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Phenolic Constituents with Inhibitory Activity against NFAT Transcription from *Desmos chinensis*

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Six phenolic constituents, 2-methoxybenzyl benzoate (**1**), negletein (**2**), 2',3'-dihydroxy-4',6'-dimethoxydihydrochalcone (**3**), 5,6-dihydroxy-7-methoxy-dihydroflavone (**4**), astilbin (**5**), and quercitrin (**6**) were isolated from the methanol extract of the dried leaves of *Desmos chinensis*. Their structures were elucidated from spectral and chemical data. Of these constituents, compounds **2** (IC_{50}: 3.89 ± 0.39 μM) and **3** (IC_{50}: 9.77 ± 0.26 μM) exhibited potent inhibitory activity against nuclear factor of activated T cells (NFAT) transcription factor, and compound **1** (IC_{50}: 28.4 ± 2.62 μM) exhibited moderate inhibitory activity.

Key words: *Desmos chinensis*, NFAT Transcription factor, Phenolic constituents

INTRODUCTION

Desmos chinensis Lour (Annonaceae) is distributed widely in Northern Vietnam and is used as an antimalarial, insecticidal, antirheumatic, antispasmodic, and analgesic folk medicine (Chi, 1997; Loi, 2001). The previously phytochemical studies of *D. chinensis* have reported several chalcones and flavonoids (Hao *et al.*, 1993; Ju *et al.*, 1999; Wu *et al.*, 1994, 2003; Zhao, 1992), C-benzylated chalcones (Rahman *et al.*, 2003), oxoaporphine alkaloids (Liu *et al.*, 2004), and essential oils (Tran *et al.*, 2003). However, only a few biological effects have been reported, e.q., antibacterial activity for the mixture of 2,4-dihydroxy-6-methoxychalcone and chrysin (Qais *et al.*, 1996), anti-HIV activity for 2-methoxy-3-methyl-4,6-dihydroxy-5-(3-hydroxy)cinnamoylbenzaldehyde and lawinal against HIV-1 replication in H9 lymphocytes (Wu *et al.*, 2003), and tyrosine kinase inhibitory effect for desmal *in situ* in epidermal growth factor receptor-overexpressing NIH3T3 cells (Kakeya *et al.*, 1993). In our continued search for new biologically active compounds from Vietnamese medicinal plants, we report herein upon the isolation and structural determination of six phenolic constituents and the inhibitory activities of these compounds against the NFAT transcription factor. Their structures were elucidated to be 2-methoxybenzyl benzoate (**1**), negletein (**2**), 2',3'-dihydroxy-4',6'-dimethoxydihydrochalcone (**3**), 5,6-dihydroxy-7-methoxy-dihydroflavone (**4**), astilbin (**5**), and quercitrin (**6**) on the basis of spectral and chemical data.

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MATERIALS AND METHODS

General experimental procedures

Melting points were determined using a Kofler microhotstage, IR spectra with a Hitachi 270-30 type spectrometer with KBr discs, optical rotations with a JASCO DIP-1000 KUY polarimeter, FAB-MS and HR FAB-MS with a JEOL JMS-DX 300 spectrometer. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) were recorded on a Bruker DRX300 spectrometer, ^{1}H-NMR (500 MHz), and ^{13}C-NMR (125 MHz) on a Bruker AM500 FT-NMR, ^{1}H-NMR (600 MHz), and ^{13}C-NMR (150 MHz) on a Bruker AM600 FT-NMR spectrometer using TMS as an internal standard. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck).

Plant material

The leaves of *D. chinensis* were collected at Tamdao Mountain, Vinhphuc province, Vietnam during December 2002 and identified by Prof. Vu Van Chuyen, Hanoi University of Pharmacy, Vietnam. A voucher specimen (VN 64) was deposited at the herbarium of the Institute of

Natural Product Chemistry, VAST, Vietnam.

Extraction and isolation

The dried and powdered leaves of *D. chinensis* (6 kg) were extracted three times with hot MeOH repeatedly to give MeOH extract (250 g), which was suspended in water and extracted sequentially using hexane, chloroform, and ethyl acetate to yield hexane (118 g), $CHCl_3$ (75 g), EtOAc (13 g) extracts, and water layer (40 g). The hexane extract (118 g) was subjected to chromatography on a silica gel column and eluted with a hexane-acetone gradient from 0 : 1 to 1 : 0, which yielded five distinct fractions [Fr. A (18 g), Fr. B (6 g), Fr. C (13 g), Fr. D (21 g), and Fr. E (60 g)] by TLC. Fr. C (13 g) was chromatographed on a silica gel column using hexane-ethyl acetate (50 : 1, 2.5 L) as eluent, yielding compound **1** (56 mg). Fr. E (60 g) and the $CHCl_3$ extract (75 g) were combined and chromatographed on a silica gel column eluted with a $CHCl_3$-MeOH gradient [100% $CHCl_3$ (2 L), 100 : 1 (2 L), 50 : 1 (2 L), 10 : 1 (2 L), 5 : 1 (2 L), 1 : 1 (2 L)] to yield six fractions Fr. F (15 g), Fr. G (50 g), Fr. H (13 g), Fr. I (25 g), Fr. J (18 g), and Fr. K (14 g), respectively. Compound **2** (35 mg) was obtained as yellow crystals from Fr. G. Fr. H was chromatographed on a silica gel column using $CHCl_3$-MeOH (50 : 1, 3 L) as eluent, to yield compounds **3** (113 mg) and **4** (20 mg). The EtOAc extract was chromatographed on a silica gel column using $CHCl_3$-MeOH-H_2O (80 : 20 : 2, 3.5 L) as eluent, to yield compounds **5** (66 mg) and **6** (26 mg).

2-Methoxybenzyl benzoate (1)

White amorphous powder; m.p. 80-84°C; IR (KBr): ν_{max}= 1720 (s, C=O), 1495 cm^{-1} (C=C), 1050 (C-O-C); FAB-MS (positive): *m/z* = 265.08 $[M+Na]^+$; HR-FAB-MS observed: *m/z* = 265.0841 (Calcd. for $C_{15}H_{14}O_3Na$: 265.0841); ^{13}C-NMR (150 MHz) and ^{1}H-NMR (600 MHz) in $CDCl_3$ see Table I.

Negletein (2)

Yellow crystals, m.p. 235-238°C; FAB-MS (positive): *m/z* = 285 $[M+H]^+$, 307 $[M+Na]^+$; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 12.49 (s. OH-5), 8.05 (dd. *J* = 8.0, 1.6 Hz, H-2' and H-6), 6.95 (s, H-3), 6.90 (s, H-8), 7.56 (m, H-3', H-4' and H-5'), and 3.14 (3H. s, OCH_3-7).

2',3'-Dihydroxy-4',6'-dimethoxy-dihydrochalcone (3)

Yellow crystals, m.p. 130-133°C; FAB-MS (positive): *m/z* = 303 $[M+H]^+$, 325 $[M+Na]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 13.03 (s, OH-2'), 8.00 (s, OH-3'), 7.25 (H-2, H-3, H-4, H-5 and H-6), 6.25 (s, H-5), 3.87 (OCH_3-4'), and 4.00 (OCH_3-6'), 3.30 (t, *J* = 3.6 Hz, H-β) and 2.90 (t, *J* = 3.6 Hz, H-α); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 205.5 (s, C=O), 155.7 (s, C-4'), 154.3 (s, C-6'), 152.7 (s, C-2'), 142.3 (s, C-1), 129.2 (d, C-2, C-3, C-5 and C-6), 128.3 (s, C-3'), 126.7 (d, C-4), 106.7 (s, C-1'), 89.0 (d, C-5'), 57.0 (q, OCH_3), 56.8 (q, OCH_3), 46.2 (t, C-β), and 30.9 (t, C-α).

5,6-Dihydroxy-7-methoxy-flavanone (4)

Yellow crystals, m.p. 150-155°C; $[\alpha]_D^{25}$ -35.0 (MeOH, *c*=0.5); FAB-MS (positive): *m/z* = 286.97 $[M+H]^+$, 308.93 $[M+Na]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 11.80 (s, OH-5), 8.20 (s, OH-6), 7.56 (m, H-2', H-3', H-4', H-5' and H-6'), 6.20 (s, H-8), 5.50 (dd, *J* = 13.0, 3.0 Hz, H-2), 3.93 (s, CH_3O), 3.12 (dd, *J* = 13.0, 13.0 Hz, H-3a), 2.85 (dd, *J* = 13.0, 3.0 Hz, H-3b); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 197.0 (s, C-4), 156.2 (s, C-7), 155.1 (s, C-9), 148.3 (s, C-5), 138.8 (s, C-1'), 129.3 (d, C-3', C-4' and C-5'), 127.8 (s, C-6), 126.5 (d, C-2' and C-6'), 103.4 (s, C-10), 91.9 (d, C-8), 80.0 (d, C-2), and 43.9 (t, C-3).

Astilbin (5)

Yellow crystals, m.p. 190-192°C; $[\alpha]_D^{25}$ +14.8 (MeOH, c= 1.00); FAB-MS (positive): *m/z* = 451 $[M+Na]^+$.

Quercitrin (6)

Yellow powder, m.p. 178-180°C; FAB-MS (positive): *m/z* = 449 $[M+H]^+$.

Selection of NFAT dependent reporter cell line

The sense and antisense oligonucleotides which containing the NFAT binding site were synthesized, annealed and ligated to get the repeated NFAT binding site. The digested DNA fragment was transfected into T Jurkat cell and then the growing clones resistant to G418 (0.8 mg/mL) were selected.

Preparation of buffers and reagents

RPMI 1640 without phenol red (11835-030, Gibco. BRL) was mixed with 0.5% fetal bovine serum and 1% penicillin-streptomycin. Phobol 12-myristate 13-acetate (25 μg/mL) and ionomycin (0.5 μM) as a stimulator were dissolved in DMSO. *p*-Nitro-phenylphosphate (120 mM) as a substrate was dissolved with secreted alkaline phosphatase (SEAP) buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, 10 mM homoarginine).

Preparation of cells and samples

The selected Jurkat T cell line was maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The harvested cells were resuspended in RPMI 1640 without phenol red. Each sample was dissolved in DMSO and diluted in RPMI 1640 without phenol red.

Determination of inhibitory activity against NFAT transcription factor

NFAT transcription factor inhibitory activity was deter-

mined using a modified secreted alkaline phosphatase (SEAP) assay (Yang *et al.*, 1997). For this assay, 100 μL of cells (1×10^4 cells/well) were incubated with 50 mL of sample and 50 μL of stimulator at 37°C for 18 h. The reaction mixture was centrifuged and 100 μL of the supernatant was heated at 65°C for 1 h. The heated sample was then incubated with 50 mL of SEAP buffer and 50 μL of substrate at 37°C for 4 h. After incubating the reaction mixture, optical density was measured at 405 nm. NFAT transcription factor inhibitory activity was expressed as percentage inhibition of the control, and statistical significance was evaluated using the Students *t*-test. Cyclosporin A was used as a positive control. This blocks the phosphatase activity of calcineurin, thereby preventing the subsequent dephosphorylation and translocation of NFAT to the nucleus (Jain *et al.*, 1995). Cell viability was determined using an MTT cell proliferation Kit (1465007, Roche).

RESULTS AND DISSCUSSION

Compound **1** was obtained as a white amorphous powder. Its IR spectrum revealed a carbonyl group (1720 cm^{-1}), C-O-C (1050 cm^{-1}), and an aromatic ring (1495 cm^{-1}). Its HR-FAB-MS spectrum provided the molecular formula $C_{15}H_{14}O_3$ (observed *m/z* = 265.0841; Calcd. for $C_{15}H_{14}O_3Na$: 265.0841). The ^{1}H-NMR spectrum of **1** showed one aromatic methoxy (δ 3.89, s), one carbinol methylene (δ 5.45, s) and nine aromatic protons (δ 6.98-8.13). The ^{1}H coupling pattern of the five aromatic protons showed the presence of one monosubstituted phenyl ring. The ^{13}C-NMR indicated the presence of 15 carbons including one carbonyl (δ 167.0), one methoxy (δ 55.9), one carbinol methylene (δ 62.6), and two aromatic rings. The H-C assignments of **1** were made from HMQC spectra. The HMBC spectrum showed correlations between the carbinol methylene protons H-7' (δ 5.45) and carbon C-7 (δ 167.0)/ C-'1 (δ 124.9)/C-2' (δ 158.0)/C-6' (δ 128.7); between methoxy protons (δ 3.89) and carbon C-2' (δ 158.0); and between aromatic protons H-2, 6 (δ 8.13) and carbons C-1 (δ 130.9)/C-7 (δ 167.0), confirming that the methylene and methoxy groups were connected to C-1' and C-2' of the aromatic ring, and that the carbonyl group was connected to the other aromatic ring. The NMR assignments and the HMBC correlations of **1** are shown in Table I. These data led to identification of **1** as 2-methoxybenzyl benzoate which was reported already (Asakawa *et al.*, 1986; Lasswell *et al.*, 1977; Nkunya, 1985). But previous data was not enough to elucidate the chemical structure, herein we report ^{1}H-, ^{13}C-NMR, and HMBC data.

Compound **3** was obtained as yellow crystals. The positive FAB-MS of **3** showed ion peaks at *m/z* = 303 $[M+H]^+$ and 325 $[M+Na]^+$, corresponding to the molecular formula $C_{17}H_{18}O_5$. ^{1}H- and ^{13}C-NMR revealed the presence of the 17 carbons of a dihydrochalcone and of two methoxy and two hydroxyl groups. The NMR data of **3** (measured in DMSO-d_6) was assigned by comparison with that of 2'-hydroxy-3',4',6'-trimethoxydihydrochalcone (Lien *et al.*, 2000), and by tracing the connectivities in the HMQC and HMBC spectra of **3**. The HMBC spectrum showed correlations between the methoxy protons (δ 3.87) and carbon C-4' (δ 155.7); between the methoxy protons (δ 4.00) and carbon C-6' (δ 154.3); between proton OH-2' (δ 13.03) and carbons C-1' (δ 106.7)/C-2' (152.7)/C-3' (128.3); between proton OH-3' (δ 8.00) and carbon C-2' (δ 152.7)/C-3' (128.3); and between proton H-5' (δ 6.25) and carbons C-1' (δ 106.7)/C-3' (δ 128.3)/C-6' (δ 154.3), thus confirming that hydroxyl groups were connected to both C-2' and C-3', and that methoxy groups were connected to both C-4' and C-6'. Therefore, compound **3** was concluded to be 2',3'-dihydroxy-4',6'-dimeth-

Table I. ^{1}H- and ^{13}C-NMR spectral data for **1**

No.	δ_C (ppm)[a,b]	δ_H (ppm)[a,c]	HMBC
1	130.9 (s)	-	
2	129.8 (d)	8.13 (dd. 8.4. 2.1 Hz)	C-7, C-4, C-6
3	130.1 (d)	7.45 (ddd. 8.4. 8.0. 1.7 Hz)	C-1
4	133.2 (d)	7.58 (dd. 8.0. 2.1 Hz)	C-2, C-6
5	130.1 (d)	7.45 (ddd. 8.4. 8.0. 1.7 Hz)	C-1
6	129.8 (d)	8.13 (dd. 8.4. 2.1 Hz)	C-7, C-4, C-2
7	167.0 (s)	-	
1	124.9 (s)	-	
2	158.0 (s)	-	
3	110.9 (d)	6.98 (dd. 8.0. 1.8 Hz)	C-1', C-5'
4	129.8 (d)	7.35 (ddd. 8.0. 7.9. 2.0 Hz)	C-6', C-2'
5	120.9 (d)	7.01 (ddd. 7.9. 7.5. 1.8 Hz)	C-3', C-1'
6	128.7 (d)	7.45 (dd. 7.5. 2.0 Hz)	C-2', C-4'
7	62.6 (t)	5.45 (s)	C-1', C-2', C-6', C-7'
OCH_3	55.9 (q)	3.89 (s)	C-2'

[a] In $CDCl_3$. [b] 150 MHz. [c] 600 MHz.

Table II. IC_{50} values of compounds **1-6** on NFAT transcription factor

Compounds	IC_{50} value[a]
2-Methoxybenzyl benzoate (**1**)	28.4 ± 2.62 μM
Negletein (**2**)	3.89 ± 0.39 μM
2',3'-Dihydroxy-4',6'-dimethoxydihydrochalcone (**3**)	9.77 ± 0.26 μM
5,6-Dihydroxy-7-methoxy-dihydroflavone (**4**)	> 50 μM
Astilbin (**5**)	> 50 μM
Quercitrin (**6**)	> 50 μM
Cyclosporin A[b]	0.29 ±0.06 μM

[a] Values of IC_{50} are presented as mean ±S .E. of three experiments.
[b] This compound was used as the positive control.

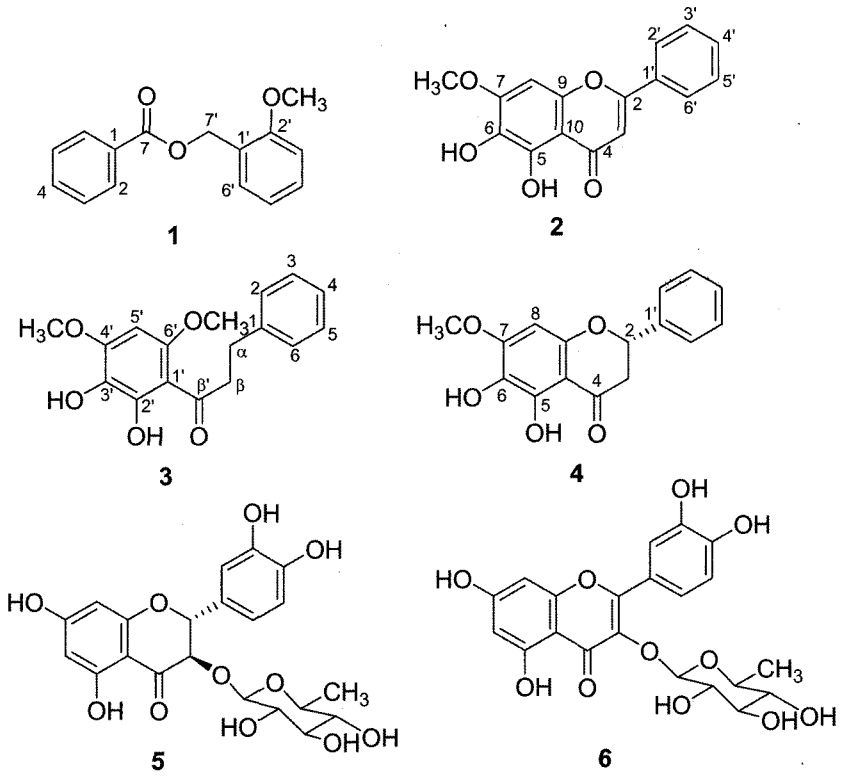

Fig. 1. Structures of compounds **1-6**

oxydihydrochalcone.

Compound **4** was obtained as yellow crystals. The positive FAB-MS of **4** showed ion peaks at *m/z* = 286.97 $[M+H]^+$ and 308.93 $[M+Na]^+$, corresponding to the molecular formula $C_{16}H_{14}O_5$. ^1H- and ^{13}C-NMR confirmed the presence of 16 carbons of a flavanone, including two hydroxyl and one methoxy group. Analysis of the spectroscopic data obtained for **4** indicated a close similarity with negletein (**2**). The ^1H coupling pattern of a five spin-system of aromatic protons showed the presence of one mono-substituted phenyl ring. In order to confirm the location of the hydroxyls and methoxy groups, HMQC and HMBC were performed in DMSO. The C-H long-range correlations between methoxy protons (δ 3.93) and carbon C-7 (δ 156.2); between hydroxyl proton OH-5 (δ 11.80) and carbons C-10 (δ 103.4)/C-5 (148.3)/C-6 (δ 127.8); between hydroxyl proton OH-6 (δ 8.20) and carbons C-5 (δ 148.3)/C-6 (δ 127.8)/ C-7 (δ 156.2); and between proton H-8 (δ 6.20) and carbons C-6 (δ 127.8)/C-10 (δ 103.4) were observed in the HMBC spectrum of **4**, which confirmed that two hydroxyl groups and one methoxy group were connected to C-5, C-6, and C-7, respectively. Consequently, **4** was determined to be 5,6-dihydroxy-7-methoxy-flavanone (Tomimori *et al.*, 1988). This is first report of **3** and **4** in *Desmos* species.

Compounds **2** (Yang *et al.*, 1996), **5** (Britto *et al.*, 1995; Kasai *et al.*, 1988) and **6** (Choi *et al.*, 1998; Xiong *et al.*, 1995) were identified as negletein, astilbin, and quercitrin, respectively by comparing their ^1H-, ^{13}C-NMR, and MS data with literature values.

Compounds **1-6** were evaluated for their inhibitory activity against NFAT transcription in an *in vitro* assay system (Yang *et al.*, 1997). Of these compounds, negletein (**2**), showed the strongest inhibitory activity against NFAT transcription factor with a 50% inhibitory concentration (IC_{50}) value of 3.89 ± 0.39 μM, and 2',3'-dihydroxy-4',6'-dimethoxydihydrochalcone (**3**) and 2-methoxybenzyl benzoate (**1**) also inhibited NFAT transcription factor with 50% inhibitory concentration (IC_{50}) values of 9.77 ± 0.26 μM and 28.4 ± 2.62 μM, respectively. Compounds **4-6** showed no inhibitory activities with IC_{50} values over 50 μM.

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