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Phenolic compounds with radical scavenging and cyclooxygenase-2 (COX-2) inhibitory activities from *Dioscorea opposita*

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ABSTRACT

Phytochemical studies of the chloroform soluble fraction of *Dioscorea opposita* resulted in the isolation of four new compounds, 3,5-dihydroxy-4-methoxybibenzyl (**1**), 3,3′,5-trihydroxy-2′-methoxybibenzyl (**2**), 10,11-dihydro-dibenz[*b*,*f*]oxepin-2,4-diol (**3**), and 10,11-dihydro-4-methoxy-dibenz[*b*,*f*]oxepin-2-ol (**4**), together with an additional fifteen known compounds. The structures of **1–4** were elucidated by spectroscopic methods including 2D NMR. All of the nineteen isolated compounds were tested in the DPPH, superoxide anion radical scavenging assays and cyclooxygenases (COXs) inhibition assay. Of those, compounds **7**, **9**, **11**, **12**, **13**, **15** and **18** exhibited radical scavenging activities and compounds **2**, **3**, **8**, **13**, **15** and **16** showed selective inhibitory activities against COX-2.

1. Introduction

Inflammatory responses occur by diverse mechanisms including biosynthesis of prostaglandins, thromboxanes, prostacyclins and leukotrienes via cyclooxygenases (COXs) and lipoxygenases (LOXs) activation.[1] Unregulated inflammation causes damage to a broad range of human organ and tissues, which can result in a variety of disorders such as atherosclerosis, rheumatoid arthritis (RA), and chronic obstructive pulmonary disease (COPD).[2] Thus, there have been many efforts to find new compounds retaining potent anti-inflammatory efficacy but less toxicity to other organs. Cyclooxygenase (COX) enzymes play key roles in the induction of inflammatory diseases via synthesis of prostaglandins H_2, and they exist as three isoforms: cyclooxygenase-1 (COX-1), expressed in most cells constitutively, inducible cyclooxygenase-2 (COX-2), triggered by pro-inflammatory stimuli, and COX-3, present mainly in the cerebral cortex and human heart.[3,4] Despite of the undesired side effects such as increased cardiovascular risk of COX-2-selective inhibitors, much research spanning many years has been focused on finding COX-2 selective inhibitors, owing to the remarkable reduction of adverse gastrointestinal and renal effects associated with conventional non-steroidal anti-inflammatory drugs (NSAIDs) as well as to their potential therapeutic benefits in several diseases, including certain types of cancer.[5–7]

According to recent studies, reactive oxygen species (ROS) are important mediators that initiate and propagate inflammatory responses by stimulating release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α).[8] It was also found that ROS induced by activated neutrophils, eosinophils, monocytes and macrophages during the inflammation process leads to tissue injury by damaging macromolecules and effecting the lipid peroxidation of membranes.[9] This indicates that free radical scavengers might be a useful means of attenuating inflammatory effects. Recently, two potent antioxidants, resveratrol and curcumin, were reported to inhibit COX-2 expression to a greater or lesser extent.[10,11]

Dioscorea opposita Thunb. (Dioscoreaceae) has been cultivated in China, Japan and Korea as a food, and widely used as a traditional medicine, for a very long time. Phytochemical investigations of *D. opposita* have revealed many chemical components such as purine derivatives, phenanthrenes, stilbenes, sapogenins and saponins.[12,13] Pharmacological studies have shown that the genus *Dioscorea* exerts significant anti-inflammatory activity against TNF-α, IL-1β, COX-2 and inhibits the formation of ROS.[14,15] However, there has been no trial conducted to find certain ingredients exhibiting anti-inflammatory activity. Therefore, it is necessary to identify the chemical constituents of *D. opposita* responsible for the beneficial inhibitory effect against COX-2 via regulation of the ROS level.

In the present study, two new dihydrostilbenes (**1**, **2**) and two new dibenzoxepins (**3**, **4**) along with fifteen known compounds (Fig. 1) were isolated from the chloroform soluble fraction of *D. opposita* rhizomes, and their antioxidative activities were evaluated

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against DPPH and superoxide anion radicals, as well as in an in vitro COX-2 inhibitory assay.

2. Results and discussion

2.1. Identification of new compounds

Compound **1** was obtained as a colorless amorphous powder having the molecular formula $C_{15}H_{16}O_3$, as determined by the observed molecular ion peak $[M]^+$ at *m/z* 244.1102 in HREIMS. The EIMS fragmentation pattern of **1** indicated the presence of a benzyl (*m/z* 91, $C_7H_7^+$) and a dihydroxymethoxybenzyl (*m/z* 153, $C_8H_9O_3^+$). It also showed typical benzenoid UV absorption at 236 nm. The ^{1}H NMR spectrum of **1** (Table 1) revealed a singlet of two equivalent protons at δ_H 6.19 (2H, s, H-2 and H-6) of ring B, five aromatic protons at δ_H 7.13 (1H, t, *J* = 7.2 Hz, H-4′), 7.14 (2H, dd, *J* = 7.2, 1.8 Hz, H-2′ and H-6′) and 7.22 (2H, t, *J* = 7.2 Hz, H-3′ and H-5′), a methoxy group at δ_H 3.75 (3H, s) and four benzylic protons at δ_H 2.66 (2H, m, H-a) and 2.83 (2H, m, H-b). The HMBC cross-peaks between the 4-OCH_3 proton and C-4 as well as between H-2/H-6 (δ_H 6.19) and C-4 proved that the methoxy group was placed between two hydroxy-group-substituted carbon atoms. The positions of H-a and H-b also were identified from an analysis of the HMBC correlations. Therefore, compound **1** was designated as 3,5-dihydroxy-4-methoxybibenzyl.

The HREIMS data of compound **2** exhibited a molecular ion peak at *m/z* 260.1032 suggesting the molecular formula $C_{15}H_{16}O_4$. The EIMS fragmentation pattern of **2** indicated fragments of a dihydroxybenzyl (*m/z* 123, $C_7H_7O_2^+$) and a hydroxy-methoxybenzyl (*m/z* 137, $C_8H_9O_2^+$). Its UV spectrum also exhibited the typical pattern of bibenzyl moiety at 243 nm. The ^{1}H NMR spectrum of **2** showed the presence of 1,3,5-trisubstituted benzene ring at δ_H 6.08 (1H, t, *J* = 2.0 Hz, H-4) and 6.16 (2H, s, H-2 and H-6), three consecutive protons of ring A at δ_H 6.63 (1H, dd, *J* = 7.5, 1.7 Hz, H-6′), 6.67 (1H, dd, *J* = 8.1, 1.7 Hz, H-4′) and 6.81 (1H, t, *J* = 8.0 Hz, H-5′), a methoxy group at δ_H 3.74 (3H, s) and typical benzylic protons at δ_H 2.66 (2H, m, H-a) and 2.85 (2H, m, H-b). The detailed assignments of ring A were confirmed by HMBC experiment. Correlations of the 2′-OCH_3 proton, H-4′ and H-b to C-2′ suggested that the methoxy group was attached to the C-2′ position. Given the correlation of H-6′ to C-b, the structure of ring A was consequently determined to be an unusual 1,2,3-trisubstituted benzyl. The positions of H-a and H-b were also assigned according to the HMBC data. On the basis of these spectral data, compound **2** was identified as 3,3′,5-trihydroxy-2′-methoxybibenzyl.

Compound **3** was isolated as a white amorphous powder. Its molecular formula, with reference to the observed HREIMS ($[M]^+$ *m/z* 228.0783, calcd 228.0786), was deduced as $C_{14}H_{12}O_3$. The presence of a bibenzyl skeleton was suggested by the UV spectrum that showed absorption at 245 nm. The ^{1}H NMR spectrum (Table 2) showed four consecutive aromatic protons of ring A at δ_H 6.98 (1H, m, H-8), 7.10 (1H, m, H-7), 7.12 (1H, dd, *J* = 8.4, 1.6 Hz, H-9) and 7.27 (1H, dd, *J* = 8.4, 1.6 Hz, H-6), two *meta*-substituted proton signals of ring B at δ_H 6.06 (1H, d, *J* = 2.9 Hz, H-1) and 6.19 (1H, d, *J* = 2.9 Hz, H-3) and four benzylic protons at δ_H 2.97 (2H, m, H-11) and 3.08 (2H, m, H-10). The ^{13}C NMR data of **3** was similar to those of 2,4-dihydroxy-9,10-dihyrdophenanthrene, except for the chemical shifts of six carbons (C-4, C-6, C-1a, C-4a, C-6a and C-9a).[16] Notably, the ^{13}C resonances of the carbon atoms directly attached to oxygen atom showed strong downfield shifts of ca. 23 ppm. These markedly downfield shifted signals at C-4a (from δ_C 115.1 to 138.40) and C-6a (from δ_C 134.8 to 157.40) were assumed to be the formation of a 4a, 6a-oxygen bridge. The assumption of this oxygen bridged structure was supported by the mass data. The molecular formula of compound **3** ($C_{14}H_{12}O_3$, *m/z* 228) showed a mass higher by 16 (one oxygen atom) compared to 2,4-dihydroxy-9,10-dihyrdophenanthrene ($C_{14}H_{12}O_2$, *m/z* 212). The HMBC correlations from H-1 to C-11/C-4a and from H-3 to C-4/C-4a enabled the completion of the locating of the hydroxy groups in this structure. The structure of **3** was thus identified as 10,11-dihydrodibenz[*b*,*f*]oxepin-2,4-diol.

The HREIMS of **4** showed a $[M]^+$ ion at *m/z* 242.0952 consistent with the molecular formula $C_{15}H_{14}O_3$. The UV spectrum showed a bibenzyl moiety at 242 nm. The ^{1}H and ^{13}C NMR spectra of **4** (Table 2) were very close to those of **3**, excepting the additional signal for the methoxy (δ_H 3.82) group. The location of the methoxy group was assigned based on the observed correlations in the HMBC and NOESY experiments. The HMBC spectrum of the methoxy proton at δ_H 3.82 and H-3 to C-4 suggested that the methoxy group was attached to C-4 in ring B. Furthermore, the NOESY correlations between the 4-OCH_3 proton and H-3 and between H-1 and H-11 (Fig. 2) confirmed a 2-hydroxy-4-methoxy benzene moiety. The structure of **4**, then, was determined to be 10,11-dihydro-4-methoxy-dibenz[*b*,*f*]oxepin-2-ol.

The 15 known compounds were identified, by comparison of the spectroscopic data (^{1}H NMR, ^{13}C NMR and MS) with the litera-

Table 1
NMR spectroscopic data (400 MHz, CD_3OD) for compounds **1** and **2**[a]

	1			**2**		
Position	δ_C [b]	δ_H (*J* in Hz)	HMBC	δ_C	δ_H (*J* in Hz)	HMBC
1	140.0, qC			146.6, qC		
2	109.5, CH	6.19 (1H, s)	3, 4, 6, a	108.7, CH	6.16 (1H, s)	3, 4, 6, a
3	152.2, qC			160.2, qC		
4	135.7, qC			102.0, CH	6.08 (1H, t, 2.0)	2, 3, 5, 6
5	152.2, qC			160.2, qC		
6	109.5, CH	6.19 (1H, s)	2, 4, 5, a	108.7, CH	6.16 (1H, s)	2, 4, a
1′	144.0, qC			137.6, qC		
2′	130.1, CH	7.14 (1H, dd, 7.2, 1.8)	1′, 6′	148.0, qC		
3′	130.3, CH	7.22 (1H, t, 7.2)	1′, 2′, 4′, 5′	152.0, qC		
4′	127.6, CH	7.13 (1H, t, 7.2)	2′, 6′	116.3, CH	6.67 (1H, dd, 8.1, 1.7)	2′, 6′
5′	130.3, CH	7.22 (1H, t, 7.2)	1′, 3′, 4′, 6′	126.0, CH	6.81 (1H, t, 8.0)	1′, 3′
6′	130.1, CH	7.14 (1H, dd, 7.2, 1.8)	1′, 2′	122.6, CH	6.63 (1H, dd, 7.5, 1.7)	4′, b
a	39.8, CH_2	2.66 (2H, m)	2, 6	39.3, CH_2	2.66 (2H, m)	1, 2, 6, 1′, b
b	39.8, CH_2	2.83 (2H, m)	2′, 6′	33.9, CH_2	2.85 (2H, m)	1, 1′, 2′, 6′, a
4-OCH_3	61.6, CH_3	3.75 (3H, s)	4			
2′-OCH_3				61.7, CH_3	3.74 (3H, s)	2′

[a] Chemical shifts are reported in ppm relative to residual CD_3OD resonance at 3.31 ppm for ^{1}H NMR and CD_3OD resonance at 49 ppm for ^{13}C NMR.
[b] Multiplicity was confirmed by HMQC.

Table 2
NMR spectroscopic data (500 MHz, CD_3OD) for compounds **3** and **4**[a]

	3			4		
Position	δ_C [b]	δ_H (*J* in Hz)	HMBC	δ_C	δ_H (*J* in Hz)	HMBC
1	106.1, CH	6.06 (1H, d, 2.9)	2, 3, 11, 4a	106.7, CH	6.18 (1H, d, 2.7)	2, 3, 11, 4a
2	153.3, qC			152.3, qC		
3	100.9, CH	6.19 (1H, d, 2.9)	1, 2, 4, 4a	98.5, CH	6.29 (1H, d, 2.7)	1, 2, 4, 4a
4	148.8, qC			152.0, qC		
6	120.7, CH	7.27 (1H, dd, 8.4, 1.6)	8, 6a, 9a	121.6, CH	7.23 (1H, dd, 8.2, 1.5)	8, 6a, 9a
7	126.7, CH	7.10 (1H, m)	9, 6a	127.1, CH	7.11 (1H, m)	9, 6a
8	123.5, CH	6.98 (1H, m)	6, 9a	123.7, CH	6.98 (1H, dd, 8.5, 1.5)	6, 9a
9	130.2, CH	7.12 (1H, dd, 8.4, 1.6)	7, 10, 6a, 9a	130.8, CH	7.07 (1H, dd, 8.5, 1.5)	7, 10, 6a, 9a
10	30.7, CH_2	3.08 (2H, m)	9, 11, 1a, 6a, 9a	31.0, CH_2	3.07 (2H, s)	9, 11, 1a, 6a, 9a
11	30.2, CH_2	2.97 (2H, m)	1, 10, 1a, 4a, 9a	30.2, CH_2	3.07 (2H, s)	1, 10, 1a, 4a, 9a
1a	133.7, qC			135.3, qC		
4a	138.4, qC			141.0, qC		
6a	157.4, qC			157.1, qC		
9a	131.6, qC			131.0, qC		
4-OCH_3				56.1, CH_3	3.82 (3H, s)	4

[a] Chemical shifts are reported in ppm relative to residual CD_3OD resonance at 3.31 ppm for ^1H NMR and CD_3OD resonance at 49 ppm for ^{13}C NMR.
[b] Multiplicity was confirmed by HMQC.

ture values, as six dihydrostilbenes, including batatasin III[17] (**5**), batatasin IV[18] (**6**), tristin[19] (**7**), 2′,3,5-trihydroxybibenzyl (**8**)[18], 2′,4-dihydroxy-3,5-dimethoxybibenzyl[20] (**9**) and 3,4-dimethoxy-2′-hydroxybibenzyl[21] (**10**); three phenanthrenes, including 3,5-dimethoxy-2,7-phenanthrenediol[22] (**11**), hircinol[23] (**12**) and 9,10-dihydro-7-methoxy-2,5-phenanthrenediol[24] (**13**); five diarylheptanoids, including (1*E*,4*E*,6*E*)-1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one[25] (**14**), (4*E*,6*E*)-7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)-4,6-heptadien-3-one[26] (**15**), (4*E*,6*E*)-1,7-bis(4-hydroxyphenyl)-4,6-heptadien-3-one[27] (**16**), (3*R*,5*R*)-3,5-dihydroxy-1, 7-bis(4-hydroxyphenyl)-3,5-heptanediol[28] (**17**) and (3*R*,5*R*)-1, 7-bis(4-hydroxy-3-methoxyphenyl)-3,5-heptanediol[28] (**18**), and apigenin[29] (**19**).

2.2. Radical scavenging activity

It is commonly considered that ROS such as hydrogen peroxide (H_2O_2), hydroxy radical ($OH^·$) and superoxide anion radical ($O_2^{·-}$) are generated in a situation of oxidative stress and play a key role in cellular damage leading to various inflammatory diseases.[30] Several papers have demonstrated that ROS are highly connected with the inflammatory process via activation of membrane phospholipase A_2 (PLA_2) which catalyzes the biotransformation of arachidonic acids (AAs) to prostaglandins and thromboxanes by COX.[31,32] In addition, ROS are directly produced by COX at the site of inflammation and amplify the acute phase of the inflammatory responses.[33] Thus, antioxidative and radical scavenging agents can attenuate COX-mediated inflammatory processes by counteracting the effects of ROS.

In the present study, 19 compounds from the chloroform-soluble fraction of *D. opposita* were tested to evaluate their antioxidative activity against both DPPH and superoxide anion radicals and to evaluate the structure–activity relationship (SAR) of each class of phenolic compounds. Among the nineteen compounds, **7**, **9**, **11**, **12**, **13**, **15** and **18** exhibited mild inhibitory activities against both DPPH and superoxide anion radicals, whereas compounds **1**, **2** and **4** just neutralized DPPH radical and compound **16** showed antioxidative activity against superoxide radical. The other compounds showed no radical-scavenging activity (see Table 3).

Two dihydrostilbenes possessing a *para*-hydroxy group in the A- or B-ring, 3,4′,5-trihydroxy-3′-methoxybibenzyl (**7**) and 2′,4-dihydroxy-3,5-dimethoxybibnezyl (**9**) showed antioxidative effects against both DPPH and superoxide radicals, but the other dihydrostilbene derivatives did not. These results indicated that the *para*-hydroxy group played a significant role in its radical scavenging capacity, and are in accordance with the results for resveratrol and its analogues.[34,35]

Among the five diarylheptanoids (**14**–**18**) having two *para*-hydroxy groups in benzene rings, just (4*E*,6*E*)-7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)-4,6-heptadien-3-one (**15**) and (3*R*,5*R*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-3,5-heptanediol (**18**) exhibited relatively strong inhibitory activities against both DPPH and $O_2^{·-}$ radicals, demonstrating that the 3′ and 3″ methoxy groups at the *ortho*-position of the benzene ring are essential to the radical scavenging activity of diarylheptanoid derivatives, which fact is supported by a previous report demonstrating that methoxy groups in curcuminoids significantly improved the radical scavenging activity.[36]

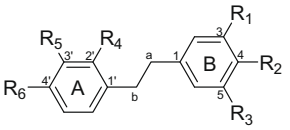
Table 3
Antioxidant activities of compounds (**1**–**19**) isolated from *D. opposita*[a]

Compound	DPPH radical IC_{50}[b] (μg/mL)	Superoxide radical IC_{50}[b] (μg/mL)
1	37.5 ± 1.8	>100
2	46.7 ± 0.1	>100
4	40.6 ± 1.2	>100
7	35.2 ± 0.5	51.6 ± 0.5
9	28.8 ± 1.0	56.3 ± 0.8
11	31.4 ± 0.8	39.6 ± 1.1
12	63.8 ± 2.3	62.4 ± 1.0
13	43.0 ± 1.8	51.5 ± 0.3
15	40.0 ± 2.1	38.8 ± 1.3
16	>100	44.1 ± 0.5
18	12.3 ± 0.2	48.7 ± 0.6
Catechin[c]	13.5 ± 0.5	13.7 ± 0.4
Ascorbic acid[c]	19.2 ± 1.1	16.7 ± 0.8

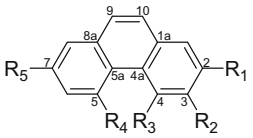
[a] Compounds **3**, **5**, **6**, **8**, **10**, **14**, **17** and **19** were considered to be inactive (IC_{50} > 100 μg/mL).
[b] Each value is expressed as the mean of triplicate experiments.
[c] Positive control.

2.3. Inhibition of COXs in vitro

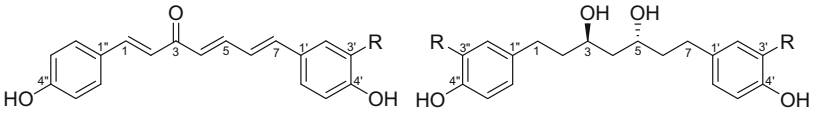
As shown in Table 4, compounds **2**, **3**, **7**, **8**, **11**, **13**, **14**, **15** and **16** showed inhibitory activity against COXs. Among these nine compounds, **2**, **3**, **8**, **13**, **15** and **16** inhibited COX-2 specifically, the new compound **2** showing the most potent inhibitory activity with the COX-2 selectivity at >55.6. Curcumin and its derivatives have been regarded as an efficient natural anti-inflammatory agent,[37] and the structures of the diarylheptanoids, isolated in this study,



1 R_1 = OH; R_2 = OCH_3; R_3 = OH; R_4 = H; R_5 = H; R_6 = H
2 R_1 = OH; R_2 = H; R_3 = OH; R_4 = OCH_3; R_5 = OH; R_6 = H
5 R_1 = OH; R_2 = H; R_3 = OCH_3; R_4 = H; R_5 = OH; R_6 = H
6 R_1 = OH; R_2 = H; R_3 = OCH_3; R_4 = OH; R_5 = H; R_6 = H
7 R_1 = OH; R_2 = H; R_3 = OH; R_4 = H; R_5 = OCH_3; R_6 = OH
8 R_1 = OH; R_2 = H; R_3 = OH; R_4 = OH; R_5 = H; R_6 = H
9 R_1 = OCH_3; R_2 = OH; R_3 = OCH_3; R_4 = OH; R_5 = H; R_6 = H
10 R_1 = OCH_3; R_2 = OCH_3; R_3 = OH; R_4 = OH; R_5 = H; R_6 = H

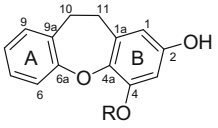


11 R_1 = OH; R_2 = OCH_3; R_3 = H; R_4 = OCH_3; R_5 = OH
12 9,10-dihydro; R_1 = OH; R_2 = H; R_3 = OCH_3; R_4 = OH; R_5 = H
13 9,10-dihydro; R_1 = OH; R_2 = H; R_3 = H; R_4 = OH; R_5 = OCH_3



14 R = H
15 1,2-dihydro; R = OCH_3
16 1,2-dihydro; R = H

17 R = H
18 R = OCH_3



3 R = H
4 R = CH_3

Figure 1. Structures of phenolic compounds **1**–**18** isolated from chloroform fraction of *D. opposita*.

Table 4
Inhibitory effects of compounds (**1–19**) isolated from *D. opposita* on ovine COX-1and COX-2 activity in vitro[a]

Compound	COX-1 IC_{50}[b] (μg/ml)	COX-2 IC_{50}[b] (μg/ml)	COX-2 selectivity[c]
2	>100	1.8 ± 0.3	>55.6
3	>100	6.4 ± 0.6	>15.6
7	4.7 ± 0.3	4.7 ± 0.1	1.0
8	>100	19.5 ± 1.5	>5.1
11	3.0 ± 0.4	2.7 ± 0.7	1.1
13	>100	10.6 ± 0.2	>9.4
14	5.8 ± 0.8	3.2 ± 0.2	1.8
15	>100	7.7 ± 1.0	>13.0
16	>100	4.9 ± 1.5	>20.4
Indomethacin[d]	0.27 ± 0.0	0.2 ± 0.1	1.4

[a] Compounds **1**, **4**, **5**, **6**, **9**, **10**, **12**, **17**, **18** and **19** were considered to be inactive (IC_{50} > 100 μg/ml) in both COX-1 and COX-2.
[b] Each value is expressed as the mean of triplicate experiments.
[c] COX-2 selectivity was determined by $IC_{50,\ COX\text{-}1}/IC_{50,\ COX\text{-}2}$.
[d] Positive control.

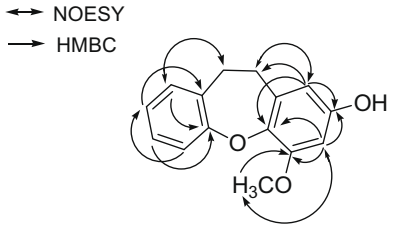


Figure 2. Selected HMBC and NOESY correlations of compound **4**.

have similar structures to the curcumin skeleton. Compound **14**, **15** and **16**, possessing ketone moiety, inhibited COXs, **15** and **16** showing selective inhibitory activity against COX-2, implying that 1,2-dihydrogenation plays an important role in COX-2 selectivity. However, compounds **17**, **18**, without the ketone group, showed no inhibitory activity against COXs.

3. Conclusion

Phytochemical and pharmacological studies on the chloroform-soluble fraction of *D. opposita* were conducted to find the molecules exhibiting antioxidative and inhibitory activity against COXs. Two new dihydrostilbenes and two new dibenzoxepins together

with fifteen known compounds, were isolated, and six of them showed selective COX-2 inhibitory activity. Among them, compound **2** showed the most potent COX-2 inhibitory activity with the selectivity at >55.6. Based on the comparison of the structures and potencies, the *para*-hydroxy substituents in the dihydrostilbene and methoxy groups at the *ortho*-position in diarylheptanoid played a key role in attenuating ROS. In the case of the diarylheptanoids, the compounds possessing both ketone moiety and hydrogenated C-1 in one molecule were more potent than the others. However, there does not appear to be any relationship between COX-2 inhibitory activity and antioxidant activity.

4. Experimental

4.1. General methods

^{1}H and ^{13}C NMR, COSY, HMQC, HMBC and NOESY spectral data were run on Bruker AVANCE 400 and 500 spectrometer. EIMS and HREIMS were recorded on JEOL JMS 700 spectrometer. UV spectra were measured on a Beckman DU 605 spectrophotometer. IR spectral data were taken on a JASCO FT/IR-4200 IR spectrophotometer. RP-MPLC was carried using a ISCO Combiflash instrument with a UV/vis detector (UA-6). A Gilson HPLC system (Gilson, USA) was used to isolate compounds, and was equipped with two 321 pumps, a UV/vis-151 detector, an autosampler 234 and a fraction collector 204. Silica gel (230–400 mesh, Merck) and Sephadex LH-20 gel (Pharmacia, Sweden) were used in column chromatography. DPPH (1,1-diphenyl-2-picrylhydrazyl), xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), catechin, ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyclooxygenase assay kits were purchased from Cayman Chemical (MI, USA).

4.2. Plant material

The rhizomes of *D. opposita* Thunb. (Dioscoreaceae) were provided by Tong Yang Moolsan Co., Ltd (Nonsan, South Korea), and identified by Prof. Gwang Jin Chang of the Korea National Agricultural College. A voucher specimen (SNUPH-0822) has been deposited in the Medicinal Herb Garden, Seoul National University.

4.3. Extraction and isolation

Fresh rhizomes of *D. opposita* (60 kg) were sliced and extracted with MeOH (20 L × 3) and evaporated under reduced pressure. The MeOH extract (1.9 kg) was suspended in distilled water (3 L) and partitioned with $CHCl_3$ (3 L), EtOAc (3 L) and *n*-BuOH (3 L), sequentially. The chloroform soluble fraction (72 g) was subjected to silica gel CC ($CHCl_3$-MeOH (30:1)→100% MeOH) to yield 12 fractions (Frs. 1-12). Fraction 2 (9.3 g) was subjected to MPLC (Redisep C-18 RP, 30 × 150 mm; detection, UV at 220 nm; flow rate, 20 mL/min) with a gradient elution of MeOH–H_2O (50:50→100% MeOH) to yield 10 subfractions (Frs. 2-1–2-10). Fraction 2-1 (0.8 g) was subjected to HPLC (YMC-pack ODS-A C18, 250 × 10 mm; eluent, MeCN–H_2O (50:50); detection, UV at 254 nm; flow rate, 4 mL/min) to yield compounds **3** (3.5 mg, t_R 29 min) and **11** (4.1 mg, t_R 24.5 min). Compounds **1** (6.9 mg, t_R 17.3 min) and **9** (4 mg, t_R 15.5 min) from fraction 2-2 (1.8 g) and compounds **4** (3.5 mg, t_R 20.5 min), **12** (41.9 mg, t_R 19 min) and **15** (7.8 mg, t_R 14 min) from fraction 2-4 (1.5 g) were isolated by HPLC (YMC-pack ODS-A C18, 250 × 10 mm; eluent, MeCN–H_2O (70:30); detection, UV at 254 nm; flow rate, 4 mL/min). Fraction 3 (7.2 g) was subjected to MPLC (Redisep C-18 RP, 30 × 150 mm; detection, UV at 220 nm; flow rate, 20 mL/min) with a gradient elution of MeOH–H_2O (50:50→100% MeOH) to obtain 9 fractions (Frs. 3-1–3-9). Fraction 3-2 (1.9 g) was subjected to HPLC (YMC-pack ODS-A C18, 250 × 10 mm; eluent, MeCN–H_2O (40:60); detection, UV at 254 nm; flow rate, 4 mL/min) to give compounds **5** (20.5 mg, t_R 23 min), **6** (39.6 mg, t_R 27.8 min), **10** (3.4 mg, t_R 27.1 min) and **13** (7.6 mg, t_R 19.7 min). Fraction 3-4 (0.5 g) was subjected to HPLC (YMC-pack ODS-A C18, 250 × 10 mm; eluent, MeCN–H_2O (40:60); detection, UV at 254 nm; flow rate, 4 mL/min) to yield compound **16** (17 mg, t_R 27 min). Fraction 4 (7.8 g) was subjected to MPLC (Redisep C-18 RP, 30 × 150 mm; detection, UV at 220 nm; flow rate, 20 mL/min) with a gradient elution of MeOH–H_2O (50:50→100% MeOH) to yield 8 fractions (Frs. 4-1–4-8). Fraction 4-1 (1.2 g) and 4-3 (0.8 g) were subjectd to HPLC (YMC-pack ODS-A C18, 250 × 10 mm; eluent, MeCN–H_2O (45:55); detection, UV at 254 nm; flow rate, 4 mL/min) to yield compounds **18** (14 mg, t_R 16 min) and **19** (6.4 mg, t_R 20.4 min). Compound **14** (2.2 mg, t_R 27 min) was isolated from fraction 4-4 (520 mg) by HPLC (YMC-pack ODS-A C18, 250 × 10 mm; eluent, MeCN–H_2O (50:50); detection, UV at 254 nm; flow rate, 4 mL/min). Fraction 5 (6.5 g) was subjected to MPLC (Redisep C-18 RP, 30 × 150 mm; detection, UV at 220 nm; flow rate, 20 mL/min) with a gradient elution of MeCN–H_2O (20:80→90:10) to yield 5 fractions (Frs. 5-1–5-5). Compounds **2** (13.8 mg, t_R 34.4 min) and **7** (6.4 mg, t_R 30.6 min) were isolated from fraction 5-1 (0.8 g) by HPLC (YMC-pack ODS-A C18, 250 × 10 mm; eluent, MeCN–H_2O (30:70); detection, UV at 254 nm; flow rate, 4 mL/min). Fraction 6 (4.3 g) was subjected to MPLC (Redisep C-18 RP, 30 × 150 mm; detection, UV at 220 nm; flow rate, 20 mL/min) with a gradient elution of MeOH–H_2O (20:80→100% MeOH) to yield 4 fractions (Frs. 6-1–6-4). Fraction 6-2 (1.2 g) was fractionated into 5 fractions (Frs. 6-2-1–6-2-5) using a Sephadex LH-20 CC (MeOH). Subfractions 6-2-2 and 6-2-4 were purified separately by HPLC (YMC-pack ODS-A C18, 250 × 10 mm; eluent, MeCN–H_2O (30:70); detection, UV at 254 nm; flow rate, 4 mL/min) to yield compounds **8** (4.5 mg, t_R 15.7 min) and **17** (23.7 mg, t_R 20.2 min).

4.3.1. 3,5-Dihydroxy-4-methoxy-bibenzyl (1)

Colorless amorphous powder (MeOH); UV (MeOH) λ_{max} (log ε) 236 (4.36) nm; IR (neat) ν_{max} 3408, 2935, 1596, 1454, 1357, 1170 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) see Table 1; EIMS *m/z* 244 [M]$^+$ (29), 153 (100), 138 (19), 110 (36), 91 (72); HREIMS *m/z* 244.1102 (calcd for $C_{15}H_{16}O_3$, 244.1099).

4.3.2. 3,3′,5-Trihydroxy-2′-methoxy-bibenzyl (2)

Brownish powder (MeOH); UV (MeOH) λ_{max} nm (log ε) 243 (4.62); IR (neat) ν_{max} 3376, 2927, 1599, 1471, 1155 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) see Table 1; EIMS *m/z* 260 [M]$^+$ (90), 229 (28), 137 (100), 123 (51), 109 (99), 77 (44); HREIMS *m/z* 260.1032 (calcd for $C_{15}H_{16}O_4$, 260.1049).

4.3.3. 10,11-Dihydro-dibenz[*b,f*]oxepin-2,4-diol (3)

White amorphous powder (MeOH); UV (MeOH) λ_{max} nm (log ε) 245 (4.40); IR (neat) ν_{max} 3420, 2924, 1616, 1457 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) see Table 2; EIMS *m/z* 228 [M]$^+$ (100), 211 (17), 199 (30), 181 (27), 153 (35), 128 (47), 115 (37), 69 (68); HREIMS *m/z* 228.0783 (calcd for $C_{14}H_{12}O_3$, 228.0786).

4.3.4. 10,11-Dihydro-4-methoxy-dibenz[*b,f*]oxepin-2-ol (4)

Brownish powder (MeOH); UV (MeOH) λ_{max} nm (log ε) 242 (4.96); IR (neat) ν_{max} 3388, 2923, 1606, 1454, 1227 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) see Table 2; EIMS *m/z* 242 [M]$^+$ (100), 227 (8), 211 (5), 199 (18), 181 (38), 153 (16), 128 (16), 115 (15), 69 (13); HREIMS *m/z* 242.0952 (calcd for $C_{15}H_{14}O_3$, 242.0943).

4.4. Radical scavenging assay

4.4.1. DPPH assay

The free radical scavenging activity of the compounds were evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method with modifications.[38] Each compound (5 μL) was added to 316 μM DPPH (95 μL) solution in 96 well plates. All of the compounds **1–19** were tested in final concentrations of 1, 2, 5, 10, 20, 50, and 100 μg/mL, respectively. The reaction mixture (100 μL) was mixed for 1 min and incubated at room temperature (37 °C) for 30 min. Then, the absorbance was measured at 515 nm on a microplate reader. The DPPH radical scavenging activity was calculated according to the equation

$$\text{DPPH radical scavenging activity } (\%) = [1 - (A_1 - A_2)/A_0] \times 100$$

where A_0 was the absorbance of the control (without the test compound), A_1 was the absorbance in the presence of the test compound, and A_2 was the absorbance sample blank (without DPPH). Using a calibration curve with different concentrations of samples, the IC_{50} was calculated. The IC_{50} is the sample concentration reducing 50% of the initial DPPH radicals under the given experimental conditions.

4.4.2. Superoxide radical ($O_2^{\cdot-}$) assay

The superoxide radical scavenging assay was performed by published methods with some modification.[39] $O_2^{\cdot-}$ was generated by xanthine/xanthine oxidase and measured in reference to the nitro blue tetrazolium (NBT) reduction. The final results were expressed as the percent inhibition of $O_2^{\cdot-}$ production in the presence of test compound. Different concentrations of test compound (0–0.1 mg/ml) in DMSO were added to 80 μL of a mixture of 0.1 mM xanthine and 0.2 mM NBT in 0.05 M phosphate buffer (pH 7.4) containing 0.1 mM EDTA. Then, 10 μL of 0.8 U/ml xanthine oxidase diluted in 0.05 M phosphate buffer (pH 7.4) was added and incubated at 37 °C for 20 min. The reaction was stopped by adding 1 M HCl. The absorbance was measured at 560 nm on a microplate reader. The inhibitory effect was calculated as

$$\text{Inhibitory rate } (\%) = [1 - (A_1 - A_2)/A_0] \times 100$$

where A_0 was the absorbance of the control (without the test compound), A_1 was the absorbance in the presence of the test compound, and A_2 was the absorbance sample blank (without xanthine oxidase). Using a calibration curve with different concentrations of samples, the IC_{50} was calculated. The IC_{50} is the sample concentration reducing 50% of the initial superoxide radicals ($O_2^{\cdot-}$) under the given experimental conditions.

4.5. Cyclooxygenase (COX) inhibitor screening assay

The inhibitory activities against COX-1 and COX-2 were determined using a colorimetric COX (ovine) Inhibitor Screening Assay Kit (Cayman Chemical Co., Cat. No. 760111) according to the manufacturer's protocol.[40] The inhibitory activities of the compounds were measured by monitoring the production of oxidized *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm followed by incubation of either ovine COX-1 or COX-2 with arachidonic acid. The enzymes were preincubated for 5 min at 25 °C with the test compounds prior to addition of arachidonic acid (final concentration 1.1 mM) and TMPD and incubation for 5 min at 25 °C. The COX-inhibiting activity was calculated according to the equation

$$\text{COX inhibiting activity } (\%) = [1 - (A_1 - A_2)/A_0] \times 100$$

where A_0 was the absorbance of the control (without the test compound), A_1 was the absorbance in the presence of the test compound, and A_2 was the absorbance sample blank (without TMPD). The IC_{50} is the concentration of tested compounds reducing 50% of ovine COX-1 or COX-2 under the given experimental conditions, and was calculated using a calibration curve with different concentrations of samples.

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