-p*-coumaroyl, the 2D NMR spectra of **1** were obtained. In the HMBC spectrum, the proton signal at $\delta$ 5.04 (1H, ddd, $J$=11.8, 10.0, 4.6 Hz, H-2) showed long-range correlations with the carbon signals at $\delta$ 81.1 (C-3) and 169.3 (C-9′), indicating that the *O-trans-p*-coumaroyl was located at C-2. In the NOESY spectrum, the $\delta$ 5.04 (H-2) was correlated with $\delta$ 0.88 (H-24) and 1.09 (H-25). Thus the relative stereochemistry of the *O-trans-p*-coumaroyl was determined to be 2$\alpha$. Hydrolysis of **1** with 10% KOH in MeOH yielded maslinic acid (**1a**)[15] and *p*-coumaric acid (**1b**).[16] The ester substituent was placed at C-2 also as result of the downfield shifts observed for H-2 and C-2 in the $^1$H- and $^{13}$C-NMR spectrum. Furthermore, based on the HMQC, HMBC, and COSY spectral data, the structure of compound **1** was determined to be 2-*O-trans-p*-coumaroyl maslinic acid.

Compound **2** was obtained as an amorphous powder. The molecular formula of **2** was established to be $C_{39}H_{54}O_7$ based on negative HR-FAB-MS. This UV spectrum exhibited absorption maxima at 245 and 327 nm, suggesting the presence of aromatic rings in the molecule. The IR spectrum contained absorption bands for hydroxyls (3550—3100 cm$^{-1}$), $\alpha,\beta$-unsaturated carbonyl (1695 cm$^{-1}$), and aromatic (1600, 1515 cm$^{-1}$) functionalities. The $^1$H-NMR spectrum of **2** was similar to that of **1**, except for the aromatic region (Table 1). Compound **2** indicated seven tertiary methyls [$\delta$ 0.83, 0.88, 0.89, 0.94, 1.06, 1.09, 1.17 (each 3H, s)], a trisubstituted olefinic double bond [$\delta$ 5.24 (1H, t, $J$=3.8 Hz)], two hydroxymethine protons [$\delta$ 5.04 (1H, ddd, $J$=11.8, 10.0, 4.4 Hz) and $\delta$ 3.24 (1H, d, $J$=10.0 Hz)], and a 1,2,4-trisubstituted benzene ring [$\delta$ 7.03 (1H, d, $J$=2.1 Hz), $\delta$ 6.93 (1H, dd, $J$=8.6, 2.1 Hz), and $\delta$ 6.77 (1H, d, $J$=8.6 Hz)]. In addition, the coupling constant of the proton signals at $\delta$ 7.55 and $\delta$ 6.27 were 15.8 Hz, indicating the presence of two *trans*-olefinic protons. Thus it was presumed that there was an *O*-caffeoyl group in **2**.

The $^{13}$C-NMR spectrum of **2** showed 39 carbon atoms which were classified as seven methyls, nine methylenes, 11 methines, and 12 quaternary carbon atoms using DEPT spectrum analysis. In addition, there were one ester carbonyl carbon at $\delta$ 169.3 (C-9′), one carboxylic acid carbon at $\delta$ 181.8 (C-28), a pair of olefinic carbons at $\delta$ 123.4 (C-12) and $\delta$ 145.3 (C-13), four double-bond carbons at $\delta$ 115.2 (C-2′), 115.9 (C-5′), 116.5 (C-8′), 122.8 (C-6′), 127.9 (C-1′), 146.6 (C-3′), 146.8 (C-7′), 149.5 (C-4′), and two hydroxylated carbons at $\delta$ 73.8 (C-2) and $\delta$ 81.2 (C-3). The negative HR-FAB-MS give the molecular formula of $C_{39}H_{54}O_7$, in agreement with the NMR spectral data. Thus **2** has an *O*-caffeoyl group and an oleanane-type skeleton containing 30 carbons. The framework of this compound was deduced from the carbon number and the presence of seven tertiary-linked methyls and double bond and two hydroxymethines. To confirm the location of the *O*-caffeoyl, the 2D NMR spectra of **2** were obtained. In the HMBC spectrum, the proton signal at $\delta$ 5.04 (1H, ddd, $J$=11.8, 10.0, 4.4 Hz, H-2) showed long-range correlations with the carbon signals at $\delta$ 81.2 (C-3) and 169.3 (C-9′), indicating that the *O*-caffeoyl was located at C-2. In the NOESY spectrum, the $\delta$ 5.04 (H-2) was correlated with $\delta$ 0.88 (H-24) and $\delta$ 1.09 (H-25). Thus the relative

Table 1. $^1$H- and $^{13}$C-NMR Spectral Data of **1**, **2** [(600/125 MHz, $CD_3OD$, TMS, $\delta$ (ppm) ($J$=Hz)] and $^{13}$C-NMR Spectral Data of **1a** [75 MHz, $CD_3OD$, TMS, $\delta$ (ppm)]

| | **1** | | **2** | | **1a** |
|---|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_C$ |
| 1 | 2.03, 1.00 m | 45.2 | 2.03, 1.00 m | 45.2 | 48.1 |
| 2 | 5.04 (ddd 11.8, 10.0, 4.6) | 73.9 | 5.04 (ddd 11.8, 10.0, 4.4) | 73.8 | 68.4 |
| 3 | 3.24 (d 10.0) | 81.1 | 3.24 (d 10.0) | 81.2 | 83.3 |
| 4 | | 41.0 | | 41.0 | 39.6 |
| 5 | 0.93 m | 56.6 | 0.93 m | 56.6 | 55.7 |
| 6 | 1.60, 1.46 m | 19.6 | 1.60, 1.48 m | 19.6 | 18.7 |
| 7 | 1.72, 1.52 m | 33.8 | 1.72, 1.51 m | 33.8 | 33.0 |
| 8 | | 43.0 | | 43.0 | 39.6 |
| 9 | 1.66 m | 49.3 | 1.67 m | 49.1 | 47.4 |
| 10 | | 39.5 | | 39.5 | 38.3 |
| 11 | 1.98, 1.84 m | 24.6 | 1.98, 1.85 m | 24.6 | 23.7 |
| 12 | 5.24 (t 3.7) | 123.4 | 5.24 (t 3.8) | 123.4 | 122.2 |
| 13 | | 145.4 | | 145.3 | 144.0 |
| 14 | | 40.6 | | 40.6 | 41.8 |
| 15 | 1.78 m | 28.8 | 1.78 m | 28.8 | 28.0 |
| 16 | 2.00, 1.60 m | 24.1 | 2.00, 1.60 m | 24.1 | 23.2 |
| 17 | | 47.6 | | 47.6 | 46.7 |
| 18 | 2.85 (dd 14.1, 4.1) | 42.8 | 2.84 (dd 13.8, 3.8) | 42.7 | 42.0 |
| 19 | 1.66, 1.11 m | 47.3 | 1.66, 1.12 m | 47.3 | 46.3 |
| 20 | | 31.6 | | 31.6 | 30.7 |
| 21 | 1.39, 1.20 m | 34.9 | 1.38, 1.20 m | 34.9 | 34.0 |
| 22 | 1.35, 1.52 m | 33.8 | 1.36, 1.52 m | 33.8 | 33.0 |
| 23 | 1.06 s | 29.2 | 1.06 s | 29.2 | 29.0 |
| 24 | 0.88 s | 17.4 | 0.88 s | 17.4 | 17.2 |
| 25 | 1.09 s | 17.0 | 1.09 s | 16.9 | 16.9 |
| 26 | 0.82 s | 17.7 | 0.83 s | 17.7 | 17.5 |
| 27 | 1.18 s | 26.4 | 1.17 s | 26.4 | 26.0 |
| 28 | | 182.0 | | 181.8 | 180.2 |
| 29 | 0.90 s | 33.6 | 0.89 s | 33.5 | 33.0 |
| 30 | 0.93 s | 24.0 | 0.94 s | 24.0 | 24.0 |
| 1′ | | 127.3 | | 127.9 | |
| 2′ | 7.45 (d 8.6) | 131.1 | 7.03 (d 2.1) | 115.2 | |
| 3′ | 6.80 (d 8.6) | 116.8 | | 146.6 | |
| 4′ | | 161.2 | | 149.5 | |
| 5′ | 6.80 (d 8.6) | 116.8 | 6.77 (d 8.6) | 115.9 | |
| 6′ | 7.45 (d 8.6) | 131.1 | 6.93 (dd 8.6, 2.1) | 122.8 | |
| 7′ | 7.62 (d 16.0) | 146.3 | 7.55 (d 15.8) | 146.8 | |
| 8′ | 6.34 (d 16.0) | 116.0 | 6.27 (d 15.8) | 116.5 | |
| 9′ | | 169.3 | | 169.3 | |

Table 2. Inhibitory Activities of Compounds **1**—**6** on the NO Production in RAW 264.7 and Scavenging Activities of DPPH Radical

| Compounds | $IC_{50}$ ($\mu$M) | |
|---|---|---|
| | NO | DPPH |
| **1** | 43.8 | >200 |
| **1a** | 50.7 | >200 |
| **1b** | 75.0 | >200 |
| **2** | 7.6 | 34.7 |
| **3** | 40.2 | >200 |
| **4** | 51.3 | >200 |
| **5** | 32.6 | 23.0 |
| **6** | 24.8 | 55.0 |

stereochemistry of the *O*-caffeoyl was determined to be 2$\alpha$. Furthermore, based on the HMQC, HMBC, and COSY spectral data, the structure of compound **2** was determined to be 2-*O*-caffeoyl maslinic acid.

In addition, oleanolic acid (**3**),[10] 3-*O*-*trans*-*p*-coumaroyl oleanolic acid (**4**),[11] 3-*O*-caffeoyl oleanolic acid (**5**),[12] 6-methoxy-2*H*-1-benzopyran (**6**),[13] and $\beta$-sitosterol (**7**)[14] were confirmed by comparison with the spectral data reported.

Compounds **1**—**6** were examined for their inhibitory activities on NO production stimulated by LPS and interferon-$\gamma$ in RAW 264.7 cells. As shown Table 2, 2-*O*-caffeoyl maslinic acid (**2**) showed the most potent on suppression of NO production, with $IC_{50}$ values of 7.6 $\mu$M. Compounds **5** and **6** showed moderate NO inhibitory activity, with $IC_{50}$ values of 32.6 and 24.8 $\mu$M, respectively. Compounds **1**, **1a**, **1b**, **3**, and **4** showed weak NO inhibitory activity, with $IC_{50}$ values of 43.8, 50.7, 75.0, 40.2, and 51.3 $\mu$M, respectively. Quercetin, used as a positive control, had an $IC_{50}$ value of 20.6 $\mu$M. Comparing **1** with **4** and **2** with **5**, compounds **1** and **2** contained a phenylpropanoid ester at C-2, and **4** and **5** contained a phenylpropanoid ester at C-3. These results suggest that the phenylpropanoid ester at C-2 may enhanced their activities. Furthermore, comparing **1** with **2** and **4** with **5**, compounds **2** and **5** contained caffeoyl groups, These results indicate that the caffeoyl ester may enhanced their activities.

Next, compounds **1**—**6** were examined for their DPPH radical-scavenging activities. Compounds **2**, **5**, and **6** showed scavenging activities, with $IC_{50}$ values of 34.7, 23.0, and 55.0 $\mu$M, respectively. However, other compounds showed no radical-scavenging activity. L-Ascorbic acid, a well-known antioxidant, used as a positive control, had an $IC_{50}$ value of 12.0 $\mu$M. The antioxidant abilities of the triterpenoids are mainly due to the presence of an aromatic ring bearing hydroxyl groups in these compounds and these effects depend on the number of hydroxyl groups.[17] Therefore **2** showed higher activity than **1** and **1a**, and **5** showed higher activity than **3** and **4**.

## Experimental

**General Experiment Procedures** The UV spectra were obtained in MeOH on a Shimadzu UV-160 spectrophotometer, and IR spectra were recorded on a JASCO IR A-2 spectrophotometer. The NMR spectra were recorded on Mercury-300BB Varian and JEOL GL-600 FT NMR spectrometers, with TMS as an internal standard. The MS were obtained on a JEOL GC mate spectrometer. Column chromatography was carried out on silica gel (Wako gel C-300, Wako Pure Chemical Industry Ltd.) and ODS (Chromatorex 100—200 mesh, Fuji Silysia Chemical Ltd.). TLC was performed on Merck TLC plates (0.25-mm thickness), with compounds visualized by spraying with 5% (v/v) $H_2SO_4$ in ethanol solution and then heating on a hot-plate. HPLC was performed on a JASCO PU-2089 apparatus equipped with a JASCO UV-2075 and RI-101 (Shodex). Shiseido SIL-SG80 (10$\phi$×250 mm) columns, YMC-PAK Pro-C18 (10$\phi$×250 mm), and CAPCELL-PAK-C18 AQ (10$\phi$×250 mm) were used for preparative purposes.

**Plant Material** The dried branch bark of *H. rhamnoides* L. was collected in Neimenggu, People's Republic of China, in October 2004 and was identified by Dr. Sheng-Li Wei, Beijing University of Chinese Medicine, Beijing, China. Voucher specimens have been deposited at the College of Pharmacy, Nihon University.

**Extraction and Isolation** The dried branch bark of *H. rhamnoides* L. (3.0 kg) was extracted with 80% acetone (10 l) and concentrated to give the extract. The extract [929 g, NO inhibitory activity 33% (100 $\mu$g/ml)] was dissolved in water and successively partitioned with an *n*-hexane layer (21 g, 89%), chloroform layer (29 g, 90%), ethyl acetate layer (37 g, 67%), *n*-butanol layer (180 g, 25%), and an aqueous fraction (632 g, 0.8%). The chloroform layer was subjected to silica gel column chromatography and eluted with hexane–EtOAc to afford 5 fractions. Fraction 5 was purified using ODS column chromatography and eluted with $CH_3OH$–$H_2O$ to give **1** (18.0 mg), **4** (8.0 mg), and **5** (32.7 mg). Fraction 4 was separated using ODS column chromatography and eluted with $CH_3OH$–$H_2O$ to give **2** (6.0 mg) and **6** (12.8 mg). Fraction 3 was purified using normal-phase HPLC (hexane–EtOAc 70 : 30) and reverse-phase HPLC ($CH_3OH$–$H_2O$, 95 : 5) to give **3** (24.9 mg). Fraction 2 was isolated using ODS column chromatography and eluted with $CH_3OH$–$H_2O$ to give **7** (12.9 mg).

2-*O*-*trans*-*p*-Coumaroyl Maslinic Acid (**1**): Colorless powder, $[\alpha]_D^{25}$ +32.0° ($c$=0.15, MeOH). UV (MeOH) $\lambda_{max}$ nm (log $\varepsilon$): 225 (3.93), 310 (4.79). IR (KBr) $\nu_{max}$ cm$^{-1}$: 3422, 1696, 1605, 1515, 1380, 1170, 956, 831. $^1$H- and $^{13}$C-NMR data, see Table 1. Negative HR-FAB-MS *m*/*z*: 617.3833 (Calcd for $C_{39}H_{53}O_6$: 617.3842).

2-*O*-Caffeoyl Maslinic Acid (**2**): Colorless powder, $[\alpha]_D^{25}$ +40.1° ($c$=0.10, MeOH). UV (MeOH) $\lambda_{max}$ nm (log $\varepsilon$): 245 (4.21), 300 (4.26), 327 (4.30). IR (KBr) $\nu_{max}$ cm$^{-1}$: 3430, 1695, 1600, 1515, 1170, 957. $^1$H- and $^{13}$C-NMR data, see Table 1. Negative HR-FAB-MS *m*/*z*: 633.3806 (Calcd for $C_{39}H_{53}O_7$: 633.3791).

**Alkaline Hydrolysis of 1** Compound **1** (7.0 mg) was hydrolyzed with 10% KOH in MeOH, neutralized with 10% HCl, and purified using reverse-phase HPLC ($CH_3OH$–$H_2O$, 90 : 10) to give **1a** (3.5 mg) and **1b** (0.7 mg). $^{13}$C-NMR data of **1a** see Table 1.

**Inhibitory Activity on NO Production from Activated Macrophage-Like Cell Line, RAW 264.7**[18,19] The cells were seeded at $1.2\times10^6$ cells/ml onto a 96-well flat-bottomed plate (Sumitomo Bakelite, #8096R, Tokyo) and then incubated at 37 °C for 2 h. Then the test compound was added to the culture simultaneously with both *Escherichia coli* LPS (100 ng/ml) and recombinant mouse IFN-$\gamma$ (0.33 ng/ml), and the cells were incubated at 37 °C, usually for 16 h. After incubation, the cells were chilled on ice. A 100-$\mu$l aliquot of the culture supernatant was placed in a well in duplicate 96-well flat-bottomed plates. A standard solution of $NaNO_2$ was also placed in other wells on the same plate. To quantify nitrite, Griess reagent 50 $\mu$l, 1% sulfanilamide in 5% $H_3PO_4$, and 0.1% *N*-1-naphthylethylenediamine dihydrochloride were added to each well. After 10 min, the reaction products were colorimetrically quantified at 550 nm with subtraction of the background absorbance at 630 nm, using a Model 3550 microplate reader (Bio-Rad). Cytotoxity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bomide (MTT) assay method.

**DPPH Radical-Scavenging Activity**[20] Acetic acid buffer 0.2 M (40 $\mu$l), 12% methanol (40 $\mu$l), and sample (0.4 $\mu$l) were mixed with DPPH 0.5 mM (40 $\mu$l). The resulting solution was thoroughly mixed and the absorbance was measured at 520 nm after 30 min. The scavenging activity was determined by comparing the absorbance with that of the control (100%) containing only DPPH and solvent.

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