Secondary Metabolites from the Mycelia of the Fungus *Monascus pilosus* BCRC 38072

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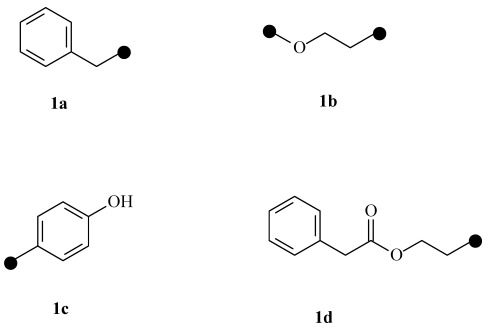
Three new compounds, including two phenylacetic acid derivatives, monaspilosin (1) and monaspiloindole (2), and one pyranoindole alkaloid, monaspyranoindole (3), were isolated from the EtOAc-soluble fraction of the MeOH extract of the mycelia of *Monascus pilosus* BCRC 38072. Twelve known compounds were also obtained in this study. The structures were elucidated by 1D and 2D NMR spectroscopy and mass spectrometry. This is the first report of *Monascus* metabolites with an indole ring. All isolates were also evaluated for their scavenging properties toward the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) in TLC autographic and spectroscopic assays.

Key words *Monascus pilosus*; Eurotiaceae; phenylacetic acid; pyranoindole; 2,2-diphenyl-1-picrylhydrazyl

Monascus has been used in oriental fermented foods for thousands of years.[1] Red mold rice fermented with *Monascus* spp. produces bioactive metabolites such as γ-aminobutyric acid (GABA), polyketides monacolin K, and some pigments, which, respectively, function as an anti-hypertension agent,[2] a cholesterol-lowering drug,[3—5] and possess antibacterial activity.[6] *Monascus* pigments, secondary metabolites possessing mainly azaphilone skeletons, have traditionally been used as natural food colorants.[1] Many other metabolites have also been reported in previous research,[7—15] most of them isolated from red mold rice obtained from solid fermentation. In contrast, the metabolites contained in the mycelia pellets from submerged cultures have rarely been investigated. The antioxidant effects exhibited in the methanol extract fraction of the mycelia pellets of *M. pilosus* BCRC 38072 were monitored using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method.[16,17] Subsequent chemical examination of the methanol extract of mycelia from *M. pilosus* led to the isolation of two new phenylacetic acid derivatives, monaspilosin (**1**) and monaspiloindole (**2**), and one new pyranoindole alkaloid, monaspyranoindole (**3**), along with twelve known compounds. This paper reports on the isolation, structural elucidation and the DPPH free radical scavenging activity of these fungal metabolites.

Results and Discussion

Extensive chromatographic purification of the EtOAc-soluble fraction of the MeOH extract of the pellets of *M. pilosus* afforded fifteen compounds. The new compound **1**, isolated as colorless oil, was assigned the molecular formula $C_{16}H_{16}O_3Na$ by ESI-MS ($[M+Na]^+$, *m/z* 279) and HR-ESI-MS. IR absorptions were observed at 3403, 1712, 1612, 1514 cm^{-1} pointing to the presence of hydroxyl, an ester carbonyl group, and a benzene ring. The UV spectrum showed maximum absorption at 277 nm, and a bathochromic shift in alkaline solution indicated the presence of a phenol derivative.[18] This was confirmed by the ^{1}H-NMR spectrum, which showed one proton at δ_H 4.90 (1H, br s) assigned to OH-15, which disappeared upon addition of D_2O. The ^{1}H-NMR



spectrum of **1** showed a mono-substituted phenyl moiety at δ_H 7.09 (5H, m, H-2—6). An AA′XX′ pattern at δ_H 6.72, 7.00 (each 2H, d, J=8.8 Hz, H-14, 16 and H-13, 17) suggested a 1,4-disubstituted benzene ring in **1**. In addition, the ^{1}H-NMR and HSQC spectra revealed the presence of a benzylic methylene group [δ_H 3.60 (2H, s)]. An oxymethylene unit [δ_H 4.26 (2H, t, J=7.0 Hz, H-10)] split into a triplet due to coupling with another methylene group [δ_H 2.84 (2H, t, J=7.0 Hz, H-11)]. The above signals and the COSY spectrum established the presence of the partial substructures: fragments, **1a**, **1b**, and **1c**, for compound **1**. The entire skeleton of **1** was constructed from the HMBC spectrum (Fig. 2). The ^2J and ^3J correlations of the signal at δ_H 3.60 (H-7) and δ_H 4.26 (H-10), with the carbon signal at δ_C 171.6 (C-8), helped to establish the connections of fragments **1a** and **1b** with the carbonyl group at C-8. That is, fragments **1a** and **1b** can be connected to produce **1d**. In addition, the cross peak between δ_H 2.84 (H-11) and δ_C 130.0 (C-13, 17), as well as δ_H 4.26 (H-10) and δ_C 129.6 (C-12), suggest that fragments **1c** and **1d** were linked together at C-11. The structure was further confirmed by ^{13}C-NMR, DEPT, COSY, NOESY (Fig. 1), HSQC, and HMBC (Fig. 2) experiments. Thus, the structure of **1** was determined to be a phenylacetic acid 2-(4-hydroxyphenyl)ethyl ester, and was given the name monaspilosin.

Compound **2**, also a colorless oil, was assigned the molecular formula $C_{18}H_{17}NO_2$, as deduced by ESI-MS and HR-ESI-MS. The presence of an ester group was revealed by an

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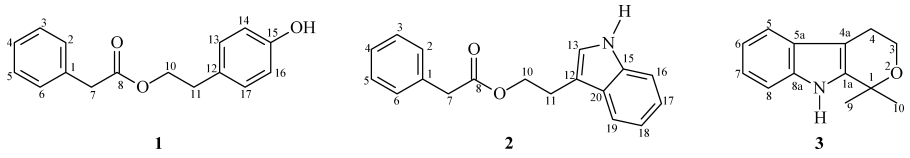

Fig. 1. New Compounds Isolated from *Monascus pilosus*

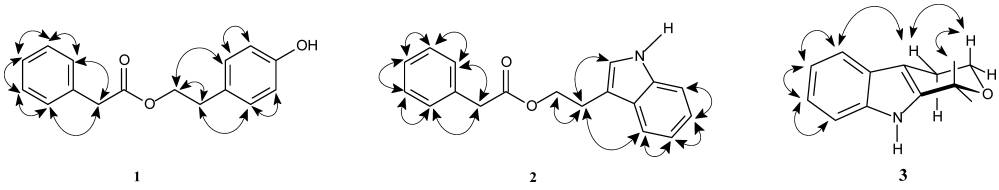

Fig. 2. NOESY Interactions of Monaspilosin (**1**), Monaspiloindole (**2**), and Monaspyranoindole (**3**)

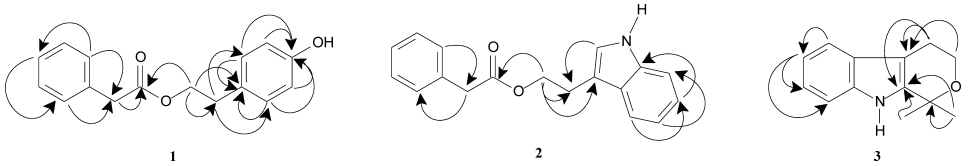

Fig. 3. Major HMBC Connectivities of Monaspilosin (**1**), Monaspiloindole (**2**), and Monaspyranoindole (**3**)

IR absorption at 1716 cm^{-1} and by a resonance signal in the ^{13}C-NMR spectrum at δ_C 171.3. The presence of a NH group in the molecule was revealed by a band at 3410 (br) cm^{-1} in the IR spectrum, which was confirmed by the signal at δ_H 8.00 (1H, br s), which disappeared upon addition of D_2O. The UV spectrum showed a maximum absorption at 280 nm, indicating the presence of a phenylacetic acid skeleton.[18] The ^{1}H-NMR spectrum of compound **2** was similar to the above compound, monaspilosin (**1**), except that the substitutent at C-11 in **2** was a 1*H*-indol-3-yl moiety in place of the *p*-hydroxyphenyl group in **1**. Signals for the indol-3-yl moiety appeared at δ_H 7.13 (1H, br t, J=7.8 Hz, H-18), 7.20 (1H, br t, J=7.8 Hz, H-17), 7.36 (1H, br d, J=7.8 Hz, H-16), and 7.61 (1H, br d, J=7.8 Hz, H-19), suggesting four coupling aromatic protons along with a nitrogen-bearing olefinic proton at δ_H 6.93 (1H, t, J=1.8 Hz, H-13). The above assignments were verified by NOESY correlations between δ_H 3.09 (H-11)/δ_H 6.93 (H-13) and δ_H 7.61 (H-19), respectively. The olefinic proton signal at δ_H 6.93 (H-13) was correlated with a methylene carbon at δ_C 24.7 (C-11), as well as δ_H 4.38 (H-10) and δ_C 112.0 (C-12) from the HMBC spectrum, suggesting that the indole moiety linked at C-11. The structure was further confirmed by ^{13}C-NMR, DEPT, COSY, NOESY (Fig. 2), HSQC, and HMBC (Fig. 3) experiments. Thus, the structure of **2** was determined to be a phenylacetic acid 2-(1*H*-indol-3-yl)ethyl ester, and designated monaspiloindole. Compound **2** was first isolated from a natural source, although it has been mentioned by Yamamoto as a reactant for synthesizing a new class of anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) and anti-vancomycin-resistant enterococci (anti-VRE) agents.[19]

Compound **3** was also isolated as colorless oil. The HR-ESI-MS spectrum gave a molecule ion $[M+Na]^+$ at *m*/*z* 224.3139, consistent with a molecular formula of $C_{13}H_{15}NONa$. UV spectrum showed maximum absorption at 288 nm, indicating the presence of an indole skeleton.[18] Its IR spectrum revealed NH absorption at 3320 cm^{-1}. Analysis of the ^{1}H-NMR spectrum of **3** revealed four typical mutually coupling aromatic protons of indole alkaloid at δ_H 7.11 (1H, td, J=7.6, 1.0 Hz, H-7), 7.17 (1H, td, J=7.6, 1.0 Hz, H-6), 7.33 (1H, dd, J=7.6, 1.0 Hz, H-5), 7.49 (1H, dd, J=7.6, 1.0 Hz, H-8) and one NH group at δ_H 7.66 (1H, br s, exchangeable with D_2O). In addition, the appearance of a set of A_2X_2 pattern signals at δ_H 2.80 (2H, t, J=5.4 Hz, H-4) and 4.05 (2H, t, J=5.4 Hz, H-3), accompanied by a singlet of two methyl groups at δ_H 1.57 (6H, s, CH_3-9, 10). The HMBC correlations (Fig. 3) from δ_H 1.57 (CH_3-9, 10) to δ_C 138.9 (C-1a); δ_H 2.80 (H-4) to δ_C 106.9, 138.9 (C-4a, 1a), and δ_H 4.05 (H-3) to δ_C 106.9 (C-4a) verify the junction of the 1,1-dimethyldihydropyrano ring to the indole moiety at C-1a, and 4a. The other key correlations of HMBC are illustrated in Fig. 3. Based on the above data, the structure of **3**, named monaspyranoindole, was elucidated as 1,1-dimethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole, which was further confirmed by ^{13}C-NMR, COSY, NOESY (Fig. 2), HSQC and HMBC (Fig. 3) experiments. Compound **3** was first isolated from a natural source, though it has since been synthesized.[20]

The other known isolates, β-sitosteryl stearate,[21] a mixture of β-sitosterol and stigmasterol,[22] ergosterol,[23] *p*-hydroxybenzoic acid,[24] methylparaben,[25] *trans*-caffeic acid,[26] linoleic acid,[27] cyclo-(L-Pro-L-Tyr),[28] 5-(hydroxymethyl)furfural,[29] (*Z*)-pulchellalactam,[30] and (4*R*,5*S*)-5-hydroxyhexan-4-olide,[31] were readily identified by comparison of their physico-chemical, spectroscopic, and mass-spectrometric data with the literature. Except for linoleic acid, this is the first time any of the compounds described above have been isolated from *Monascus* spp. The radical-scavenging properties of the fifteen compounds were evaluated against the DPPH radical.[16] By using DPPH as a TLC spray reagent, *p*-hydroxybenzoic acid and *trans*-caffeic acid (5, 10 μg) appeared as strong yellow spots against a purple background, while compound **1** displayed moderate yellow spots, and

Table 1. ^{1}H- and ^{13}C-NMR Data for Compounds **1**—**3** in $CDCl_3$ (400 MHz)

No.	**1**		**2**		**3**	
	δ_C	δ_H (mult; *J*, Hz)	δ_C	δ_H (mult; *J*, Hz)	δ_C	δ_H (mult; *J*, Hz)
1	133.9	—	134.1	—	71.8	—
1a	—	—		—	138.9	—
2	129.3	7.09 (m)	129.3	7.30 (m)		—
3	127.1	7.09 (m)	127.0	7.30 (m)	60.5	4.05 (t, 5.4)
4	128.5	7.09 (m)	128.5	7.30 (m)	22.4	2.80 (t, 5.4)
4a	—	—		—	106.9	—
5	127.1	7.09 (m)	127.0	7.30 (m)	110.8	7.33 (dd, 7.6, 1.0)
5a	—	—		—	127.0	—
6	129.3	7.09 (m)	129.3	7.30 (m)	121.7	7.17 (td, 7.6, 1.0)
7	41.4	3.60 (s)	41.5	3.63 (s)	119.6	7.11 (td, 7.6, 1.0)
8	171.6	—	171.6	—	118.3	7.49 (dd, 7.6, 1.0)
8a	—	—		—	135.7	—
9	—	—		—	27.9	1.57 (s)
10	65.6	4.26 (t, 7.0)	65.0	4.38 (t, 7.0)	27.9	1.57 (s)
11	34.1	2.84 (t, 7.0)	24.7	3.09 (td, 7.0, 1.8)	—	—
12	129.6	—	112.0	—	—	—
13	130.0	7.00 (d, 8.8)	122.0	6.93 (t, 1.8)	—	—
14	115.3	6.72 (d, 8.8)		—	—	—
15	154.2	—	136.1	—	—	—
16	115.3	6.72 (d, 8.8)	111.1	7.36 (br d, 7.8)	—	—
17	130.0	7.00 (d, 8.8)	122.1	7.20 (br t, 7.8)	—	—
18	—	—	119.4	7.13 (br t, 7.8)	—	—
19	—	—	118.8	7.61 (br d, 7.8)	—	—
20	—	—	127.4	—	—	—
OH-15	—	4.90 (br s)		—	—	—
NH	—	—		8.00 (br s)	—	7.66 (br s)

other compounds did not react with the radical. The phenol derivatives *p*-hydroxybenzoic acid and *trans*-caffeic acid were more active than **1** in the above concentration range. The free radical scavenging effects of the isolates (compound **1**, *p*-hydroxybenzoic acid and *trans*-caffeic acid), corresponding to the intensity of quenching of the DPPH radical, were evaluated by spectroscopic assay. At a concentration of 50 μM, the test compounds showed moderate DPPH radical scavenging activity, with 40, 65, and 87% inhibition for compound **1**, *p*-hydroxybenzoic acid and *trans*-caffeic acid, respectively, with *trans*-caffeic acid the most active compound in this study.

In summary, most of recent studies on secondary metabolites from *Monascus* have investigated red mold rice and whole broth. These metabolites are azaphilone,[15] furanoisophthalides,[15] amino acid,[13,15] polyketides,[1] and fatty acids.[1] Nevertheless, the chemical characteristics, as well as the biological activities, of many *Monascus* metabolites still remain unclear. In this study we focus on the secondary metabolites appearing in the mycelia of *Monascus* submerged culture, which has seldom been reported on. The three metabolites **1**, **2** and **3**, phenyl ester, indole, and pyranoindole alkaloid found in this study are new, naturally occurring compounds. Interestingly, this is the first report of an indole alkaloid isolated from this *Monascus* spp. Compound **1**, *p*-hydroxybenzoic acid and *trans*-caffeic acid were found to have significant antioxidant properties, as determined by experiments with 2,2-diphenyl-1-picrylhydrazyl (DPPH), which suggests their ability to efficiently scavenge free radicals. Compounds **1** and **2** have similar structures, but the latter does not show the ability to efficiently scavenge free radicals, which implies that the presence of a phenol group is crucial for activity. The hydrogen donating ability was associated with the radical scavenging effects of antioxidants on the DPPH radical. Structures containing phenolic hydroxyl and carboxylic acid moieties have long been recognized to function as electron or hydrogen donors. Thus, the higher DPPH radical scavenging activity of *p*-hydroxybenzoic acid and *trans*-caffeic acid may be related to the phenolic and carboxylic acid groups present in the molecules. These results suggest that *Monascus* has distinct and diverse metabolites which arise under different fermentation conditions. It may therefore be possible to find more new bioactive natural products by cultivating *Monascus* under different conditions.

Experimental

General All melting points were determined on a Yanaco micro-melting point apparatus and were uncorrected. Optical rotations were measured on a Jasco P-1020 digital polarimeter, UV spectra were obtained on a Jasco UV-240 spectrophotometer in MeOH, and IR spectra (KBr or neat) were taken on a Perkin-Elmer System 2000 FT-IR spectrometer. 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, NOESY, HSQC, HMBC) NMR spectra using $CDCl_3$ and CD_3OD as solvent were recorded on a Varian Unity Plus 400 (400 MHz for ^{1}H-NMR, 100 MHz for ^{13}C-NMR) and Varian INOVA-500 (500 MHz for ^{1}H-NMR, 125 MHz for ^{13}C-NMR) spectrometer. Chemical shifts were internally referenced to the solvent signals in $CDCl_3$ (^{1}H, δ 7.26; ^{13}C, δ 77.0) with Tetramethylsilane (TMS) as the internal standard. Low-resolution ESI-MS spectra were obtained on an API 3000 (Applied Biosystems) and high-resolution ESI-MS spectra on a Bruker Daltonics APEX II 30e spectrometer. Low-resolution EI-MS spectra were recorded on a Quattro GC/MS spectrometer having a direct inlet system. Silica gel (70—230, 230—400 mesh) (Merck) was used for column chromatography, and silica gel 60 F-254 (Merck) was used for TLC and prep. TLC. For radical scavenging TLC autographic assay, DPPH (Sigma) was used as spray reagent.

Microorganism *Monascus pilosus* BCRC 38072 was used throughout this study, and specimens deposited at the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute.

Media The inoculum's medium contained: malt extract, 3 g; yeast extract, 3 g; glucose, 5 g; agar, 1.5 g; and distilled water 1 l. The initial pH of the medium was 8. The synthetic culture medium contained: glucose, 20 g; monosodium L-glutamate (MSG), 10 g; K_2HPO_4, 5 g; KH_2PO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; KCl, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; and $MnSO_4 \cdot H_2O$, 0.003 g per liter of distilled water. The initial pH of the medium was adjusted to 5.5.

Cultivation Methods The slant culture was kept on PDA (potato dextrose agar) Difco. Spores of strains were prepared by growth on PDA slants for 14 d at 28 °C. Spores were washed with sterile water. A suspension of 10^7 spores was used to incubate a 5 l Erlenmeyer flask containing 2 l inoculum medium, which was incubated at 28 °C on a rotary shaker for 3 d. This inoculum was then transferred to a 50 l fermentor (B. Braun, Germany) containing 30 l of synthetic medium, operated at 100 rpm and 30 °C, with an aeration rate of 0.3 vvm. After 14 d of cultivation, the pellet mycelia harvested from the culture broth were used as samples for further extraction.

Extraction and Isolation The dried mycelia of the *M. pilosus* BCRC 38072 (2 kg) were extracted three times with MeOH at room temperature. The methanol syrup extract was partitioned between EtOAc and H_2O (1 : 1) to afford EtOAc (2.5 g) and H_2O (10.2 g) soluble fractions. The EtOAc-soluble fraction (2.5 g) was chromatographed over silica gel (75 g, 70—230 mesh), eluting with *n*-hexane and enriched with EtOAc to produce ten fractions (A1—A10). Fraction A4 (0.56 g) was subjected to a silica gel (18 g) chromatography by eluting with *n*-hexane–EtOAc (100 : 1), enriched with EtOAc, to furnish 20 fractions (A4-1—A4-20). Fraction A4-11 (21.0 mg) was purified by preparative TLC (*n*-hexane–EtOAc, 5 : 1) to give monaspyranoindole (**3**) (1.3 mg), (4*R*,5*S*)-5-hydroxyhexan-4-olide (6.2 mg), and ergosterol (2.9 mg). Fraction A5 (125.5 mg) was subjected to a silica gel (3 g) chromatography by eluting with *n*-hexane–EtOAc (10 : 1), enriched with EtOAc, to furnish 8 fractions (A5-1—A5-8). Fraction A5-4 (7.5 mg) was purified by preparative TLC (*n*-hexane–EtOAc, 15 : 1) to furnish monaspilosin (**1**) (2.0 mg). Fraction A5-5 (12.5 mg) was purified by preparative TLC (*n*-hexane–EtOAc, 5 : 1) to yield β-sitosteryl stearate (4.0 mg), and linoleic acid (9.8 mg). Fraction A6 (214.3 mg), eluting with *n*-hexane–acetone (20 : 1), was further separated using silica gel column chromatography and preparative TLC (*n*-hexane–EtOAc (5 : 1)) and gave monaspiloindole (**2**) (2.7 mg), (*Z*)-pulchellalactam (21.7 mg), and a mixture of β-sitosterol and stigmasterol (8.8 mg). Fraction A7 (312.4 mg) was repeatedly chromatographed over silica gel and purified by preparative TLC to afford *p*-hydroxybenzoic acid (4.2 mg), 5-(hydroxymethyl)furfural (14.1 mg), and methylparaben (2.5 mg). Fraction A9 (290.3 mg) was chromatographed on a silica gel (9 g) column, eluting with $CHCl_3$–EtOAc (20 : 1) to yield 12 fractions (A9-1—A9-12). Fraction A9-1 (26.2 mg) was subjected to further silica gel column chromatography and purified by preparative TLC to afford *trans*-caffeic acid (3.2 mg) and cyclo-(L-Pro-L-Tyr) (4.1 mg).

Monaspilosin (**1**): Colorless oil. ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz): see Table 1. IR (Neat) cm^{-1}: 3403 (OH), 1712 (C=O), 1612, 1514 (aromatic ring C=C stretch). UV λ_{max} (MeOH) nm (log ε): 277 (3.42). HR-ESI-MS *m/z* 279.0997 $[M+Na]^+$ (Calcd for $C_{16}H_{16}O_3Na$, 279.0995). ESI-MS *m/z* 279 $[M+Na]^+$.

Monaspiloindole (**2**): Colorless oil. ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz): see Table 1. IR (Neat) cm^{-1}: 3410 (OH), 1716 (C=O), 1617, 1517 (aromatic ring C=C stretch). UV λ_{max} (MeOH) nm (log ε): 280 (3.74). HR-ESI-MS *m/z* 302.1159 $[M+Na]^+$ (Calcd for $C_{18}H_{17}NO_2Na$, 302.1157). ESI-MS *m/z* 302 $[M+Na]^+$.

Monaspyranoindole (**3**): Colorless oil. ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz): see Table 1. IR (Neat) cm^{-1}: 3320 (NH). UV λ_{max} (MeOH) nm (log ε): 288 (3.60). HR-ESI-MS *m/z* 224.3137 $[M+Na]^+$ (Calcd for $C_{13}H_{15}NONa$, 224.3139). ESI-MS *m/z* 224 $[M+Na]^+$.

Reduction of DPPH Radical After developing and drying, TLC plates were sprayed with a 0.2% DPPH (Aldrich-Sigma) solution in MeOH. The plates were examined 30 min after spraying. Compounds showing a yellow-on-purple spot were regarded as having antioxidant qualities.[16] The intensity of the yellow color depends upon the amount and nature of radical scavenger present in the sample.

Determination of the Scavenging Effect on DPPH Radical The radical scavenging activity of the test compounds was examined with the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, as described previously.[17] α-Tocopherol (=vitamin E) (Sigma) was used as control. Fifty microliters of a solution containing the different compounds (final concentration was 50 μM) to be tested was added to 5 ml of a 1.0×10^{-4} M MeOH solution of DPPH. The reaction mixture was shaken vigorously, and its absorbance at 517 nm was determined after 30 min incubation in a dark area. Decreasing DPPH solution absorbance indicates an increase in DPPH radical-scavenging activity. The DPPH solution, without sample solutions, was used as a control. All tests were run in triplicate and averaged. This activity is given as % DPPH radical-scavenging and is calculated in the equation: % DPPH radical-scavenging=(control absorbance−sample absorbance/control absorbance)×100.

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