Biosci. Biotechnol. Biochem., **67** (2), 410–414, 2003

Note

Antioxidative Compounds from *Crotalaria sessiliflora*

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Received August 2, 2002; Accepted October 17, 2002

Seven antioxidative compounds were isolated from the EtOAc extract of the aerial part of *C. sessiliflora* (Japanese name, tanukimame) by activity-guided fractionation with 2,2-diphenyl-1-picrylhydrazyl (DPPH). Among the isolated compounds, hydroxyeucomic acid showed the strongest free radical-scavenging activity, which was almost identical to that of epigallocatechin gallate, against DPPH. Orientin and isoorientin showed strong anti-peroxidative activities toward linoleic acid and protective effects against the bactericidal action of the *tert*-butyl peroxyl radical. Their activities were nearly equal to that of epigallocatechin gallate.

Key words: antioxidative compound; *Crotalaria sessiliflora*; free radical; *tert*-butyl peroxyl radical

Free radicals have been reported as a cause of such diseases as cancer, heart disease and inflammation.[1,2] Free radicals are also a major factor in food deterioration.[3] The generation of free radicals can be inhibited by enzymatic and non-enzymatic mechanisms. Antioxidants such as phenolic compounds play an important role in the non-enzymatic mechanism and have been reported to have biological activities such as anticancer, antiinflamation and prevention of coronary heart disease.[4,5] For these reasons, the utilization of antioxidants is an important strategy to prevent the progression of diseases caused by free radicals and to maintain food quality by preventing lipid peroxidation. New potent antioxidants can also serve as leading compounds for designing new active substances.

In the course of our search for potent antioxidants from plants, we have focused on *C. sessiliflora*, because the extract showed many spots which had strong radical-scavenging activity against DPPH on a paper chromatogram. In this study, we describe the isolation and identification of the active compounds and their antioxidative activities toward DPPH radicals, the linoleic acid peroxidation induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and the protective effect against the bactericidal action of the *tert*-butyl peroxyl radical.

Compound **1** was identified as hydroquinone on the basis of IR, MS and NMR spectral data and by comparison with those of the authentic compound. On the basis of the spectral data, **2** was determined to be eucomic acid.[6] The ^{1}H-NMR and ^{13}C-NMR spectra of **3** were almost identical to those of **2**, except for the aromatic signals. The ^{1}H-NMR spectrum of **3** showed a typical ABX system at δ_H 6.72 (1H, d, J=2.13 Hz), δ_H 6.67 (1H, d, J=7.90 Hz), and δ_H 6.57 (1H, dd, J=7.90 and 2.13 Hz). Moreover, the molecular ion peak of **3** was larger than that of **2** by 16 amu. These data indicated the presence of an *o*-dihydroxyl group in the aromatic ring. Therefore, compound **3** was determined to be hydroxyeucomic acid.[7] Although **1**–**3** are known substances, they were isolated from the genus *Crotalaria* for the first time. In particular, **3** is a rare substance on which only one study has reported.[7]

Compound **4** was assigned to be eriodictyol-7-*O*-*β*-D-glucopyranoside on the basis of UV, MS, ^{1}H-NMR and ^{13}C-NMR spectral data.[8] The CD spectrum of **4** showed a positive Cotton effect at 292 nm and a negative Cotton effect at 333 nm. This observation indicated the chiral center at C-2 to be (*R*).[9] Accordingly, the structure of **4** was elucidated as (2*R*)-eriodictyol-7-*O*-*β*-D-glucopyranoside.

Compounds **5**, **6** and **7** were assigned as vitexin,[10] orientin and isoorientin,[11] respectively, on the basis of UV,[12] ^{1}H-NMR and ^{13}C-NMR spectral data. The chemical structures of the antioxidative compounds isolated from *C. sessiliflora* are indicated in Fig. 1.

Some phenolic substances were detected from BuOH extract by paper chromatography. However, the antioxidative activities of those substances against DPPH were negligible, so no further investigation was carried out.

Table 1 shows the effect of the isolated compounds and positive controls on scavenging the DPPH radical. Among the isolated compounds, hydroxyeucomic acid showed the strongest free radical-scavenging

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; *tert*-BuOOH, *tert*-butyl hydroperoxide; BHT, butylated hydroxytoluene; CD, circular dichroism; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid

Table 1. Free Radical-scavenging Activities of the Isolated Compounds against DPPH and Their Protective Effect on the Bactericidal Action of the *tert*-Butyl Peroxyl Radical

Compound	DPPH	*tert*-BuOOH
	IC_{50} (μM)[a]	MIC (μM)[b]
Eucomic acid	>90	125
Hydroxyeucomic acid	5.3	125
Hydroquinone	27	nt[c]
(2*R*)-Eriodictyol-7-*O*-*β*-D-glucopyranoside	12.3	125
Vitexin	>60	250
Orientin	9.5	62
Isoorientin	9.5	62
BHT	25	>1000
Epigallocatechin gallate	4.2	40

[a] The concentration of a sample required to reduce the DPPH radical by 50%. Each value represents the mean of two separate experiments.
[b] The minimum concentration of a tested compound still exhibiting a protective effect against the bactericidal action of the peroxyl radical.
[c] nt = not tested.

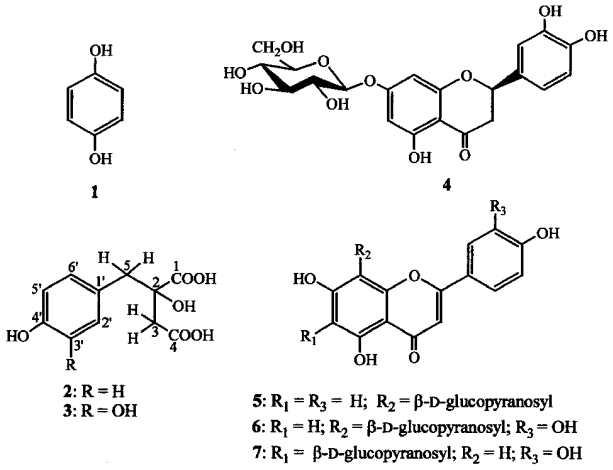

Fig. 1. Chemical Structures of the Antioxidative Compounds from *C. sessiliflora*.

1, hydroquinone; **2**, eucomic acid; **3**, hydroxyeucomic acid; **4**; (2*R*)-eriodictyol-7-*O*-*β*-D-glucopyranoside; **5**, vitexin; **6**, orientin; **7**, isoorientin.

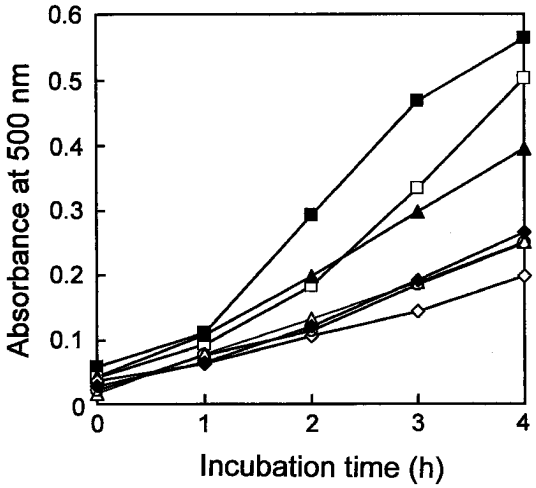

Fig. 2. Antioxidative Activity of the Isolated Compounds Measured by the Inhibition of Linoleic Acid Peroxidation.

Control (0 μM sample, ■), BHT (□), hydroxyeucomic acid (▲), isoorientin (◆), orientin (○), (2*R*)-eriodictyol-7-*O*-*β*-D-glucopyranoside (△), epigallocatechin gallate (◇). Each value represents the mean of two separate experiments. See the experimental section for details of the assay.

activity which was almost identical to that of epigallocatechin gallate used as a positive control.

The antioxidative activities of the isolated compounds to inhibit AAPH-induced linoleic acid peroxidation are presented in Fig. 2. As expected, the antioxidative activities of orientin, isoorientin, hydroxyeucomic acid and (2*R*)-eriodictyol-7-*O*-*β*-D-glucopyranoside were stronger than that of BHT. Orientin, isoorientin and (2*R*)-eriodictyol-7-*O*-*β*-D-glucopyranoside had similar antioxidative activities in this analysis, their activities being nearly equal to that of epigallocatechin gallate. Although hydroxyeucomic acid showed the strongest free radical-scavenging activity to the DPPH radical, in this analysis, the activity was lower than that of orientin, isoorientin or (2*R*)-eriodictyol-7-*O*-*β*-D-glucopyranoside.

We further investigated the protective effect of the isolated compounds against the bactericidal action of free radicals on *Staphylococcus aureus*. This system involves the bacteria receiving fatal damage through lipid peroxidation of the membranes, and the DNA single strand breaking.[13] Table 1 shows the protective activity of the isolated compounds against the peroxyl radical. The present study demonstrates that orientin and isoorientin significantly protected bacteria from the bactericidal action of peroxyl radicals, although their activities were lower than that of epigallocatechin gallate. Hydroxyeucomic acid, eucomic acid and (2*R*)-eriodictyol-7-*O*-*β*-D-glucopyranoside showed similar activity, while BHT showed very low activity.

Depending upon the testing method, the isolated compounds showed different antioxidative activities. Orientin and isoorientin showed strong activities toward DPPH and linoleic acid peroxidation (Fig. 2).

They also had a strong protective effect on the bactericidal action of the peroxyl radical (Table 1). Both compounds seemed to protect the cell by quenching of the peroxyl radical and the inhibition of lipid peroxidation. On the other hand, hydroxyeucomic acid showed low inhibitory activity toward lipid peroxidation and a low protective effect on the bactericidal action of the peroxyl radical, although it had the strongest radical-scavenging activity against DPPH (Table 1). Differences in the antioxidative activity by the testing systems were probably due to different reaction mechanisms. In the DPPH radical system, the antioxidative substance reacts directly with the DPPH radical by hydrogen atom donation.[14] On the other hand, in the lipid peroxidation system, breaking of the chain reaction by the antioxidative compounds may have occurred.

The utilization of the aerial part of *C. sessiliflora* has not previously been described, although the seeds, which contain monocrotaline (pyrrolizidine alkaloid), have been used as an antitumor agent in Chinese folk medicine.[15] Considering that this plant contains several antioxidative compounds with strong activities, it may be used as a medicinal plant to protect body from free radical-mediated damage.

Experimental

Paper chromatography for detecting free radical scavengers. Two-dimensional paper chromatography was carried out on ADVANTEC No. 5B paper with the following solvents: (1) 2% AcOH and (2) *n*-BuOH:AcOH:H_2O (4:1:5, upper layer). The radical-scavenging compounds on the paper chromatogram were detected by spraying with a 0.2% DPPH solution in MeOH. The paper was examined 30 min later, the active compounds appearing as yellow spots against a purple background.

Isolation. Freshly harvested aerial parts of *C. sessiliflora* (*ca.* 40 kg) were refluxed with MeOH. After evaporating MeOH under reduced pressure, the aqueous concentrate was filtered to remove chlorophyll. The filtrate was successively partitioned with EtOAc and *n*-BuOH. The organic layers were concentrated under reduced pressure to give an EtOAc extract (96.9 g) and a BuOH extract (132.9 g).

Part of the EtOAc extract (35.9 g) was chromatographed on a Sephadex LH-20 column (42 × 7 cm) with 50% MeOH. The eluate, which was collected in 18-ml aliquots, was monitored by UV at 280 and 350 nm. Based on the elution pattern, the eluate was separated into 19 fractions. Fractions 3–5 (tube nos. 98–142), which gave a similar pattern upon separation and contained several free radical scavengers on the paper chromatogram, were subjected to silica gel partition column chromatography (Wakogel Q-23, 42 × 3.5 cm), using EtOAc saturated with H_2O as an eluent, to give subfractions A and B. The concentrate of subfraction A was treated by a Bond Elute® cartridge (6 cc/500 mg), using 5% MeOH as the eluent to remove colored substances, and then separated by ODS column chromatography (Wakosil 25C18, 33 × 2 cm) with a stepwise MeOH gradient (17–22%) containing 0.01% TFA to afford compounds **1** (48 mg) and **2** (1.04 g). The concentrate of subfraction B was rechromatographed on a Sephadex LH-20 column (40 × 3 cm, 38% MeOH) and a Bond Elute® column (2% MeOH), and freeze-dried to give a colorless hygroscopic powder of **3** (107 mg). The precipitate of fraction 12 (tube nos. 251–274) from the first Sephadex LH-20 column was filtered and recrystallized from MeOH to give **4** (46 mg). The yellow powder from fraction 14 (tube nos. 287–346) was recrystallized from MeOH-pyridine to give **5** (35 mg). Compounds **6** (28 mg) and **7** (19 mg) were obtained from fraction 15 (tube nos. 347–390) by semipreparative HPLC, using 32% MeOH (0.01% TFA) as the eluent at a flow rate of 1.0 ml/min.

Eucomic acid (2). Colorless needles (EtOAc). FAB-MS *m/z*: 239.1 $[M-1]^-$. IR ν_{max} (KBr) cm^{-1}: 3307 (OH), 1737 (C=O), 1516 (Ar), 1205, 838 (Ar). NMR δ_H (CD_3OD): 7.06 (2H, d, J=8.5 Hz, H-2′, H-6′), 6.69 (2H, d, J=8.5 Hz, H-3′, H-5′), 2.95 (1H, d, J=13.4 Hz, H-5b), 2.94 (1H, d, J=16.2 Hz, H-3b) 2.86 (1H, d, J=13.4 Hz, H-5a), 2.57 (1H, d, J=16.2 Hz, H-3a). NMR δ_C (CD_3OD): 177.7 (C-4), 174.2 (C-1), 157.4 (C-4′), 132.6 (C-2′, C-6′), 127.7(C-1′), 115.9 (C-3′, C-5′), 76.9 (C-2), 45.4 (C-3), 43.7 (C-5).

Hydroxyeucomic acid (3). Colorless hygroscopic powder. FAB-MS *m/z*: 255.1 $[M-1]^-$. IR ν_{max} (KBr) cm^{-1}: 3390 (OH), 1737 (C=O), 1712 (C=O), 1521 (Ar), 1288, 1209, 1119, 798 (Ar). NMR δ_H (CD_3OD): 6.72 (1H, d, J=2.13 Hz, H-2′), 6.67 (1H, d, J=7.90 Hz, H-5′), 6.57 (1H, dd, J=7.90, 2.13 Hz, H-6′), 2.94 (1H, d, J=16.4 Hz, H-3b), 2.90 (1H, d, J=14.0 Hz, H-5b), 2.81 (1H, d, J=13.7 Hz, H-5a), 2.57 (1H, d, J=16.4 Hz, H-3a). NMR δ_C (CD_3OD): 177.8 (C-4), 174.3 (C-1), 145.8 (C-3′), 145.3 (C-4′), 128.4 (C-1′), 123.1 (C-6′), 118.8 (C-2′), 116.0 (C-5′), 76.9 (C-2), 45.7 (C-3), 43.7 (C-5).

(2R)-Eriodictyol-7-O-β-D-glucopyranoside (4). FAB-MS *m/z*: 449.0 $[M-1]^-$, 451.2 $[M+H]^+$, 473.0 $[M+Na]^+$. $[\alpha]_D^{26}$ −50.6° (*c* 0.17, MeOH). CD: $[\theta]_{292}+2.212\times10^4$, $[\theta]_{333}-1.145\times10^4$. IR ν_{max} (KBr) cm^{-1}: 3402 (OH), 1643 (C=O), 1299, 1195, 1172, 1076. UV λ_{max} (MeOH) nm (ε): 326 (3,720), 284 (21,400); +NaOH: 430, 363, 286; +$AlCl_3$: 383, 305; +$AlCl_3$+HCl: 385, 305; +NaOAc: 327, 284; +NaOAc+H_3BO_3: 327, 284. NMR δ_H (CD_3OD): 6.92 (1H, s, H-2′), 6.79 (2H, s, H-5′, H-6′), 6.20 (1H, d, J=1.8 Hz, H-8), 6.18 (1H, d, J=1.8 Hz,

H-6), 5.32 (1H, dd, J=3.1, 12.2 Hz, H-2), 4.98 (1H, d, J=7.3 Hz, H-1″), 3.88 (1H, d, J= 12.2 Hz, H-6″), 3.69 (1H, dd, J=12.2, 5.5 Hz, H-6″), 3.5–3.4 (4H, m, H-2″, 3″, 4″, 5″), 3.11 (1H, dd, J=17.1, 12.2 Hz, H-3b), 2.76 (1H, dd, J=17.1, 3.1 Hz, H-3a). NMR δ_C (CD_3OD): 198.6 (C-4), 167.0 (C-7), 164.9 (C-5), 164.6 (C-8a), 147.0 (C-4′), 146.6 (C-3′), 131.5 (C-1′), 119.4 (C-6′), 116.3 (C-5′), 114.9 (C-2′), 105.0 (C-4a), 101.3 (C-1″), 98.0 (C-8), 97.0 (C-6), 80.7 (C-2), 78.3 (C-3″), 77.9 (C-5″), 74.7 (C-2″) 71.2 (C-4″), 62.4 (C-6″), 44.1 (C-3).

Vitexin (5). Yellow triangular crystals (MeOH-pyridine). UV λ_{max} (MeOH) nm (ε): 334 (18,600), 278 (19,100); +NaOH: 395, 329, 280; +$AlCl_3$: 388, 348, 305, 277; +$AlCl_3$+HCl: 385, 344, 304, 278; +NaOAc: 387, 279; +NaOAc+H_3BO_3: 341, 271. NMR δ_H (DMSO-d_6): 13.16 (1H, s, OH-5), 10.80 (1H, s, OH-7), 10.31 (1H, s, OH-4′), 8.03 (2H, d, J=8.5 Hz, H-2′, 6′), 6.89 (2H, d, J=8.5 Hz, H-3′, 5′), 6.77 (1H, s, H-3), 6.28 (1H, s, H-6), 4.70 (1H, d, J=9.8 Hz, H-1″), 3.85 (1H, m, H-2″) , 3.77 (1H, m, H-6″), 3.53 (1H, m, H-6″), 3.4–3.2 (3H, m, H-3″, 4″, 5″). NMR δ_C (DMSO-d_6): 182.0 (C-4), 163.9 (C-2), 162.4 (C-7) 161.0 (C-4′), 160.3 (C-5), 155.9 (C-8a), 128.8 (C-2′, C-6′), 121.5 (C-1′), 115.7 (C-3′, C-5′), 104.5 (C-8), 104.0 (C-4a), 102.3 (C-3), 98.1 (C-6), 81.7 (C-5″), 78.6 (C-3″), 73.3 (C-1″), 70.8 (C-2″), 70.5 (C-4″), 61.2 (C-6″).

Orientin (6). Yellow needles (MeOH). UV λ_{max} (MeOH) nm (ε): 349 (20,900), 257 (15,800); +NaOH: 405, 270; +NaOAc: 400; +NaOAc+H_3BO_3: 373, 264; +$AlCl_3$: 427, 275; +$AlCl_3$+HCl: 388, 361, 277. NMR δ_H (DMSO-d_6): 13.16 (1H, s, OH-5), 10.83 (1H, s, OH-7), 10.03 (1H, s, OH-4′), 9.07 (1H, s, OH-3′), 7.53 (1H, dd, J=7.9, 1.8 Hz, H-6′), 7.48 (1H, d, J=1.8 Hz, H-2′), 6.87 (1H, d, J=7.9 Hz, H-5′), 6.64 (1H, s, H-3), 6.27 (1H, s, H-6), 4.69 (1H, d, J=9.7 Hz, H-1″), 3.9–3.8 (2H, m, H-2″, 6″), 3.56 (1H, m, H-6″), 3.5–3.2 (3H, m, H-3″, 4″, 5″). NMR δ_C (DMSO-d_6): 181.9 (C-4), 164.0 (C-2), 162.4 (C-7) 160.3 (C-5), 155.9 (C-8a), 149.5 (C-4′), 145.7 (C-3′), 121.9 (C-1′), 119.3 (C-6′), 115.6 (C-5′), 114.0 (C-2′), 104.4 (C-8), 103.9 (C-4a), 102.3 (C-3), 98.0 (C-6), 81.9 (C-5″), 78.7 (C-3″), 73.3 (C-1″), 70.7 (C-2″), 70.6 (C-4″), 61.5 (C-6″).

Isooerientin (7). Yellow needles (MeOH). UV λ_{max} (MeOH) nm (ε): 350 (1,590), 213 (28,200); +NaOH: 407, 338 sh, 269, 239, 221; +$AlCl_3$: 437, 333, 277; +$AlCl_3$+HCl: 379, 364, 278; +NaOAc: 402, 331, 275, 240; +NaOAc+H_3BO_3: 376, 264. NMR δ_H (DMSO-d_6): 13.53 (1H, s, OH-5), 7.42 (1H, d, J=7.9 Hz, H-6′), 7.41 (1H, s, H-2′), 6.93 (1H, d, J=7.9 Hz, H-5′), 6.67 (1H, s, H-3), 6.53 (1H, s, H-8), 4.62 (1H, d, J=9.8 Hz, H-1″), 4.2–3.6 (2H, m, H-4″, 6″), 3.5 (1H, m, H-6″), 3.3–3.2 (3H, m, H-2″, 3″, 5″). NMR δ_C (DMSO-d_6): 182.3 (C-4), 164.2 (C-2), 164.1 (C-7), 161.0 (C-5), 156.8 (C-8a), 150.3 (C-4′), 146.2 (C-3′), 121.8 (C-1′), 119.5 (C-6′), 116.6 (C-5′), 113.6 (C-2′), 109.2 (C-6), 103.7 (C-4a), 103.2 (C-3), 94.2 (C-8), 81.8 (C-5″), 79.3 (C-3″), 73.5 (C-1″), 70.9 (C-2″), 70.7 (C-4″), 61.9 (C-6″).

Radical-scavenging activity against DPPH. The radical-scavenging assay was performed according to the method reported by Blois[14] with some modifications. Epigallocatechin gallate and BHT were used as positive controls. In a test tube, a sample in ethanol (2 ml) was diluted with 2 ml of 0.1 M acetate buffer (pH 5.5) and then 1.0 ml of 0.5 mM DPPH in ethanol was added. The reaction mixture was incubated in the dark at 30°C for 30 min, and the absorbance was measured by a spectrophotometer at 517 nm. The radical-scavenging activity is expressed in terms of IC_{50} (the concentration of a sample required to reduce the DPPH radical by 50%).

Linoleic acid oxidation. The antioxidative activity of the isolated compounds was assayed according to the method reported by Azuma *et al.*[16] with some modifications. An ethanolic solution of each sample (0.5 ml, final concentration of 20 μM) was added to 2.5 ml of 0.2 M phosphate buffer (pH 7.0). This solution was mixed with 1.3% (w/v) linoleic acid in ethanol (2.5 ml) and 1 ml of H_2O. Peroxidation was initiated by the addition of 0.25 ml of 46.6 mM AAPH in a 40% ethanolic solution. The reaction mixture was incubated at 50°C in dark, sampling being carried out every 1 h for 4 h. The degree of oxidation was measured according to the ferric thiocyanate method.[17] The reaction mixture (0.1 ml) was diluted with 75% ethanol (9.7 ml) and then mixed with 0.1 ml of 20 mM $FeCl_2$ solution in 3.5% HCl and 0.1 ml of 10% ammonium thiocyanate solution. The mixture was shaken vigorously and, after three minutes, the absorbance was measured at 500 nm.

tert-Butyl peroxyl radical-scavenging activity. The antioxidative activity of the isolated compounds was assayed according to the method reported by Akaike *et al.*[18] with a slight modification. *S. aureus* IFO 12732 suspension (0.1 ml) was placed in a test tube containing 0.6 ml of phosphate-buffered saline (PBS, pH 7.3). Serially diluted antioxidant solutions in DMSO (0.1 ml) and 0.1 ml of a hemoglobin solution (1 mg/ml) were then added to the bacterial suspension. Next, 0.1 ml of 0.2 mM *tert*-butyl hydroperoxide solution in PBS was added, and the mixture was incubated at 37°C for 30 min to generate ROO$^{\bullet}$. After that, 2 ml of a two-fold concentration of the mannitol-phenol red medium was added to the reaction mixture to stop the reaction. The reaction mixture was serially diluted with the mannitol-phenol red medium in a 96-microwell plate and incubated at

37°C for 16 h. The amount of bacteria was quantified with a colorimetric method with a multiplate reader at the wavelength of 620 nm. The antioxidative activity is expressed as MIC, which means the minimum concentration of a tested compound still exhibiting a protective effect on the bactericidal action of the peroxyl radical.

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