Biologically Active Bergenin Derivatives from *Bergenia stracheyi*

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New bergenin derivatives, bergecins A and B (**1** and **2**, resp.), have been isolated from the AcOEt-soluble fraction of *Bergenia stracheyi*, along with bergenin (**3**), and their structures were elucidated on the basis of ^{1}H- and ^{13}C-NMR spectra, and by COSY, HMQC, and HMBC experiments. Compound **2** showed potent inhibitory potential against the enzyme lipoxygenase, while **1** was moderately active. On the other hand, both compounds exhibited significant antioxidant activities in 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay.

Introduction. – The genus *Bergenia* belongs to the family Saxifragaceae and comprises of over 34 species. This genus is represented in Pakistan by six species [1]. The plants of *Bergenia* have diverse medicinal properties and are used for the treatment of diarrhea, vomiting, fever, cough, pulmonary infections, menorrhagia, excessive uterine hemorrhage, kidney stones, and ulcer of large intestines [2]. These are also applied externally for wounds, boils, and eyesores [3][4]. One of the species of this genus is *Bergenia stracheyi*, which grows abundantly in northern hilly areas of Pakistan. Although some secondary metabolites have previously been reported from *B. stracheyi* [5–8], no systematic phytochemical or pharmacological studies have been carried out on this species. The chemotaxonomic and ethnopharmacological importance of the genus *Bergenia* prompted us to carry out phytochemical investigation on *B. stracheyi*. We herein report the isolation of bergenin, and two new bioactive bergenin derivatives named as bergecins A and B (**1** and **2**, resp.; see *Fig. 1*).

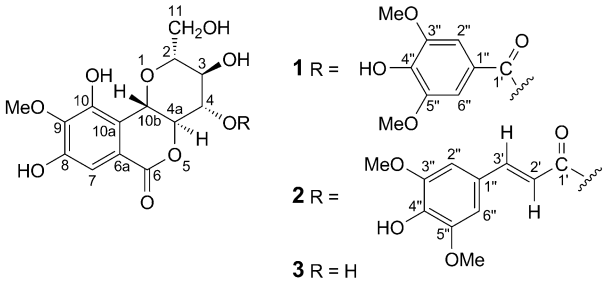

Fig. 1. *Stuctures of compounds* **1**–**3**

Results and Discussion. – *Chemistry.* The EtOH extract of the whole plant of *B. stracheyi* was processed as described in the *Exper. Part* to afford bergenin, and two of its new derivatives named as bergecins A and B (**1** and **2**, resp.). Both of these gave violet coloration with $FeCl_3$ for a phenol.

Bergecin A (**1**) was obtained as an off-white amorphous solid. The UV spectrum showed characteristic absorptions at 220 and 274 nm for methyl 4-*O*-methylgallate [9]. It did not display any shift with either AcONa or $B(OH)_3$. The IR spectrum revealed the presence of OH group (3320 cm^{-1}) and ester CO groups (1710 cm^{-1}), and aromatic (1600, 1530, 1510 cm^{-1}) and MeO (1220 cm^{-1}) moieties. The molecular formula was established as $C_{23}H_{24}O_{13}$ on the basis of HR-EI-MS (*m/z* 508.1220 (M^+; calc. 508.1216)). The molecular formula was further supported by the ^{13}C-NMR spectra (broad band (BB) and distortionless enhancement by polarization transfer (DEPT)), which exhibited 23 signals attributed to three Me, one CH_2, eight CH groups, and eleven quaternary C-atoms (*Table 1*). The most downfield signals at δ(C) 167.7 and 165.6 were assigned to the ester CO groups, while six other downfield signals are due to the O-bearing aromatic C-atoms. The O-bearing CH groups resonated at δ(C) 81.3, 80.5, 75.4, 74.4, and 71.8 while O-bearing CH_2 gave rise to a signal at δ(C) 64.9.

Table 1. *^{13}C-NMR Data of* **1**–**3**. At 125 MHz, in CD_3OD; δ in ppm.

C-Atom	**1**	**2**	**3**	C-Atom	**1**	**2**	**3**
C(2)	81.3	81.0	81.1	C(1″)	126.1	126.4	–
C(3)	71.8	71.8	71.3	C(2″)	110.3	109.9	–
C(4)	75.4	74.9	72.1	C(3″)	149.2	148.6	–
C(4a)	80.5	80.4	80.0	C(4″)	141.4	140.9	–
C(6)	165.6	165.6	165.2	C(5″)	149.2	148.6	–
C(7)	111.2	111.0	111.1	C(6″)	110.3	109.9	–
C(8)	152.3	152.1	152.4	C(1′)	167.7	168.5	–
C(9)	142.3	142.3	142.7	C(2′)	–	116.0	–
C(10)	151.7	151.6	151.2	C(3′)	–	143.6	–
C(10a)	116.9	116.9	116.3	*Me*O–C(3″) and	60.9	–	61.0
C(10b)	74.4	75.0	74.8	*Me*O–C(5″)			
CH_2(11)	64.9	64.6	64.3	*Me*O–C(9)	60.7	–	60.8

The ^{1}H-NMR spectrum showed signals for a 3,5-dimethoxygalloyl moiety (δ(H) 7.09 (*s*, 2 H) and δ(H) 3.89 (*s*, 2 MeO)) in addition to those of another aromatic H-atom at δ(H) 7.08 (*s*, 1 H) and of a MeO group at δ(H) 3.85 (*s*, 3 H). The chemical shifts of the O-bearing CH and O-bearing CH_2 H-atoms are compiled in *Table 2*, and their correlations are shown in *Fig. 2*. A six-C-atom aliphatic system, therefore, was established, and the large coupling constants (10.0 Hz) indicated that the CH H-atoms were all *trans* and diaxial [10].

The signals remaining after substraction of the signals of 3,5-dimethylgalloyl moiety from the ^{13}C-NMR spectrum of **1** displayed a very close resemblance to those of bergenin (*Table 1*). The presence of bergenin moiety was also supported by a major peak in EI-MS at *m/z* 208 [10]. Further diagnostic fragments were observed at *m/z* 327 [*M* – dimethylgalloyl]$^+$ and 181 [dimethylgalloyl]$^+$. The *singlet* of aromatic H-atom at δ(H) 7.08 was assigned to H–C(7), as it must be *ortho* to the CO group [11]. The

Table 2. *^{1}H-NMR Data of* **1** *and* **2**. At 500 MHz, in CD_3OD; δ in ppm; J in Hz.

	1	**2**
H–C(2)	3.82–3.84 (*m*)	3.81–3.83 (*m*)
H–C(3)	3.55 (*dd*, *J* = 10.0)	3.56 (*dd*, *J* = 10.0)
H–C(4)	4.07 (*dd*, *J* = 10.0)	4.02 (*dd*, *J* = 10.0)
H–C(4a)	4.38 (*dd*, *J* = 10.0)	4.38 (*dd*, *J* = 10.0)
H–C(7)	7.08 (*s*)	7.08 (*s*)
H–C(10b)	5.01 (*d*, *J* = 10.0)	5.04 (*d*, *J* = 10.0)
CH_2(11)	4.11 (*dd*, *J* = 7.2, 11.0),	4.11 (*dd*, *J* = 7.2, 11.0),
	3.91 (*dd*, *J* = 7.0, 11.0)	3.91 (*dd*, *J* = 7.0, 11.0)
H–C(2″)	7.09 (*s*)	7.09 (s)
H–C(6″)	7.09 (*s*)	7.09 (*s*)
H–C(2′)	–	6.09 (*d*, *J* = 12.4)
H–C(3′)	–	6.72 (*d*, *J* = 12.4)
MeO–C(3″) and MeO–C(5″)	3.89 (*s*)	3.91 (*s*)
MeO–C(9)	3.85 (*s*)	3.85 (*s*)

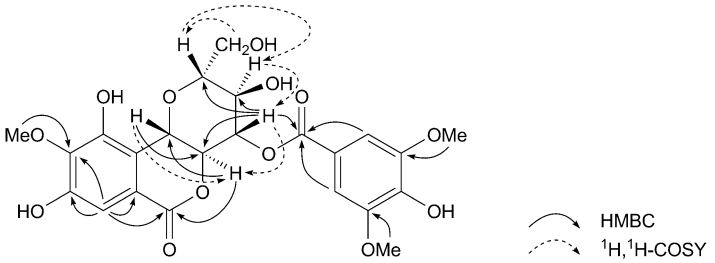

Fig. 2. *Key HMBC and ^{1}H,^{1}H-COSY correlations of bergecin A* (**1**)

assignment was further confirmed by the HMBC spectrum in which H–C(7) showed ^2J correlations with C(6a) and C(8), as well as ^3J correlations with C(6) and C(9). Controlled acid hydrolysis and subsequent methylation provided methyl tri-*O*-methylgallate and dimethylbergenin. Thus, **1** was a dimethylgalloyl derivative of bergenin. The esterification of the OH group at C(4) was reflected by downfield shift of the C(4) signal in ^{13}C-NMR compared to bergenin. Conclusive evidence was provided by HMBC experiments in which H–C(4) exhibited ^2J correlations with C(3) and C(4a), and ^3J correlations with C(2), C(10b) and C(1′). The MeO H-atom signal at δ(H) 3.89 showed ^3J correlations with C(3″) and C(5″). In the NOESY spectrum, the MeO–C(3″) and MeO–C(5″) H-atom signals displayed correlations those of with H–C(2″) and H–C(6″). On the other hand, no such correlation was observed in the case of MeO–C(9) H-atoms, confirming the presence of OH groups in the adjacent positions C(8) and C(10). Moreover, the correlations between the signals of β-oriented H–C(2), H–C(4) and H–C(10b) as well as those of α-oriented H–C(3) and H–C(4a) were similar to the correlations observed for bergenin. All these evidences were in complete agreement with the assigned structure of bergecin A (**1**) as 4-*O*-(3,5-di-*O*-methylgalloyl)bergenin (*Fig. 1*).

Bergecin B (**2**) was obtained as a light yellow amorphous solid. The UV spectrum was similar to that of **1**. The IR spectrum was also similar to that of **1** except for the additional band indicating a conjugated C=C bond (1630 cm^{-1}). The molecular formula was established as $C_{25}H_{26}O_{13}$ on the basis of HR-EI-MS (*m/z* 534.1369 (M^+; calc. 534.1373). The ^{13}C-NMR (BB and DEPT) showed 25 signals comprising those for three Me, one CH_2, and ten CH groups, and eleven quaternary C-atoms (*Table 1*). It exhibited signals similar to those of bergecin A (**1**) except signals of two additional olefinic C-atoms at δ(C) 143.6 and 116.0. The ^{1}H-NMR spectrum is also similar to **1** except additional olefinic H-atoms at δ(H) 6.72 ($d, J = 12.4$, 1 H) and 6.09 ($d, J = 12.4$, 1 H). The larger coupling constant allowed us to assign (*E*) configuration to the C=C bond. In the EI-MS, major peaks at *m/z* 208 and 327 were common with those of **1**, revealing similarity in bergenin moiety. The acid hydrolysis and subsequent methylation provided dimethylbergenin and methyl (*E*)-3,4,5-trimethoxycinnamate. The esterification of OH group at C(4) was evidenced by the downfield chemical shift of the signal of C(4), compared to bergenin, and further confirmed by HMBCs; H–C(4) signal showed ^2J correlations with the signals of C(3) and C(4a), and ^3J correlations with those of C(2), C(10b), and C(1′). The H–C(3′) signal showed ^2J correlations with signals of C(1″), C(2′), and ^3J correlations with C(1′), C(2″), and C(6″), respectively. The positions of MeO groups were confirmed by ^3J correlations of MeO H-atom signals at δ(H) 3.91 with those of C(3″) and C(5″). The NOESY correlations were similar to those of **1**, thereby confirming the structure of bergecin B (**2**) as 4-*O*-[(*E*)-4-hydroxy-3,5-dimethoxycinnamoyl]bergenin (*Fig. 1*).

Biology. Lipoxygenases (LOX; EC 1.13.11.12) constitute a family of non-heme iron containing dioxygenases that are widely distributed in animals and plants. In mammalian cells, these are key enzymes in the biosynthesis of a variety of non-regulatory compounds. It has been found that the lipoxygenase products play a role in a variety of disorders such as inflammation [12] and bronchial asthma [13]. Lipoxygenases are, therefore, potential target for the rational drug design and discovery of mechanism-based inhibitors for the treatment of inflammation, bronchial asthma, cancer, and autoimmune diseases.

Compounds **1** and **2** inhibited the LOX enzyme in a concentration-dependent fashion with IC_{50} values of 49.78 and 24.3 μM, respectively. The *Lineweaver–Burk* (*Fig. 3*) as well as *Dixon* (*Fig. 4*) plots indicate that the nature of inhibition is purely non-competitive, with K_1 values 40.2 and 19.2 μM, respectively (*Table 3*). The K_1 values are calculated in two ways. First, the slopes of each line in the *Lineweaver–Burk* plot were plotted *vs.* different concentrations of compounds **1** and **2**. Second, K_1 is directly measured from the *Dixon* plot as an intercept on the *x*-axis. Determination of the inhibition type is critical for identifying the mechanism of inhibition and the sites of inhibitor binding. The *Lineweaver–Burk* and *Dixon* plots, and their re-plots indicate a pure non-competitive type of inhibition of both **1** and **2** against the LOX enzyme. In other words, we can state that both these compounds and linoleic acid bind randomly and independently at the different sites of LOX. This indicates that the inhibition depends only on the concentrations of compounds **1** and **2**, and the dissociation constant (K_1) [14]. The compound **2**, therefore, shows potent inhibitory potential against the enzyme LOX compared to positive control, while **1** is moderately active.

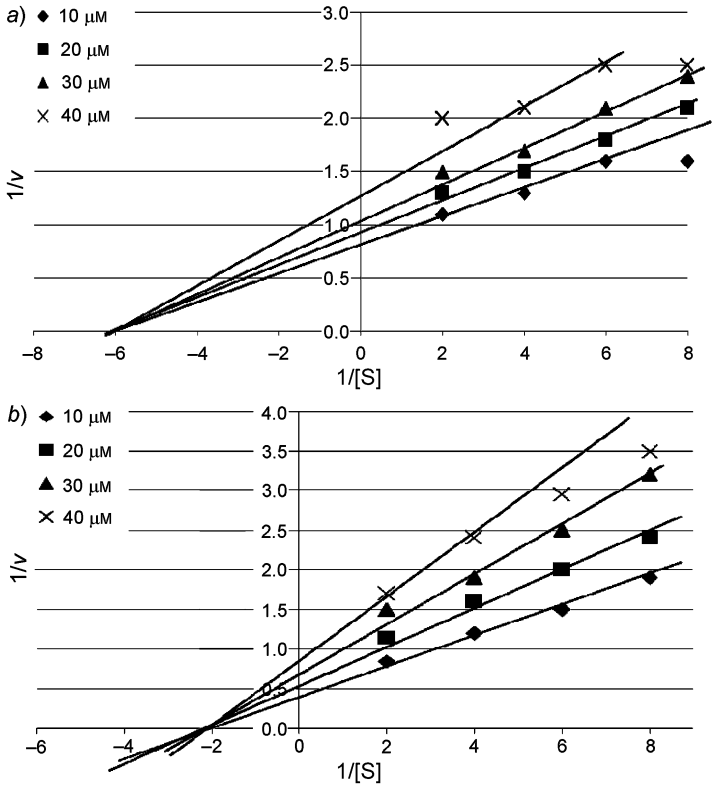

Fig. 3. Lineweaver–Burk *plots of compounds* a) **1** *and* b) **2** *of the reciprocal of initial velocities* [1/(μM/l/min)] vs. *the reciprocal of four fixed linoleic acid* (substrate) *concentrations [S]* [mM] *in the presence of 10, 20, 30, and 40 μM of compounds* **1** *and* **2**

Table 3. In vitro *Inhibition of Lipoxygenase and DPPH-Scavenging Activity of* **1** *and* **2**. For details, see *Exper. Part*.

Compound	Lipoxygenase inhibition IC_{50} [μM]	K_1 [μM] S.E.M[a])	Type of inhibition	DPPH Scavenging assay IC_{50} [μM]
1	49.78 ± 0.10	40.2 ± 0.21	Non-competitive	62.2 ± 0.19
2	24.3 ± 0.26	19.2 ± 0.18	Non-competitive	60.4 ± 0.17
Baicalein[b])	22.6 ± 0.08	18.0 ± 0.03	Mixed type	–
BHA[b])	–	–	–	44.2 ± 0.12

[a]) Standard error of the mean of 3–5 assays. [b]) Positive control, butylated hydroxyanisole (= 2-(*tert*-butyl)-4-methoxyphenol).

For the evaluation of antioxidant activity of the isolated compounds, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay was adopted. Both compounds exhibited significant antioxidant activities compared with the positive control (*Table 3*).

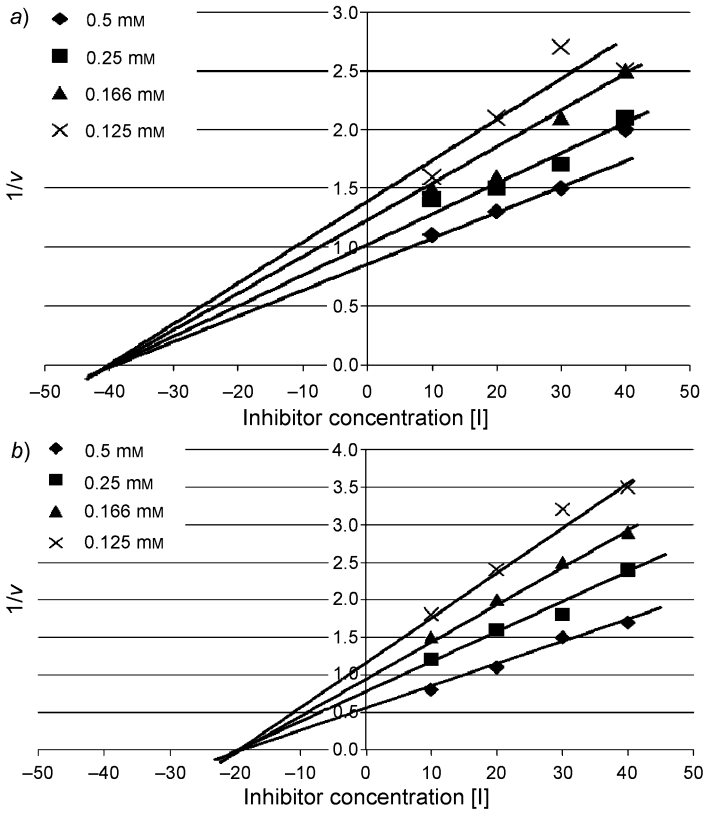

Fig. 4. Dixon *plots of compounds* a) **1** *and* b) **2** *of the reciprocal of initial velocities* [1/(μM/l/min)] vs. *inhibitor concentration [I]* [μM] *at four fixed linoleic acid concentrations, 0.5, 0.25, 0.16, and 0.12 m*M. Each point in the plots represents the mean of three experiments.

Experimental Part

General. TLC: Pre-coated silica gel F_{254} plates (*E. Merck*, D-Darmstadt); detection at 254 nm and by spraying with ceric sulfate reagent. Column chromatography (CC): silica gel (SiO_2; 230–400 mesh; *E. Merck*, D-Darmstadt). Optical rotations: *Jasco-DIP-360* digital polarimeter, 1-cm tube. UV Spectra: *Hitachi-3200* spectrophotometer; in MeOH; λ_{max} (log ε) in nm. IR Spectra: *Jasco-320-A* spectrophotometer; in cm^{-1}. ^1H- and ^{13}C-NMR, and 2D-NMR, spectra: *Bruker-AMX-400* spectrometer; δ in ppm rel. to Me_4Si internal standard, *J* in Hz. EI- and HR-EI-MS: electron impact (EI) mode with *Finnigan* MAT 12 and MAT 312 spectrometers; in *m/z* (%).

Plant Material. The whole plant of *B. stracheyi* Hook. f. & Thorns was collected from Gilgit and identified by *Sher Wali Khan*, Department of Biological Science, Karakoram International University Gilgit. A voucher specimen has been deposited with the Herbarium of the Department of Botany, University of Karachi (No. 156).

Extraction and Isolation. The whole plant (10 kg) was shade dried, ground, and extracted with EtOH (3×40 l). The combined EtOH (275 g) extract was divided into hexane- (70 g), $CHCl_3$- (40 g), AcOEt- (60 g), and H_2O-soluble (80 g) subfractions. The AcOEt-soluble fraction was chromatographed over SiO_2 and eluted with mixtures of $CHCl_3$/MeOH in increasing order of polarity to obtain five fractions: *Fr.* E_A ($CHCl_3$/MeOH 9.9:0.1), *Fr.* E_B ($CHCl_3$/MeOH 9.7:0.3), *Fr.* E_C ($CHCl_3$/MeOH 9.5:0.5), *Fr.* E_D

($CHCl_3$/MeOH 9.3 : 0.7), and *Fr.* E_E ($CHCl_3$/MeOH 9.0 : 1.0). The *Frs.* E_A and E_B could not be worked up due to paucity of material. The *Fr.* E_C contained a major compound with lingering traces of impurities, which could be removed through HPLC on *JAIGEL ODS-M 80* column with H_2O/MeOH 1 : 1 at a flow rate of 3 ml/min. The pure compound **1** was obtained as an off-white amorphous solid (t_R 40 min; 14 mg). The *Fr.* E_D was also subjected to HPLC on same column, mobile phase, and flow rate to furnish compound **2** as light-yellow amorphous solid (t_R 31 min; 11 mg). The *Fr.* E_E contained a major compound, which crystallized from MeOH, m.p. 238°, and could be identified as bergenin (**3**) by comparison of physical and spectral data with those reported in the literature [10].

Bergecin A (=*4*-O-*(3,5-Di*-O-*methylgalloyl)bergenin*; **1**). Off-white amorphous solid. $[\alpha]_D^{25} = -7.5$ ($c = 0.056$, MeOH). UV (MeOH): 220 (4.39), 274 (3.95). IR (nujol): 3320, 1710, 1600, 1530, 1510, 1220. ^{1}H- and ^{13}C-NMR: see *Tables 2* and *1*, resp. EI-MS: 508 (100), 327 (37), 208 (77), 181 (52). HR-EI-MS: 508.1220 (M^+, $C_{23}H_{24}O_{13}^+$; calc. 508.1216).

Bergecin B (=*4*-O-*[(*E*)-4-Hydroxy-3,5-dimethoxycinnamoyl]bergenin*; **2**). Light-yellow amorphous solid. $[\alpha]_D^{25} = -48.1$ ($c = 0.042$, MeOH). UV (MeOH): 218 (4.41), 275 (3.98). IR (nujol): 3330, 1710, 1630, 1600, 1525, 1500, 1220. ^{1}H- and ^{13}C-NMR: see *Tables 2* and *1*, resp. EI-MS: 534 (100), 327 (40), 208 (81), 207 (40). HR-EI-MS: 534.1369 (M^+, $C_{25}H_{26}O_{13}^+$; calc. 534.1373).

Acid Hydrolysis of **1** *and* **2**. Controlled acid hydrolysis was carried out according to a previously reported protocol [15]. To **1** (10 mg) in H_2O (1 ml) was added CF_3CO_2H (0.1 ml), and the mixture was refluxed for 40 h. The residue obtained after evaporation was methylated with CH_2N_2/Et_2O at 4° for 3 h. Prep. TLC (C_6H_6/Me_2CO 1 : 1) afforded dimethyl bergenin (2.1 mg), m.p. 206°, and methyl tri-*O*-methylgallate (2 mg), m.p. 79–81°. Analogus hydrolysis of **2** gave dimethylbergenin (2.2 mg) and methyl (*E*)-3,4,5-trimethoxycinnamate (2.3 mg), m.p. 99–100°. All the hydrolysis products were identified by comparison with authentic samples (m.p., IR, and ^{1}H-NMR).

In vitro *Lipoxygenase Inhibitory Assay.* Lipoxygenase inhibitory activity was determined by slightly modifying the spectrometric method developed by *Tappel* [16]. Lipoxygenase (1.13.11.12) type I-B (from soybean) and linoleic acid were purchased from *Sigma Chemicals.* A mixture of 160 ml of 100 μM phosphate buffer, pH 5.0, 10 ml of test compound, and 20 ml of lipoxygenase soln. was incubated for 10 min at 25°. The reaction was then initiated by the addition of 10 ml of linoleic acid (substrate) soln. [17], resulting in the formation of (9*Z*,11*E*,13*S*)-13-hydroperoxyoctadeca-9,11-dienoate. The change in absorbance was followed for 6 min at 234 nm. Test compounds and the control were dissolved in MeOH or 50% EtOH. All the reactions were performed in triplicate on a 96-well plate reader *Spectromax 384 plus* (*Molecular Devices*, USA). The IC_{50} values were calculated using the *EZ-Fit Enzyme Kinetics Program* (*Perrella Scientific Inc.*, Amherst, USA). The percentage [%] inhibition was calculated by the formula $(E - S)/E \times 100$, where E is the activity of the enzyme without test compound, and S is the activity of enzyme with test compound.

Dissociation constant/inhibition constant (K_1) was determined by the interpretation of *Dixon* and *Lineweaver–Burk* plots and their secondary replots using initial velocities obtained over a substrate (linoleic acid) concentration range between 0.06–0.5 mM.

Antioxidant Assay. The DPPH (=1,1-diphenyl-2-picrylhydrazyl) assay was performed essentially according to the protocol in [18]: 95 μl of 3.2 mM DPPH soln. in abs. EtOH and 5 μl of sample soln. in DMSO were mixed in a 96-well plate. The optical density was measured at 515 nm after incubation of the plate for 1 h at 37°. The DPPH control contained no sample but was otherwise identical.

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