Catechin Derivatives from *Parapiptadenia rigida* with *in Vitro* Wound-Healing Properties

Cleber A. Schmidt,[†] Renato Murillo,[‡] Torsten Bruhn,[§] Gerhard Bringmann,[§] Marcia Goettert,[⊥] Berta Heinzmann,[∥] Volker Brecht,[▽] Stefan A. Laufer,[⊥] and Irmgard Merfort*[,†]

Department of Pharmaceutical Biology and Biotechnology, Albert-Ludwigs-Universität, 79104 Freiburg, Germany, Escuela de Quimica and CIPRONA, Universidad de Costa Rica, 2060 San José, Costa Rica, Institute of Organic Chemistry, Julius-Maximilians-Universität, D-97074 Würzburg, Germany, Institute of Pharmacy, Eberhard-Karls-Universität, D-72076 Tübingen, Germany, Department of Pharmaceutical Industry, Universidade Federal de Santa Maria, 97015-900 Santa Maria, Brazil, and Department of Pharmaceutical and Medicinal Chemistry, Albert-Ludwigs-Universität, 79104 Freiburg, Germany

Received July 29, 2010

Analysis of the ethanolic extract of the bark from *Parapiptadenia rigida* resulted in the isolation of the new catechin derivatives 4′,3″-di-*O*-methylapocynin-D (**10**), 4′,3″-di-*O*-methylapocynin-B (**11**), epigallocatechin-3-*O*-ferulate (**8**), and 4′-*O*-methylepigallocatechin-3-*O*-ferulate (**9**) and the catechins 4′-*O*-methylepigallocatechin-3-*O*-gallate (**6**) and 4′-*O*-methylepicatechin-3-*O*-gallate (**7**). These compounds, isolated for the first time from a natural source, are accompanied by the five known catechins 4′-*O*-methylgallocatechin (**1**), 4′-*O*-methylepigallocatechin (**2**), 3′-*O*-methylepicatechin (**3**), epigallocatechin-3-*O*-gallate (**4**), and epicatechin-3-*O*-gallate (**5**). Compounds **5** and **7** displayed promising wound-healing effects in a scratch assay. Some of the catechin derivatives showed inhibitory effects on NF-κB DNA binding and p38α MAPK activity.

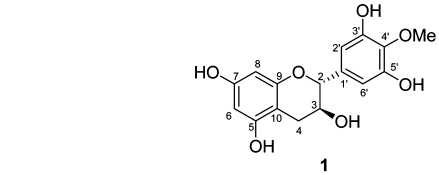
Parapiptadenia rigida (Benth.) Brenan (Fabaceae), popularly known in Brazil as "Angico-vermelho" (red Angico), is a perennial shrub or tree native to South America.[1,2] Preparations from its bark are used in traditional medicine because of its anti-inflammatory, astringent, expectorant, antidiarrheic, antihemorrhagic, antimicrobial, and wound-healing properties.[3−5] To date, the bark has been known for its content of about 15% tannins, but a detailed analysis has not been undertaken.[2] Bioguided fractionation of the ethanolic extract led to the isolation of five known compounds (**1**−**5**)[6−11] and six new *O*-methylated catechin derivatives (**6**−**11**). The isolated compounds, except for **3**, **9**, and **10**, as well as the ethanolic extract of *P. rigida* were studied in cell-based assays for their wound-healing properties using a scratch assay. Additionally, their anti-inflammatory activity was investigated in the NF-κB electrophoretic mobility shift (EMSA) and p38 MAPK assays.

Results and Discussion

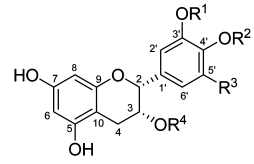
Fractionation of the ethanolic bark extract from *P. rigida* afforded five known catechin derivatives. On the basis of 1D and 2D NMR (^{1}H, ^{13}C, COSY, HSQC, HMBC), as well as MS (ESI, EI, CI) analyses the compounds were identified as 4′-*O*-methylgallocatechin (**1**),[6] 4′-*O*-methylepigallocatechin (**2**),[7] 3′-*O*-methylepicatechin (**3**),[8] epigallocatechin-3-*O*-gallate (**4**),[9,10] and epicatechin-3-*O*-gallate (**5**).[11] The diastereomeric character of **1** and **2** was confirmed by differences observed for H-2 as well as C-2 and C-3 in the ^{1}H and ^{13}C NMR spectra. Compound **1** (2,3-*trans*) showed a doublet for H-2 (δ_H 4.58) and signals for C-2 at δ_C 82.6 and for C-3 at δ_C 68.7, while **2** exhibited a broad singlet (δ_H 4.81) for H-2 and upfield shifts for C-2 (δ_C 79.7) and C-3 (δ_C 67.4) characteristic of 2,3-*cis*-flavan-3-ols.[12,13]

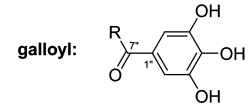
The 1D and 2D NMR data (^{1}H, ^{13}C, COSY, HSQC, HMBC), the MS (ESI, CI, HRESI), and optical rotation data of **6** were found to be identical with those of 4′-*O*-methylepigallocatechin-3-*O*-gallate.[14,15] The ^{1}H NMR data of **7** agreed well with those reported for 4′-*O*-methylepicatechin-3-*O*-gallate.[16] However, its ^{13}C NMR and HRESI are presented here for the first time. Compounds **6** and **7** have been reported as products from chemical synthesis (**6**)[15,17] and/or as a biliary metabolite in rats (**6**, **7**),[14,16] but not as native natural products.

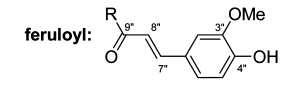
The molecular formula of **8**, which was obtained as a brownish amorphous powder, was deduced as $C_{25}H_{23}O_{10}$ from the HRESIMS ion at *m*/*z* 483.1286 $[M + H]^+$. ^{1}H and ^{13}C NMR data revealed the presence of a flavan-3-ol derivative with a 3,5,7,3′,4′,5′-hexasubstitution pattern (Table 1). The 2,3-*cis* configuration was confirmed



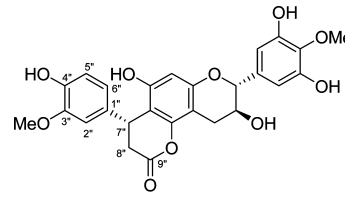
1



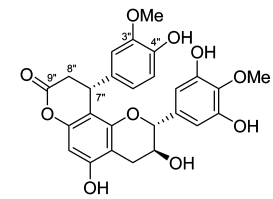




2: R^1 = H R^2 = CH_3 R^3 = OH R^4 = H
3: R^1 = CH_3 R^2 = H R^3 = H R^4 = H
4: R^1 = H R^2 = H R^3 = OH R^4 = galloyl
5: R^1 = H R^2 = H R^3 = H R^4 = galloyl
6: R^1 = H R^2 = CH_3 R^3 = OH R^4 = galloyl
7: R^1 = H R^2 = CH_3 R^3 = H R^4 = galloyl
8: R^1 = H R^2 = H R^3 = OH R^4 = feruloyl
9: R^1 = H R^2 = CH_3 R^3 = OH R^4 = feruloyl



10



11

* To whom correspondence should be addressed. Phone: +49 0761 203 8373. Fax: +49 0761 203 8383. E-mail: irmgard.merfort@pharmazie.uni-freiburg.de.

[†] Department of Pharmaceutical Biology and Biotechnology, Albert-Ludwigs-Universität.

[‡] Universidad de Costa Rica.

[§] Julius-Maximilians-Universität.

[⊥] Eberhard-Karls-Universität.

[∥] Universidade Federal de Santa Maria.

[▽] Department of Pharmaceutical and Medicinal Chemistry, Albert-Ludwigs-Universität.

10.1021/np100523s
Published on Web 11/16/2010

Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectroscopic Data for Compounds **8** and **9** in Methanol-d_4 (δ in ppm, J in Hz)

	8			**9**		
position	δ_C, mult.	δ_H (J in Hz)	HMBC[a]	δ_C, mult.	δ_H (J in Hz)	HMBC[a]
2	78.5 d	4.95, brs	3, 9, 2′, 6′, 1′	76.8 d	4.99, brs	3, 2′, 6′, 1′
3	69.8 d	5.49, m		68.4 d	5.5, m	
4	26.7 t	β 2.85, dd (2.4, 17.4)	2, 3, 10	25.3 t	β 2.86, dd (2.5, 17.3)	2, 3, 5, 9, 10
		α 2.99, dd (4.5, 17.3)	9, 10		α 3.0, dd (4.6, 17.3)	9, 10
5	157.9 s			156.5 s		
6	96.5 d	5.99, d (2.3)	5, 7, 8, 10	95.2 d	5.99, d (2.3)	5, 7, 8, 10
7	157.9 s			156.5 s		
8	95.8 d	5.97, d (2.3)	6, 7, 9, 10	94.5 d	5.98, d (2.3)	6, 7, 9, 10
9	157.1 s			155.6 s		
10	99.3 s			97.9 s		
1′	130.7 s			134.4 s		
2′	106.8 d	6.54, s	2, 1′, 3′, 4′, 6′	105.7 d	6.56, s	2, 1′, 3′, 4′, 6′
3′	146.7 s			150.0 s		
4′	133.8 s			134.9 s		
5′	146.7 s			150.0 s		
6′	106.8 d	6.54, s	2, 1′, 2′, 4′, 5′	105.7 d	6.56, s	2, 1′, 2′, 4′, 5′
1″	127.9 s			129.9 s		
2″	111.7 d	7.15, d (1.8)	3″,4″, 6″, 7″	110.3 d	7.17, d (1.85)	3″, 4″, 6″, 7″
3″	149.3 s			147.9 s		
4″	150.5 s			149.1 s		
5″	116.3 d	6.79, d (8.2)	1″, 3″, 4″	114.9 d	6.79, d (8.20)	1″, 3″, 4″
6″	124.2 d	7.04, dd (1.8, 8.2)	4″, 5″, 7″	122.9 d	7.04, dd (1.85, 8.20)	2″, 4″, 5″, 7″
7″	147.1 d	7.51, d (15.9)	2″, 6″, 8″, 9″	145.8 d	7.50, d (15.85)	2″, 6″, 8″, 9″
8″	115.5 d	6.30, d (15.9)	1″, 9″	114.0 d	6.3, d (15.85)	1″, 9″
9″	168.6 s			167.2 s		
OMe-4′				59.5 q	3.80, s	4′
OMe-3″	56.4 q	3.87, s	3″	55.1 q	3.88, s	3″

[a] HMBC correlations are from proton(s) stated to the indicated carbon.

by the presence of a broad singlet at δ_H 4.95 for H-2 and by the upfield shift (δ_C 78.5) of C-2. NMR and optical rotation data were similar to those reported for epigallocatechin.[18,19] However, the H-3 multiplet showed a strong downfield shift (δ_H 5.49) suggesting a substitution with an acyl moiety at C-3. Additional aromatic ABX-type set signals at δ_H 7.15 (H-2″, d, J = 1.8 Hz), 7.04 (H-6″, dd, J = 1.8, 8.2 Hz), and 6.79 (H-5″, d, J = 8.2 Hz) together with mutually coupled *trans*-olefinic signals at δ_H 6.30 (d, J = 15.9 Hz) and 7.51 (d, J = 15.9 Hz) were observed. A long-range correlation between the methoxy group at δ_H 3.87 (s) and C-3″ (δ_C 149.3) revealed the occurrence of a ferulate moiety. Its C-3 location was confirmed by a long-range correlation between the carboxylic carbon at δ_C 168.6 and H-3. Thus, **8** was identified as a new compound, epigallocatechin-3-*O*-ferulate.

The molecular formula of **9** was calculated as $C_{26}H_{24}O_{10}$ from the HRESIMS measurements, which exhibited an $[M + H]^+$ ion at m/z 497.1442. The ^{1}H and ^{13}C NMR spectra were similar to those of **8** except for the presence of an additional methoxy group, which was located at C-4′ (Table 1) due to a long-range correlation between this carbon and the methoxy group. Hence, **9** was identified as the new 4′-*O*-methylepigallocatechin-3-*O*-ferulate. Whereas the 3-*O*-caffeate, 3-*O*-coumarate, and 3-*O*-cinnamate of epigallocatechin had been isolated from leaves of *Camellia sinensis*,[20] the occurrence of a 3-*O*-feruloylcatechin derivative is reported here for the first time. In general, esterification at C-3 in catechins has rarely been found.

The molecular formula of compound **10**, $C_{26}H_{24}O_{10}$, was concluded from its HREIMS, which showed an $[M]^+$ ion at m/z 496.1377. Analysis of the ^{1}H and the ^{13}C NMR spectra confirmed **10** to be a gallocatechin derivative, *O*-methylated at C-4′ (see Table 2). The 2,3-*trans* configuration was established by the doublet of H-2 at δ_H 4.76 and the coupling constant ($J_{2,3}$ = 6.2 Hz) together with the chemical shifts of C-2 and C-3 at δ_C 81.1 and 66.4, respectively (Table 2). A trisubstitution of the A ring was deduced from the occurrence of a one-proton aromatic singlet at δ_H 6.28, indicating a substituent either at C-6 or at C-8. Resonances attributable to mutually coupled benzylic methine (δ_H 4.55, brd) and methylene signals (δ_H 2.96, dd, J = 2, 15.6; 3.08, dd, J = 6.8, 15.6) together with an aromatic 4″-hydroxy-3″-methoxy substitution pattern (δ_H 6.55, dd; 6.71, d; 6.78, d; and 3.80, s) were also observed. The additional occurrence of a carboxylic carbon signal at δ_C 168.9 suggested the presence of a phenylpropanoid-substituted 4′-*O*-methylgallocatechin. A long-range correlation between the benzylic methine (C-7″) and the *ortho*-protons of the disubstituted aromatic ring system (H-2″ and H-6″) confirmed the occurrence of a phenyldihydro-2(*3H*)-pyranone system. The ^{13}C NMR chemical shift of the A-ring methine at δ_C 98.3 was in accordance with those of C-6 phenylpropanoid-substituted rather than those with C-8 phenylpropanoid-substituted catechins and epicatechins.[21−23] In the latter case the C-6 chemical shift is more upfield and the C-10 shift more downfield. Additional confirmation for a C-6 substitution was obtained from long-range correlations between the methine proton (H-7″) at δ_H 4.55 and C-5, C-6, and C-7 at δ_C 150.5, 105.8, and 153.4, respectively (Table 2). The signal for H-8 was unequivocally assigned at δ_H 6.28 because of the observed long-range correlation with C-9 at δ_C 154.1. The C-9 chemical shift was proven by a long-range correlation with H-2 at δ_H 4.76. Thus, compound **10** was very similar to apocynin D isolated from leaves of *Apocynum venetum*, but with additional *O*-methyl groups.[21] Finally, the configuration of C-7″ was determined by comparing CD data with those of apocynin D[21] and a phenylpropanoid-substituted catechin isolated from *Castanopsis hystrix*, which differed from apocynin D by the missing hydroxy group at C-5′.[22] CD data (positive Cotton effects (CEs) at 233 and 291 nm and a negative CE at 253 nm) agreed well with a 2*R*,3*S*,7″*R* configuration, the 7″*R* absolute configuration also evident for the two above-mentioned flavan-3-ols. According to the structural similarities, the new compound **10** was named 4′,3″-di-*O*-methylapocynin-D.

The ESIMS of compound **11** in the positive and negative modes showed quasimolecular ions at m/z 497 $[M + H]^+$ and 495 $[M - H]^-$, respectively, consistent with the molecular formula $C_{26}H_{24}O_{10}$. This was confirmed by its HREIMS, which showed an $[M]^+$ ion at m/z 496.1370. Detailed ^{1}H, ^{13}C, and HMBC spectra suggested **11** to be a gallocatechin derivative similar to **10**, but with differences

Table 2. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Spectroscopic Data of Compounds **10** and **11** in Methanol-d_4 (δ in ppm, J in Hz)

	10			**11**		
position	δ_C, mult.	δ_H (J in Hz)	HMBC[a]	δ_C, mult.	δ_H (J in Hz)	HMBC[a]
2	81.1 d	4.76, d (6.2)	3, 4, 9, 2′, 6′, 1′	83.1 d	4.66, d (7.0)	3, 4, 9, 2′, 6′, 1′
3	66.4 d	4.12, ddd (4.6, 6.2, 6.7)		68.2 d	3.97, ddd (7.0, 7.6, 5.2)	
4	25.5 t	β 2.66, dd (6.7, 16.5)	3, 9, 10	28.3 t	β 2.64, dd (7.6, 16.5)	3, 10
		α 2.90, dd (4.6, 16.5)	2, 3, 9, 10		α 2.89, dd (5.2, 16.5)	2, 3, 5, 9, 10
5	150.5 s			156.9 s		
6	105.8 s			96.4 d	6.24, s	5, 7, 8, 10
7	153.4 s			152.1 s		
8	98.3 d	6.28, s	6, 7, 9, 10	105.8 s		
9	154.1 s			152.9 s		
10	98.9 s			106.0 s		
1′	134.9 s			136.1 s		
2′	105.5 d	6.42, s	2, 1′, 3′, 4′, 6′	107.4 d	6.27, s	2, 1′, 3′, 4′, 6′
3′	150.3 s			151.5 s		
4′	134.9 s			136.5 s		
5′	150.3 s			151.5 s		
6′	105.5 d	6.42, s	2, 1′, 2′, 4′, 5′	107.4 d	6.27, s	2, 1′, 2′, 4′, 5′
1″	133.4 s			135.3 s		
2″	110.3 d	6.78, d (2.1)	3″, 4″, 6″, 7″	111.9 d	6.74, d (1.6)	1″, 3″, 4″, 6″, 7″
3″	147.6 s			149.0 s		
4″	145.0 s			146.3 s		
5″	114.8 d	6.71, d (8.1)	1″, 3″	116.3 d	6.71, d (8.2)	1″, 3″
6″	118.6 d	6.55, dd (8.1, 2.1)	2″, 4″, 7″	119.9 d	6.53, dd (1.6, 8.2)	2″, 4″, 7″
7″	33.9 d	4.55, brd (2, 6.8)	5, 6, 7, 1″, 2″, 6″, 8″, 9″	35.5 d	4.45, brd (7.1)	8, 7, 9, 1″, 2″, 6″, 8″, 9″
8″	36.7 t	2.96, dd (2, 15.6)	1″, 7″, 9″	38.3 t	2.85, brd (15.9)	8, 9″
		3.08, dd (6.8, 15.6)	6, 9″		3.04, dd (7.1, 15.9)	1″, 7″, 9″
9″	168.9 s			170.8 s		
OMe-4′	59.3 q	3.82, s	4′	60.7 t	3.79, s	4′
OMe-3″	54.8 q	3.80, s	3″	56.3 t	3.75, s	3″

[a] HMBC correlations are from proton(s) stated to the indicated carbon.

in the chemical shifts for C-5, -6, -8, and -10. Thus, the signal for C-6 was shifted upfield to δ_C 96.4, whereas those for C-5 (δ_C 156.9), C-8 (δ_C 105.8), and C-10 (δ_C 106) exhibited downfield shifts compared to **10** (Table 2), suggesting the pyranone ring was bound to C-8 and 7-OH of the flavan A ring. The signal for H-6 was unambiguously assigned to the chemical shift at δ_H 6.24, confirmed by the long-range correlation with C-5 at δ_C 156.9. Additionally, the chemical shift of C-5 was proven by a long-range correlation with H-4α at δ_H 2.89. Hence, compound **11** was the dimethyl ether derivative of either apocynin-A or -B.[21] To define the absolute configuration of **11** at C-7″, a circular dichroism investigation was undertaken. Taking into account that the relative configuration at C-2 versus C-3 was elucidated as *trans* by the NMR measurements, only four possible diastereomers (2*R*,3*S*,7″*R*, 2*S*,3*R*,7″*S*, 2*R*,3*S*,7″*S*, and 2*S*,3*R*,7″*R*) had to be considered. Comparison of the quantum chemically predicted CD spectra of these possible diastereomers with the experimental CD curve showed that only the spectrum computed for the 2*R*,3*S*,7″*R* configuration had the same CEs measured for **11**, with positive CEs at 230 and 286 nm and a negative CE around 250 nm (Figure 1, Figure 3S in the Supporting Information). Hence, the absolute configuration at C-7″ was determined to be *R*. The measured Cotton effects also agreed with those of apocynin B, which has a similar structure, but lacking the *O*-methyl groups.[21] Therefore, **11** was named 4′,3″-di-*O*-methyl-apocynin-B, reported here for the first time.

All isolated compounds could be assigned to peaks in the HPLC chromatogram obtained from the ethanolic extract of the bark from *P. rigida* (Figure 2). Quantification of the main phenolics was performed using a calibration curve with the respective isolated compound. 4′-*O*-Methylgallocatechin (**1**) was the main phenolic compound in the ethanolic extract (Figure 2).

As preparations from *P. rigida* are also used in traditional medicine, the ethanolic extract and some of the main compounds, i.e., **1**, **2**, **4**, **5**, **6**, **7**, **8**, and **11**, were studied for their wound-healing effects in a scratch assay and their anti-inflammatory activity in the NF-κB EMSA and p38 MAPK assays.

The wound-healing properties of *P. rigida* were investigated by measuring the influence on fibroblast migration to and proliferation

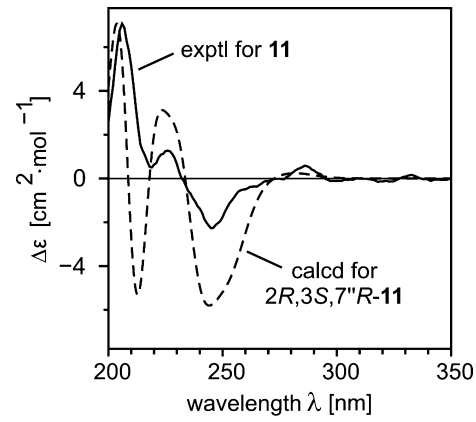


Figure 1. Calculated and experimental CD curves of 2*R*,3*S*,7″*R*-**11**.

into an artificial wounded monolayer using the scratch assay model and Swiss 3T3 mouse fibroblasts.[24] The majority of samples showed enhanced cell numbers when concentrations of 1 and 10 μM were studied, whereas 20 μM concentrations led mostly to a reduction (see Figure 3, compounds **1**, **4**, **5**, **6**, **7**, and **11**). Compounds **5** and **7** were the most active ones at a 1 μM concentration, but showed reduced activity at a concentration of 10 μM. This decrease in activity may be explained by the possible antiproliferative effect at higher concentrations, as observed for **4** in keloid fibroblasts. Studies provided evidence that the antiproliferative effect may be due to inhibition of the STAT3-signaling pathway.[25] Moreover, in rat aortic smooth muscle cells the same compound was shown to possess antiproliferative as well as antimigrating effects, which is supposed to be partly Ras/JNK mediated.[26] The ethanolic extract increased cell numbers at 10 μg/mL to about 40%, whereas 50 μg/mL showed an inhibitory effect. PDGF was used as a positive control, where 2 ng/mL showed an average stimulating effect of 59.5%.

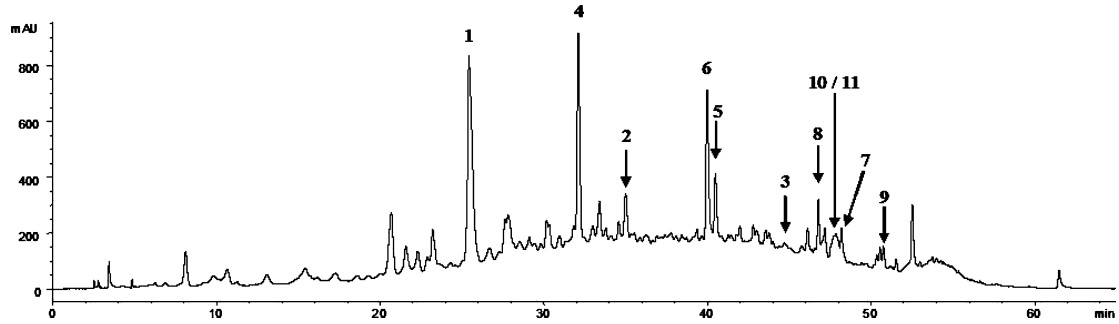


Figure 2. HPLC chromatogram of the ethanolic extract from *P. rigida* at 10 mg extract/mL MeOH 30% (HPLC conditions: see Experimental Section). Concentrations of the main compounds in the extract: **1**: 8.04%, **2**: 1.54%, **4**: 1.94%, **5**: 1.22%, **6**: 1.04%, **7**: 0.62%, **8**: 1.25%.

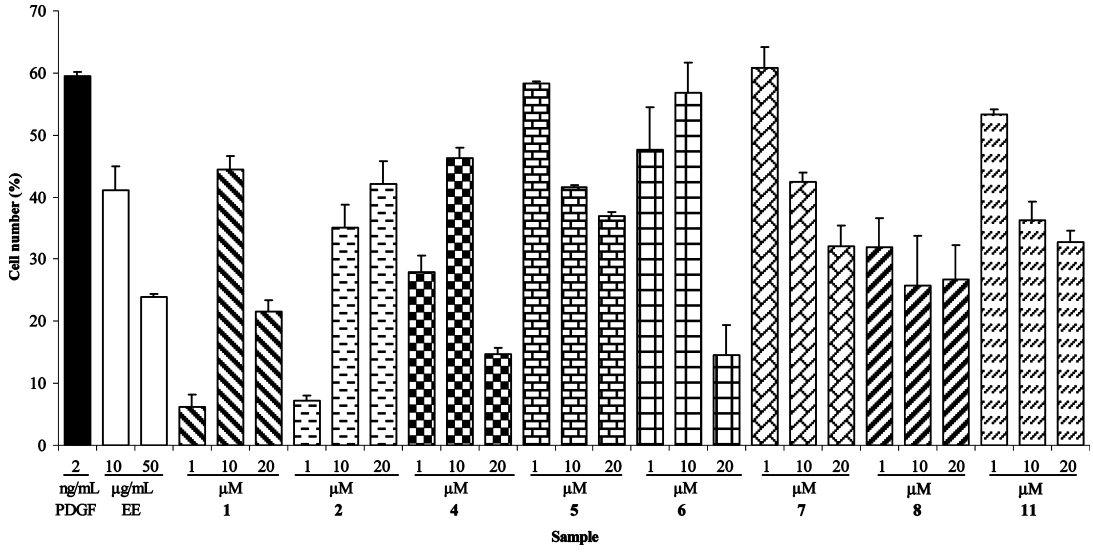


Figure 3. Effect of an ethanolic extract from the bark of *P. rigida* and isolated catechin derivatives (**1**, **2**, **4**, **5**−**8**, **11**) on the migratory and proliferative activities of 3T3 Swiss fibroblasts in the scratch assay after 12 h of incubation (37 °C; 5% CO_2). Positive control: PDGF (2 ng/mL); EtOH extract (EE) of *P. rigida* (10 and 50 μg/mL); and isolated compounds at 1, 10, and 20 μM, in parentheses concentration in μg/mL: **1** [4′-*O*-methylgallocatechin (0.3; 3.2 and 6.4 μg/mL)]; **2** [4′-*O*-methylepigallocatechin (0.32; 3.2 and 6.4 μg/mL)]; **4** [epigallocatechin-3-*O*-gallate (0.4; 4.5 and 9.2 μg/mL)]; **5** [epicatechin-3-*O*-gallate (0.4; 4.4; 8.8 μg/mL)]; **6** [4′-*O*-methylepigallocatechin-3-*O*-gallate (0.5; 4.7 and 9.4 μg/mL)]; **7** [4′-*O*-methylepicatechin-3-*O*-gallate (0.4; 4.6; 9.1 μg/mL)]; **8** [epigallocatechin-3-*O*-ferulate (0.5; 4.8 and 9.6 μg/mL)]; **11** [4′,3″-di-*O*-methylapocynin-B (0.5; 5.0 and 9.9 μg/mL)]. Data are expressed as % of cells that migrate and proliferate to the wounded area compared to the blank control. Bars represent means ± SEM of at least three experiments.

The complex pathophysiological process of wound-healing is an interplay of several cellular and biochemical processes, including the interaction of re-epithelialization, angiogenesis, granulation tissue formation, and collagen deposition, but also of inflammation, especially initially.[27] As NF-κB is a central protein regulating the transcription of many inflammatory and proinflammatory cytokines and enzymes, the influence of the crude ethanolic extract and some isolated compounds was evaluated for their effect on TNF-α-induced NF-κB activation in Jurkat cells by the electrophoretic mobility shift assay (EMSA). Except for **11**, all compounds only moderately impaired TNF-α-induced NF-κB after 24 h of incubation (see Figure 1S in the Supporting Information). In general, concentrations of about 50 μM were necessary to induce 50% inhibition. Compound **11** inhibited NF-κB at 10 and 20 μM concentrations, whereas higher concentrations led to a decrease of NF-κB inhibition (see Figure 4). Cytotoxicity studied in an MTT assay was always below 11% (see Figure 2S in the Supporting Information). The ethanolic extract (100 μg/mL) itself exhibited an NF-κB inhibition of about 40%, but this concentration also showed a cytotoxicity of 50%. Therefore, NF-κB inhibition is most likely influenced by cytotoxic effects. Inhibition of NF-κB DNA binding was already shown for epicatechin and catechin as well as for a dimeric procyanidin in Jurkat cells.[28] However, the inhibitory effects seem to be rather unspecific, as these compounds targeted the NF-κB pathway at multiple steps. Epicatechin also impairs NF-κB DNA binding in Hodgkin's lymphoma cell lines at concentrations similar to those used in our study. This inhibition was not associated with its antioxidant activity, changes in p65 phosphorylation, or NF-κB nuclear translocation, but was probably due to inhibition of NF-κB DNA binding. Further results on cell viability do not exclude the influence of cytotoxic effects.[29] Thus, as yet NF-κB inhibition of catechin derivatives should not be overestimated.

The serine/threonine kinase p38α MAPK is also known to play a central role in proinflammatory responses through its involvement in the biosynthesis of proinflammatory cytokines, such as IL-1β and TNF-α.[30,31] Therefore, compounds **2**, **4**, **5**, and **11** were investigated for their inhibitory effects on p38α MAPK in an ELISA providing the following IC_{50} values: **2**, 39.16 ± 3.92 μM; **4**, 2.21 ± 0.48 μM; **5**, 1.47 ± 0.36 μM; and **11**, 50.80 ± 4.81 μM. Our results confirm previous reports on p38α MAPK inhibition by **4** and **5**[32−34] and increase our knowledge on structural features that contribute to the inhibitory activity. Thus, trihydroxylation of the B ring and esterfication by gallic acid increased inhibition of p38α MAPK. Interestingly, introduction of an *O*-methyl group even led to a slightly increased inhibition. Hence, the observed anti-inflammatory effects of preparations from *P. rigida* observed in traditional medicine may

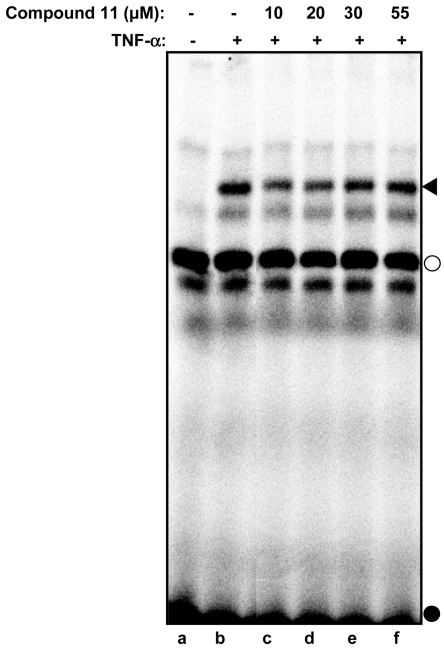


Figure 4. NF-κB EMSA of nuclear extracts prepared from Jurkat cells. *Lane a*: unstimulated control cells. *Lane b*: cells treated with 2.5 ng/mL rh-TNF-α. *Lanes c*−*f*: cells pretreated for 24 h with 10, 20, 30, and 55 μM compound **11** and subsequently stimulated for 1 h with 2.5 ng/mL rh-TNF-α. ▲: NF-κB DNA complexes. ○: Nonspecific binding to the probe. ●: Unbound oligonucleotide.

partly be explained by their influence on NF-κB and phosphorylation of p38α MAPK.

In summary, this is the first phytochemical study of the bark of *P. rigida*. We demonstrate that catechin derivatives may have beneficial properties on the combinatorial effect during inflammatory and re-epithelialization phases of the wound-repair process. However, studies should also be performed to confirm these effects on re-epithelialization *in vivo*. Recently, it has been demonstrated that compound **4** showed a significant improvement in scar formation in rats in terms of both collagen orientation and maturity during the wound-healing process.[35]

Experimental Section

General Experimental Procedures. Optical rotations were measured in MeOH at 20 °C on a Perkin-Elmer polarimeter, model 341. CD spectra were measured on a Jasco J-715 spectropolarimeter, at 190−600 nm in MeOH. Infrared spectra were recorded on a Perkin-Elmer Spectrun One FT-IR spectrometer with ATR sampling. NMR spectra were recorded in methanol-d_4 on a Bruker DRX 400 MHz instrument at 400 MHz (^{1}H) and 100 MHz (^{13}C). MS data were taken with the following instruments: EI-MS, TSQ 700 mass spectrometer (Thermo Fisher, Waltham, U.S.); ESI-MS, LCQ-Advantage mass spectrometer (Thermo Fisher); HR-ESIMS, LTQ Orbitrap XL mass spectrometer (Thermo Fisher); HR-EIMS, MAT-95XL double-focusing magnetic field mass spectrometer (Thermo Fisher). MPLC was carried out with Eurosil Bioselect 100, C-18 (20−45 μm) and open-column chromatography with Sephadex LH-20. Column fractions were monitored by TLC (silica gel 60 F 254, Merck), and detection was done at 254 and 366 nm and with anisaldehyde−H_2SO_4 and heating at 110 °C. Analytical TLC was carried out with an Automatic TLC sampler (CAMAG). HPLC analysis was performed on a Hewlett-Packard 1090 apparatus, using a Phenomenex Luna C-18 column (150 × 4.6 mm, 3 μm), with mobile phases A (H_2O−MeCN, 95:5) and B (MeOH−H_2O, 95:5), both with 0.1% HCOOH: linear gradient starting with 5% of B, increasing to 30% at 30 min, 50% at 45 min, and 100% from 50 to 55 min; re-equilibration of the column from 56 to 65 min, flow rate 0.5 mL/min, detection at λ 275 nm; sample injection of 20 μL.

Plant Material. The bark from *Parapiptadenia rigida* (Benth.) Brenan was collected from a natural habitat of the plants located on "Morro Cechela" in Santa Maria, Rio Grande do Sul, Brazil, in October 2007, and identified by Dr. Solon Jonas Longhi, Federal University of Santa Maria. A voucher specimen was deposited at the herbarium of the University, code SMDB 12309.

Extraction and Isolation. Air-dried and powdered bark (1.3 kg) was extracted with EtOH using a Soxhlet apparatus. The crude EtOH extract was concentrated under vacuum at 40 °C to yield 230.2 g of extract, which was treated with MeOH at −20 °C, giving a soluble part of 221.4 g after solvent removal. Initial fractionation of 6 g of the EtOH extract was carried out using open-column liquid chromatography on Sephadex LH-20 (60 × 6 cm) with MeOH and yielded 14 fractions. Fractions 5a (154 mL), 9a (209 mL), 10a (341 mL), and 11a (215 mL) were dried and subjected to additional MPLC on RP-18 silica gel (50 × 1.2 cm). Fraction 5a (586 mg) was chromatographed using mixtures of MeOH−H_2O (30−100%, increase of 10% MeOH per 30 min) and a flow rate of 0.8 mL/min, yielding 18 subfractions, from which fractions 3 (52 mL) and 4 (39 mL) gave **1** (254 mg). Fractions 7 (42 mL) and 8 (28 mL), 11 (31 mL) and 12 (14 mL), and 13 (18 mL) and 14 (17 mL) were combined, dried, and rechromatographed under the same conditions to give compounds **2** (fr. 2: 21 mL = 14.1 mg), **3** (fr. 2: 10.5 mL = 2 mg), and **11** (fr. 5: 19 mL = 2.8 mg), respectively. Subfraction 3 (10.5 mL = 2 mg), which mainly contained **10**, was purified by analytical HPLC (conditions: see General Experimental Procedures) and gave **10** (1.3 mg). Fraction 9a (301 mg) was fractionated using a flow rate of 0.7 mL/min of MeOH−H_2O (15−45% MeOH and increase of 5% per 20 min, 45−55% MeOH in 60 min, 55−100% MeOH and increase of 10% per 20 min) and afforded **6** (fr. 2: 31.5 mL = 22.6 mg), **7** (fr. 4: 52.5 mL = 16.14 mg), and **9** (fr. 5: 42 mL = 6.63 mg). Compound **5** (fr. 4: 52.5 mL = 18.78 mg) was isolated from fraction 10a (358 mg) with MeOH−H_2O starting from 20% to 70% (increase of 5% MeOH per 30 min), at 0.7 mL/min flow rate. Fraction 11a was subfractionated starting with H_2O−MeOH:MeCN (80:15:5) as the mobile phase and a flow rate of 0.8 mL/min, changing to 60:30:10 after 60 min. Nine subfractions were obtained, from which 5 (17.5 mL) and 7 (73.5 mL) gave **4** (24.2 mg) and **8** (18.4 mg), respectively.

Cell Culture. Jurkat T cells (ACC No 282) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco-BRL).

Swiss 3T3 albino mouse fibroblasts (Cell Line Service, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin and maintained at 37 °C in a humidified, 5% CO_2 environment (Gibco-BRL).

NF-κB Electrophoretic Mobility Shift Assay. Jurkat T cells (3 × 10^5 cells/mL) were preincubated with the crude extract (50, 100 μg/mL) and the isolated compounds (10, 20, 30, 55 μM) for 24 h and subsequently stimulated for 1 h with rh-TNF-α at 2.5 ng/mL (R&D Systems). Nuclear cell extracts were prepared as previously described.[36] NF-κB oligonucleotide (Promega) was labeled using [γ-^{33}P] dATP (3000 Ci/mmol; Amersham). The specificity of the NF-κB-DNA binding was assessed by competition with a 100-fold molar excess of unlabeled oligonucleotide containing the consensus sequence for NF-κB. The bands were quantified densitometrically using a PhosphoImager scan.

Scratch Assay. Wound-healing properties were evaluated *in vitro* using Swiss 3T3 albino mouse fibroblasts and the scratch assay as previously described.[24,37] The crude extract was tested at 10 and 50 μg/mL and the isolated compounds at 1, 10, and 20 μM concentrations. Platelet-derived growth factor (PDGF) at 2 ng/mL was used as a positive control. A negative control containing only cells and 4 μL of DMSO was used as a reference to calculate the percentual rate of the increase in cell number for each sample after 12 h incubation. The experiments were performed, at least, in triplicate. The data were analyzed using CellC software.[38]

P38α MAPK Assay. Experiments were carried out as described in the literature.[30,37] Detailed information is given in the Supporting Information.

MTT Assay. Cytotoxic activity was studied using the MTT colorimetric assay as previously described and modified to a 96-well plate.[39] Detailed information is given in the Supporting Information.

Computational Methods. Conformational analyses of the four possible diastereomers of compound **11** were performed with Gaussian03[40] using the B3LYP/6-31G*[41] method. Subsequent single-point energy calculations using RI-SCS-MP2/TZVP[42] yielded two

energetically relevant conformers for the 2*R*,3*S*,7″*R* and the 2*S*,3*R*,7″*S* diastereomers and only one each for the 2*R*,3*S*,7″*S* and the 2*S*,3*R*,7″*R* configurations. For the TDB2PLYP/SV(P)[43] computations, COSMO[44] (methanol) was used to include solvent effects. Single-point energy and TD calculations were performed with ORCA.[45] The single CD spectra predicted for the 2*R*,3*S*,7″*R* and the 2*S*,3*R*,7″*S* configurations were added up with Boltzmann weighting[46] to give the calculated overall CD spectrum (using the energies of the RI-SCS-MP2/TZVP//B3LYP/6-31G* calculations). Boltzmann weighting, Gauss curve generation (using a sigma value of 0.24 eV), and application of a UV shift[46] (24 nm) were done with SpecDis.[47]

Statistical Analysis. Statistical analyses were carried out using the Origin Scientific Graphing and Analysis Software, version 7.0, or Microsoft Office Excel 2007. Data are expressed as the mean ± SEM. Significant differences between the treated groups and the control were determined by the one-way ANOVA, at a level of $P < 0.01$.

4′-*O*-Methylepigallocatechin-3-*O*-gallate (6): brownish, amorphous powder; $[\alpha]^{20}_D$ −122 (*c* 2.0, MeOH); IR (neat) ν_{max} 3351, 1687, 1604, 1514, 1447, 1311, 1193, 1141, 1097, 1031, 820, 765, 716 cm^{-1}; HRESIMS *m*/*z* 473.1072 (calcd for $C_{23}H_{20}O_{11}$ + H, 473.1078); ESIMS (negative mode) *m*/*z* 471 [M − H]$^-$ (100); (positive mode) *m*/*z* 473 [M + H]$^+$ (100), 303 (61); CIMS (NH_3) *m*/*z* 473 [M + H]$^+$ (5), 407 (9), 391 (14), 321 (100), 303 (44), 277 (10), 233 (24), 182 (21), 153 (21), 139 (17), 127 (21).

4′-*O*-Methylepicatechin-3-*O*-gallate (7): brownish, amorphous powder; $[\alpha]^{20}_D$ −87 (*c* 1.0, MeOH); IR (neat) ν_{max} 3220, 1605, 1514, 1447, 1141, 1033, 823, 763 cm^{-1}; ^{13}C NMR (methanol-d_4, 100 MHz) 166.2 (C, CO), 156.5 (C, C-5, C-7, C-9), 147.2 (C, C-4′), 145.7 (C, C-3′), 144.9 (C, C-3″, C-5″), 138.4 (C, C-4″), 131.3 (C, C-1′), 120.0 (C, C-1″), 117.9 (CH, C-6′), 113.5 (CH, C-2′), 110.9 (CH, C-5′), 108.9 (CH, C-2″, C-6″), 98.0 (C, C-10), 95.3 (CH, C-6), 94.6 (CH, C-8), 77.1 (CH, C-2), 68.6 (CH, C-3), 59.5 (CH_3, OCH_3), 25.5 (CH_2, C-4); HRESIMS *m*/*z* 457.1129 (calcd for $C_{23}H_{20}O_{10}$ + H, 457.1129); ESIMS (negative mode) *m*/*z* 455 [M − H]$^-$ (100).

Epigallocatechin-3-*O*-ferulate (8): brownish, amorphous powder; $[\alpha]^{20}_D$ −56 (*c* 0.5, MeOH); IR (neat) ν_{max} 3365, 2839, 1686, 1604, 1514, 1449, 1266, 1144, 1032, 821 cm^{-1}; ^{1}H and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1; HRESIMS *m*/*z* 483.1286 (calcd for $C_{25}H_{22}O_{10}$ + H, 483.1286); ESIMS (positive mode) *m*/*z* 505 [M + Na]$^+$ (55), 483.1 [M + H]$^+$ (14), 289 (9), 249 (16).

4′-*O*-Methylepigallocatechin-3-*O*-ferulate (9): brownish, amorphous powder; $[\alpha]^{20}_D$ −125 (*c* 1.0, MeOH); IR (neat) ν_{max} 3372, 1686, 1602, 1513, 1450, 1356, 1265, 1144, 1048, 825, 719 cm^{-1}; ^{1}H and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1; HRESIMS *m*/*z* 497.1442 (calcd for $C_{26}H_{24}O_{10}$ + H, 497.1442); ESIMS (negative mode) *m*/*z* 495.2 [M − H]$^-$ (100).

4′,3″-Di-*O*-methylapocynin-D (10): brown, amorphous powder; CD (MeOH) $\Delta\varepsilon_{209}$ (−18.2), $\Delta\varepsilon_{233}$ (+7.3), $\Delta\varepsilon_{244}$ (0), $\Delta\varepsilon_{253}$ (−2.4), $\Delta\varepsilon_{278}$ (0), $\Delta\varepsilon_{291}$ (+0.8), $\Delta\varepsilon_{300}$ (0); $[\alpha]^{20}_D$ −25 (*c* 1.0, MeOH); IR (neat) ν_{max} 3384, 2920, 1741, 1605, 1514, 1442, 1269, 1123, 1054, 992, 827, 688 cm^{-1}; ^{1}H and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 2; HREIMS *m*/*z* 496.13770 (calcd for $C_{26}H_{24}O_{10}$, 496.136950); ESIMS (positive mode) *m*/*z* 519 [M + Na]$^+$, 497 [M + H]$^+$ (4), 457 (7), 399 (8), 327 (8); (negative mode) *m*/*z* 495 [M − H]$^-$ (59), 371 (5), 303 (5).

4′,3″-Di-*O*-methylapocynin-B (11): brownish, amorphous powder; CD (MeOH) $\Delta\varepsilon_{205}$ (+7.0), $\Delta\varepsilon_{228}$ (+1.2), $\Delta\varepsilon_{247}$ (−2.1), $\Delta\varepsilon_{286}$ (+0.7); $[\alpha]^{20}_D$ +48 (*c* 0.85, MeOH); IR (neat) ν_{max} 3212, 2923, 2852, 1602, 1515, 1447, 1112, 1053, 698 cm^{-1}; ^{1}H and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 2; HREIMS *m*/*z* 496.136996 (calcd for $C_{26}H_{24}O_{10}$, 496.136950); EIMS *m*/*z* 496 [M]$^+$ (38), 315 (22), 300 (7), 286 (13), 273 (9), 243 (6), 191 (76), 182 (100), 167 (84), 153 (37), 139 (27), 124 (54), 109 (55), 81 (57); ESIMS (positive mode) *m*/*z* 519 [M + Na]$^+$ (12), 497 [M+H]$^+$ (6); (negative mode) *m*/*z* 495 [M − H]$^-$ (100); 465 (6), 371 (6); CIMS (NH_3) *m*/*z* 497 [M + H]$^+$ (64), 373 (22), 321 (26), 301 (96), 273 (7), 212 (8), 200 (18), 182 (17), 179 (14), 151 (30), 142 (15), 124 (100).

Acknowledgment. The authors wish to thank the government of Baden-Württemberg (Zukunftsoffensive IV) for financial support. The authors are grateful to Prof. Dr. Solon J. Longhi, Department of Forest Science, for the localization and identification of the plant, and Mrs. M. da Costa Soliz for the preparation of the plant extract (both Federal University of Santa Maria, Brazil), M. Wagner and Dr. P. Bisel, Department of Pharmaceutical and Medicinal Chemistry, for measuring the optical rotation and recording the IR spectra, Dr. J. Wörth and C. Warth, Institute of Organic Chemistry (all University of Freiburg), Dr. M. Nimtz (Helmholtz Centre for Infection Research, Braunschweig) for the MS and HRMS data, D. Götz, Institute of Organic Chemistry, University of Würzburg, for recording the CD spectrum of **11**, M. Fronza, Department of Pharmaceutical Biology, for helpful advice in the scratch assay, and Dr. V. Wray (Helmholtz Centre for Infectious Research, Braunschweig) for critical reading of the manuscript.

Supporting Information Available: The results from the NF-κB EMSA, p38α MAPK, and MTT assays, as well as ^{1}H, ^{13}C, HSQC, HMBC, and COSY spectra of compounds **6**−**11**, Cartesian coordinates of the main conformers of the possible diastereomers of **11**, and calculated CD spectra of **11** are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Brenan, J. P. M. *Kew Bull.* **1963**, *17*, 227–228.
(2) Torres, C. M.; Repke, D. B. *Anadenanthera: Visionary Plant of Ancient South America*; The Haworth Press: Binghamton, 2006.
(3) Avancini, C.; Wiest, J. M.; Dall'Agnol, R.; Haas, J. S.; von Poser, G. L. *Lat. Am. J. Pharm.* **2008**, *27*, 894–899.
(4) de Souza, G. C.; Haas, A. P. S.; von Poser, G. L.; Schapoval, E. E. S.; Elisabetsky, E. *J. Ethnopharmacol.* **2004**, *90*, 135–143.
(5) Souza, G. C.; Hass, A. P. S.; Poser, G. L. V.; Elisabetsky, E. *Rev. Bras. Plant. Med.* **2004**, *6*, 83–91.
(6) Garcia, J.; Massoma, T.; Morin, C.; Mpondo, T. N.; Nyasse, B. *Phytochemistry* **1993**, *32*, 1626–1628.
(7) Delle Monache, F.; Dalbuquerque, I. L.; Chiappeta, A. D.; Demello, J. F. *Phytochemistry* **1992**, *31*, 259–261.
(8) Morimoto, S.; Nonaka, G.; Nishioka, I.; Ezaki, N.; Takizawa, N. *Chem. Pharm. Bull.* **1985**, *33*, 2281–2286.
(9) Miketova, P.; Schram, K. H.; Whitney, J. L.; Kerns, E. H.; Valcic, S.; Timmermann, B. N.; Volk, K. J. *J. Nat. Prod.* **1998**, *61*, 461–467.
(10) Seto, R.; Nakamura, H.; Nanjo, F.; Hara, Y. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 1434–1439.
(11) Sakar, M. K.; Petereit, F.; Nahrstedt, A. *Phytochemistry* **1993**, *33*, 171–174.
(12) Clark-Lewis, J. W.; Jackman, L. M.; Spotswood, T. M. *Aust. J. Chem.* **1964**, *17*, 632–648.
(13) Hemingway, R. W.; Tobiason, F. L.; Mcgraw, G. W.; Steynberg, J. P. *Magn. Reson. Chem.* **1996**, *34*, 424–433.
(14) Kida, K.; Suzuki, M.; Matsumoto, N.; Nanjo, F.; Hara, Y. *J. Agric. Food Chem.* **2000**, *48*, 4151–4155.
(15) Wan, S. B.; Dou, Q. P.; Chan, T. H. *Tetrahedron* **2006**, *62*, 5897–5904.
(16) Kohri, T.; Suzuki, M.; Nanjo, F. *J. Agric. Food Chem.* **2003**, *51*, 5561–5566.
(17) Yano, S.; Fujimura, Y.; Umeda, D.; Miyase, T.; Yamada, K.; Tachibana, H. *J. Agric. Food Chem.* **2007**, *55*, 7144–7148.
(18) Coxon, D. T.; Vora, V. C.; Tee, J. L.; Grant, M. S.; Ollis, W. D.; Holmes, A. *Tetrahedron* **1972**, *28*, 2819–2826.
(19) Sawai, Y.; Sakata, K. *J. Agric. Food Chem.* **1998**, *46*, 111–114.
(20) Hashimoto, F.; Nonaka, G.; Nishioka, I. *Chem. Pharm. Bull.* **1989**, *37*, 77–85.
(21) Fan, W. Z.; Tezuka, Y.; Xiong, Q. B.; Hattori, M.; Namba, T.; Kadota, S. *Chem. Pharm. Bull.* **1999**, *47*, 1049–1050.
(22) Chen, H. F.; Tanaka, T.; Nonaka, G. I.; Fujioka, T.; Mihashi, K. *Phytochemistry* **1993**, *33*, 183–187.
(23) Nonaka, G.; Nishioka, I. *Chem. Pharm. Bull.* **1982**, *30*, 4268–4276.
(24) Fronza, M.; Heinzmann, B.; Hamburger, M.; Laufer, S.; Merfort, I. *J. Ethnopharmacol.* **2009**, *126*, 463–467.
(25) Park, G.; Yoon, B. S.; Moon, J. H.; Kim, B.; Jun, E. K.; Oh, S.; Kim, H.; Song, H. J.; Noh, J. Y.; Oh, C.; You, S. *J. Invest. Dermatol.* **2008**, *128*, 2429–2441.
(26) Hwang, K. C.; Lee, K. H.; Jang, Y. S.; Yun, Y. P.; Chung, K. H. *J. Cardiovasc. Pharmacol.* **2002**, *39*, 271–277.
(27) Appleton, I. *Idrugs* **2003**, *6*, 1067–1072.
(28) Mackenzie, G. G.; Keen, C. L.; Fraga, C. G.; Oteiza, P. I. *Free Radical Biol. Med.* **2002**, *33*, S207–S208.
(29) Mackenzie, G. G.; Oteiza, P. I. *Free Radical Res.* **2006**, *40*, 1086–1094.
(30) Laufer, S.; Thuma, S.; Peifer, C.; Greim, C.; Herweh, Y.; Albrecht, A.; Dehner, F. *Anal. Biochem.* **2005**, *344*, 135–137.
(31) Hedges, J. C.; Singer, C. A.; Gerthoffer, W. T. *Am. J. Resp. Cell. Mol.* **2000**, *23*, 86–94.
(32) Huang, C. C.; Wu, W. B.; Fang, J. Y.; Chiang, H. S.; Chen, S. K.; Chen, B. H.; Chen, Y. T.; Hung, C. F. *Molecules* **2007**, *12*, 1845–1858.

(33) Kim, S. J.; Jeong, H. J.; Lee, K. M.; Myung, N. Y.; An, N. H.; Yang, W. M.; Park, S. K.; Lee, H. J.; Hong, S. H.; Kim, H. M.; Um, J. Y. *J. Nutr. Biochem.* **2007**, *18*, 587–596.
(34) Kundu, J. K.; Surh, Y. J. *Ann. N.Y. Acad. Sci.* **2007**, *1095*, 504–512.
(35) Kapoor, M.; Howard, R.; Hall, I.; Appleton, I. *Am. J. Pathol.* **2004**, *165*, 299–307.
(36) Mackenzie, G. G.; Carrasquedo, F.; Delfino, J. M.; Keen, C. L.; Fraga, C. G.; Oteiza, P. I. *FASEB J.* **2003**, *17*, 167–192.
(37) Schmidt, C.; Fronza, M.; Goettert, M.; Geller, F.; Luik, S.; Flores, E. M. M.; Bittencourt, C. F.; Zanetti, G. D.; Heinzmann, B. M.; Laufer, S.; Merfort, I. *J. Ethnopharmacol.* **2009**, *122*, 523–532.
(38) Selinummi, J.; Seppala, J.; Yli-Harja, O.; Puhakka, J. A. *Biotechniques* **2005**, *39*, 859–863.
(39) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–63.
(40) Frisch, M. J. Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian03, revision E.01*; Gaussian, Inc.: Wallingford, CT, 2004.
(41) (a) Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 5648–5652. (b) Lee, C.; Yang, W.; Parr, R. G. *Phys. Rev. B* **1988**, *37*, 785–789. (c) Hariharan, P. C.; Pople, J. A. *Theor. Chim. Acta* **1973**, *28*, 213–222. (d) Francl, M. M.; Pietro, W. J.; Hehre, W. J.; Binkley, J. S.; Gordon, M. S.; DeFrees, D. J.; Pople, J. A. *J. Chem. Phys.* **1982**, *77*, 3654–3665.
(42) (a) Grimme, S. *J. Chem. Phys.* **2003**, *118*, 9095–9102. (b) Schäfer, A.; Huber, C.; Ahlrichs, R. *J. Chem. Phys.* **1994**, *100*, 5829–5835.
(43) (a) Grimme, S.; Neese, F. *J. Chem. Phys.* **2007**, *127*, 154116. (b) Schäfer, A.; Horn, H.; Ahlrichs, R. *J. Chem. Phys.* **1992**, *97*, 2571–2577.
(44) Klamt, A. *COSMO-RS: From Quantum Chemistry to Fluid Phase Thermodynamics and Drug Design*; Elsevier: Amsterdam, 2005; p 246.
(45) Neese, F.; Becker, U.; Ganyushin, D.; Kossmann, S.; Hansen, A.; Liakos, D.; Petrenko, T.; Riplinger, C.; Wennmohs, F. *ORCA, version 2.7.0*; Universität Bonn: Bonn, Germany, 2009.
(46) Bringmann, G.; Bruhn, T.; Maksimenka, K.; Hemberger, Y. *Eur. J. Org. Chem.* **2009**, 2717–2727.
(47) Bruhn, T.; Hemberger, Y.; Schaumlöffel, A.; Bringmann, G. *SpecDis, version 1.45*; University Würzburg: Würzburg, Germany, 2009.

NP100523S