Studies on the Chemical Constituents of Stem Bark of *Millettia leucantha*: Isolation of New Chalcones with Cytotoxic, Anti-herpes Simplex Virus and Anti-inflammatory Activities

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Four new chalcone derivatives (1, 4, 7, 10) were isolated from the stem bark of *Millettia leucantha* Kurz (Leguminosae) along with two known ones (2, 6) and five known flavones (3, 5, 8, 9, 11). Structure elucidation and unambiguous assignment of the isolates were achieved with the aid of 1D and 2D NMR extensive studies. Correlation of 10 to 4 was successfully done by reduction with Et_3SiH/CF_3CO_2H. Moderate cytotoxic activity was observed in chalcones (1, 10), whereas dihydrochalcones (4, 6) showed moderate anti-Herpes Simplex Virus (HSV) activity. Interestingly, flavone 8 showed significant anti-inflammatory effects inhibiting both cyclooxygenase (COX)-1 and -2.

Key words *Millettia leucantha*; flavone; anti-inflammatory activity; chalcone; cytotoxic activity; anti-HSV activity

The genus *Millettia* Weight et Arn. (Leguminosae) consists of *ca.* 100 old world species of climbers and trees, and their seeds and other parts are in some cases known to show insecticidal and piscicidal properties.[1)] In previous studies[2)] flavonoids, isoflavonoids and alkaloids were isolated as chemical components. *M. leucantha* Kurz is a lofty tree, with pinnate leaves and white flowers, growing throughout Thailand[3)]; however, its medicinal use is unknown. The present investigation deals with the isolation of chalcone derivatives (**1**, **2**, **4**, **6**, **7**, **10**) including four new ones (**1**, **4**, **7**, **10**) and flavones (**3**, **5**, **8**, **9**, **11**) from the stem bark, and their cytotoxic, antiviral and anti-inflammatory activities.

Isolation of Chemical Constituents

Repeated column chromatography (CC) of the ethanolic extract of the stem bark of *M. leucantha* afforded eleven products (**1**—**11**) in the order of polarity.

Among them, seven compounds were identified to be known chalcones, 2′-hydroxy-3,4,4′,6′-tetramethoxychalcone[4,5)] (**2**) and dihydromilletenone methyl ether[6)] (**6**), known furanoflavones, karanjin[7,8)] (**3**) and lanceolatin B[4—6,9)] (**5**), and known simple flavones, desmethoxykanugin[10)] (**8**), 3′,4′-methylenedioxy-7-methoxyflavone[6)] (**9**), and 3′,4′-methylenedioxy-5,7-dimethoxyflavone[11)] (**11**), by comparison with reported data. Other chalcone derivatives (**1**, **4**, **7**, **10**) were found to be new ones. (Chart 1).

A new chalcone **1** with molecular formula $C_{18}H_{16}O_5$, was obtained as yellow needles. The ^{1}H-NMR spectrum (Table 1) showed a set of *trans*-olefinic protons at δ 7.35 and 7.60, a methylenedioxy group at δ 6.01 and two methoxy substituents at δ 3.87 and 3.91, in addition to two ABX aromatic systems. In electron impact mass spectrum (EI-MS) a relatively abundant fragment peak observed at *m*/*z* 165 [2,4-$(MeO)_2C_6H_3$–CO^+, 43%] indicated that two methoxy groups were located on the A ring connected to the ketonic function in a chalcone system.[12)] Thus, **1** was determined to be 2′,4′-dimethoxy-3,4-methylenedioxychalcone, which has been synthesized[4,5)] but is reported for the first time as a natural product.

The second product **4**, obtained as a pale yellow oil, had molecular formula $C_{19}H_{20}O_6$. The EI-MS showed that a peak at *m*/*z* 195 [2,4,6-$(MeO)_3C_6H_2CO^+$, 82%] arose from the ketonic A ring fragment substituted by three methoxy groups. In the ^{1}H-NMR spectrum (Table 1) the presence of two sets of methylene protons [δ 3.02 (2H, m, H-α), 2.91 (2H, m, H-β)] indicated that **4** was 2′,4′,6′-trimethoxy-3,4-methylenedioxydihydrochalcone.

Compound **7**, β-methoxychalcone, obtained as pale yellow needles, had molecular formula $C_{20}H_{20}O_7$. The ^{1}H-NMR spectrum (Table 1) showed an olefinic proton as a singlet at δ 6.33 assigned to H-α. Substitution of the ketonic A ring by a methylenedioxy group was deduced by the appearance of peaks at *m*/*z* 149 (3,4-OCH_2O–C_6H_3–CO^+, 27%) and 121 (3,4-OCH_2O–$C_6H_3^+$, 20%) in EI-MS. Furthermore, a base peak (*m*/*z* 341) was reasonably assigned to be a benzopyrilium cation[13,14)] produced by the loss of a methoxy group

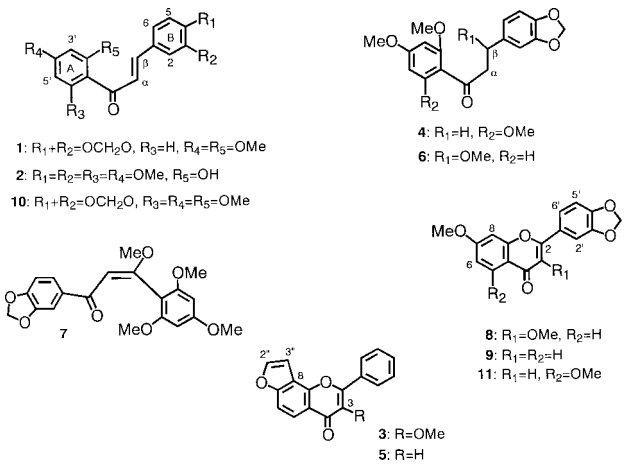

Chart 1. Chemical Constituents of *M. leucantha* Isolated by Us

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(Chart 2). Heteronuclear multiple bond connectivity (HMBC) correlation in **7** was shown in Chart 3, indicating the exclusion of an alternative possible structure **12**. Thus, **7** was determined to be 2,4,6,β-tetramethoxy-3′,4′-methylenedioxychalcone.

The (*E*)-configuration of the olefinic function was confirmed by nuclear Overhauser effect (NOE) experiment (Chart 3). An *E*-isomer is known to be the preferred isomer in naturally occurring β-methoxychalcones.[14)]

Compound **10**, molecular formula $C_{19}H_{18}O_6$, was obtained as pale yellow needles. The ^{1}H-NMR spectrum (Table 1) revealed the presence of a set of *trans*-olefinic protons at δ 6.78 and 7.28 (each d, J=16.0 Hz) in addition to 2,4,6-trimethoxy- and 3,4-methylenedioxybenzene rings. These data showed the presence of a chalcone skeleton and a similar substitution pattern of that of dihydro chalcone **4** (*vide supra*). Chemical correlation of the chalcone **10** to dihydrochalcone **4** was successfully done by 1,4-reduction with Et_3SiH/CF_3CO_2H, in which perhydrochalcone **13** was obtained as an over-reduction by-product. This 2′,4′,6′-trimethoxy-3,4-methylenedioxychalcone (**10**) was isolated for the first time from a natural source, although the synthetic product is known and has been patented.[15)]

The structures of all new chalcone derivatives (**1**, **4**, **7**, **10**) were precisely elucidated by ^{13}C-NMR (Table 2) and 2D NMR techniques (^{1}H-detected heteronuclear multiple quantum coherence (HMQC) and HMBC experiments).

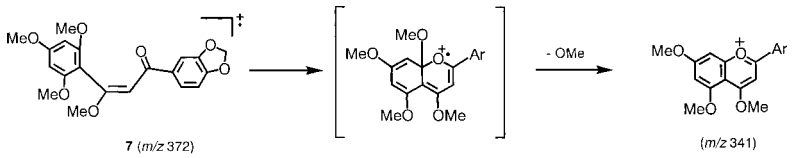

Chart 2. A Possible Formation of Benzopyrilium Cation from β-Methoxychalcone **7** in EI-MS

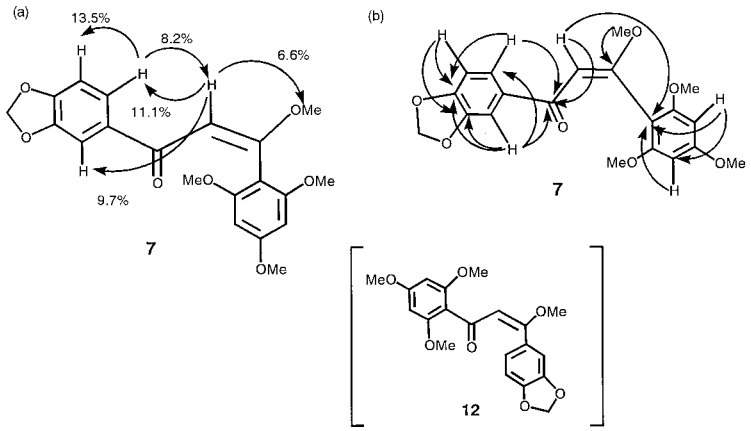

Chart 3. Selected NOE Enhancement (a) and HMBC Correlation (b) in **7**

Table 1. ^{1}H-NMR Data[a)] of New Chalcones (**1**, **4**, **7**, **10**) from *M. leucantha*

H[#]	**1**	**4**	**7**	**10**
2	7.12 (d, J=1.6)	6.71 (d, J=2)	—	7.05 (d, J=2)
3	—	—	6.10 (s)	—
5	6.82 (d, J=8)	6.71 (d, J=7.5)	6.10 (s)	6.79 (d, J=8)
6	7.07 (dd, J=8, 1.6)	6.65 (dd, J=7.5, 2)	—	6.98 (dd, J=8, 2)
2′	—	—	7.32 (d, J=2)	—
3′	6.50 (d, J=2)	6.09 (s)	—	6.16 (s)
5′	6.56 (dd, J=8.8, 2)	6.09 (s)	6.76 (d, J=8.4)	6.16 (s)
6′	7.75 (d, J=8.8)	—	7.46 (dd, J=8.4, 2)	—
OCH_2O	6.01 (s)	5.90 (s)	5.98 (s)	5.99 (s)
α	7.35 (d, J=16)	3.02 (m)	6.33 (s)	6.78 (d, J=16)
β	7.60 (d, J=16)	2.91 (m)	—	7.28 (d, J=16)
β-OMe	—	—	3.83 (s)	—
2-OMe	—	—	3.73 (s)	—
4-OMe	—	—	3.80 (s)	—
6-OMe	—	—	3.73 (s)	—
2′-OMe	3.91 (s)	3.76 (s)	—	3.77 (s)
4′-OMe	3.87 (s)	3.82 (s)	—	3.86 (s)
6′-OMe	—	3.76 (s)	—	3.77 (s)

a) ^{1}H-NMR (400 and 500 MHz in $CDCl_3$) are reported downfield from internal TMS at 0.00 ppm and peak multiplicities are quoted in Hz. The assignments are based on decoupling, HMQC, and HMBC experiments.

Table 2. ^{13}C-NMR Data[a] of New Chalcones (**1**, **4**, **7**, **10**) from *M. leucantha*

C#	1	4	7	10
1	129.9	135.4	107.0	129.4
2	106.6	108.9	158.5	106.7
3	148.2	147.4	90.8	148.2
4	149.3	145.5	162.1	149.5
5	108.5	108.0	90.8	108.4
6	124.7	121.1	158.5	124.7
1′	122.3	113.3	134.7	111.9
2′	160.3	158.2	108.2	158.7
3′	98.6	90.5	147.5	90.7
4′	164.0	162.3	150.4	162.3
5′	98.6	90.5	107.4	90.7
6′	132.8	158.2	123.4	158.7
OCH_2O	101.4	100.7	101.4	101.5
C=O	190.3	203.4	188.4	194.1
α	125.3	46.5	101.2	127.3
β	141.0	29.7	165.7	144.0
β-OMe	—	—	55.9	—
2-OMe	—	—	55.9	—
4-OMe	—	—	55.2	—
6-OMe	—	—	55.9	—
2′-OMe	55.7	55.7	—	55.9
4′-OMe	55.5	55.4	—	55.4
6′-OMe	—	55.7	—	55.9

a) ^{13}C-NMR (125 MHz in $CDCl_3$) assignments are related to internal $CDCl_3$ at 77.00 ppm. The assignments are based on decoupling, HMQC, and HMBC experiments.

Table 3. Cytotoxic Data of Chemical Constituents from *M. leucantha* against NCI-H460

Compound	IC_{50} (μg/ml)
1[a]	7.36
2[b]	>10
4[b]	>10
5[b]	>10
6[b]	>10
7[a]	>10
8[b]	>10
9[b]	>10
10[a]	3.69
11[b]	>10

a) Dissolved in EtOH, but insoluble material remains. *b*) Dissolved in DMSO.

Table 4. Anti-HSV Activity Test of Chemical Constituents from *M. leucantha*

Compound	Activity[a]
1[b]	Inactive
2[b]	Inactive
4[c]	Active
5[b]	Inactive
6[c]	Active
7[b]	Inactive
8	Inactive
10[b]	Inactive
11	Inactive

a) Antiviral activity was identified by reported method.[19] *b*) Insoluble material remains in DMSO. *c*) See Table 5.

Biological Activity

Flavonoids and chalcones have been examined a wide range of biological activities.[16—18] We examined cytotoxic, anti-viral, and anti-inflammatory activities of the above isolated products from *M. leucantha*.

Table 5. Inhibitory Effect of **4** and **6** on HSV-1 and 2

Compound	IC_{50} (μg/ml)		Cell toxicity dose to Vero cell (CC_{50}, μg/ml)
	HSV-1	HSV-2	
4	15.5±3	17.0±1	38.5±2.5
6	17.0±2	36.3±4	45.5±2
Acyclovir	0.06±0.01	0.5±0.08	—

Table 6. COX Inhibitory Test of Chemical Constituents from *M. leucantha*

Compound	% Inhibition at 10 μg/ml[a]		IC_{50} (μM)	
	COX-1	COX-2	COX-1	COX-2
1	—	NI[b]	—	—
2	—	NI[b]	—	—
5	—	NI[b]	—	—
7	—	NI[b]	—	—
8	*ca.* 50	80	—	0.96±0.003[c]
10	—	NI[b]	—	—
11	—	NI[b]	—	—
Aspirin	93.1	35.6	11.41±3.71	19.80±11.20
Indomethacin	—	—	0.005±0.003	0.006±0.002
NS-398[d]	—	—	NI[b]	0.01±0.01

a) Compounds with ≥80% inhibition were further analyzed for IC_{50} value. *b*) NI=no inhibition. *c*) Mean±S.E. (*n*). *d*) NS-398=*N*-(2-[cyclohexyloxy]-4-nitrophenyl)methanesulfonamide.

Cytotoxicity test against NCI-H460 showed moderate activity in two chalcones (**1**, **10**), between which **10** was twofold stronger than **1** (Table 3).

On the other hand, dihydrochalcones (**4**, **6**) were positive in a preliminary anti-HSV test according to the reported method[19] (Table 4). More precise examination of the anti-HSV activity indicated that **4** was active in both types of virus (HSV-1 and -2), whereas **6** was more effective in HSV-1 (Table 5).

Finally, anti-inflammatory effects were examined by inhibition of cyclooxygenase (COX)-1 and -2 using selected chemical constituents (**1**, **2**, **5**, **7**, **8**, **10**, **11**). The data are shown in Table 6. It was found that only the flavone **8** inhibited COX significantly and preferentially COX-2 more than COX-1.

Experimental

General Melting points were measured on a micro melting point hot-stage apparatus (Yanagimoto) and are given uncorrected. ^{1}H- (400, 500 MHz) and ^{13}C-NMR (100, 125 MHz) were measured on JEOL JNM-ECP400 and JEOL JNM-GSX500A (TMS as an internal standard). Mass spectra were obtained by JEOL JMS-AM20 (EI-MS) and JEOL JMS-HX110 (FAB-MS) spectrometers. Absorption spectra were measured on JASCO FT/IR-300E and Shimadzu UV-160A. TLC was performed using Merck precoated plate (Kieselgel 60 F254). Silica gel FL100D (Fuji Silysia Chemical Ltd.) was used in column chromatography (CC).

Plant Material The stem bark of *M. leucantha* Kurz (Leguminosae) was collected from the World Biosphere Reserve, Sakaeraj Environmental Research Station, Nakorn-Rachasima Province, Thailand during April–May, 1999. Authentication was achieved by comparison with the herbarium specimen (BKF No. 18009) at the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. A voucher specimen has been deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Extraction and Isolation of 1—11 The dried and pulverized stem bark (2.7 kg) was macerated twice with 95% EtOH (3, 2 l) for 3-d period and filtered. The combined filtrates were pooled and evaporated *in vacuo* until dry-

ness to yield a syrupy mass (132.6 g). The alcoholic extract (60.0 g) was chromatographed on silica gel and successively eluted with mixtures of hexane–$CHCl_3$ and then $CHCl_3$–MeOH of sequentially increasing polarity. The 80% $CHCl_3$ fraction was further purified by repeated CC using hexane–EtOAc system resulting in the isolation of **1** (46.0 mg, 5.6×10^{-3}%) and **2** (14.0 mg, 1.2×10^{-3}%). The $CHCl_3$ fraction was eluted with hexane–EtOAc system to afford **3** (4.1 mg, 0.3×10^{-3}%) and then eluted with benzene afforded **4** (29.3 mg, 2.4×10^{-3}%), **5** (10.7 mg, 0.9×10^{-3}%) and **6** (21.2 mg, 1.7×10^{-3}%). Recrystallisation of 1% MeOH in the $CHCl_3$ fraction gave **7** (347.5 mg, 28.5×10^{-3}%) and then elution with hexane–$CHCl_3$ system of the mother liquor yielded **8** (92.1 mg, 7.5×10^{-3}%) and **9** (6.6 mg, 0.5×10^{-3}%). Elution with hexane–EtOAc system of the 10% MeOH in the $CHCl_3$ fraction gave **10** (142.2 mg, 11.7×10^{-3}%). And 20% MeOH in the $CHCl_3$ fraction was eluted with benzene–EtOAc system to yield **11** (14.1 mg, 1.2×10^{-3}%).

Physical Data of Known Compounds 2′-Hydroxy-3,4,4′,6′-tetramethoxychalcone (**2**): Orange needles, mp 143—145 °C (hexane–EtOAc) (lit.[5] 150—152 °C (MeOH)). Karanjin (**3**): Colorless plates from $CHCl_3$, mp 161 °C. Lanceolatin B (**5**): Colorless needles, mp 116—121 °C. Dihydromilletenone methyl ether (**6**): Colorless oil. Desmethoxykanugin (**8**): Solid, mp 140—141 °C (lit.[10] mp 140—142 °C). 3′,4′-Methylenedioxy-7-methoxyflavone (**9**): Colorless needles, mp 202—204 °C ($CHCl_3$) [lit.[6] mp 208—210 °C (Et_2O)]. 3′,4′-Methylenedioxy-5,7-dimethoxyflavone (**11**): Colorless needles, mp 245—246 °C.

2′,4′-Dimethoxy-3,4-methylenedioxychalcone (**1**): Yellow needles, mp 129—131 °C (lit.[4] 136—138 °C for synthetic compound). UV λ_{max} (MeOH) nm (log ε): 206 (4.51), 245 (4.10), 304 (4.08), 348 (4.43). IR ν_{max} (Nujol) cm^{-1}: 3072, 2972, 2835, 1660, 1605, 1450, 1369, 1292, 1104, 976, 856. EI-MS *m/z*: 312 (M^+, 70%), 297 (23), 284 (23), 241 (5), 165 (43), 147 (7), 135 (100), 107 (33), 89 (72), 63 (40). *Anal.* Calcd for $C_{18}H_{16}O_5\cdot1/6H_2O$: C, 68.60; H, 5.21. Found: C, 68.66; H, 5.00. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

2′,4′,6′-Trimethoxy-3,4-methylenedioxydihydrochalcone (**4**): Pale yellow oil. UV λ_{max} (MeOH) nm (log ε): 207 (4.84), 233 (4.51), 285 (4.31). IR ν_{max} (neat) cm^{-1}: 2938, 2840, 1700, 1654, 1607, 1542, 1458, 1206, 1037, 928. HR-FAB-MS *m/z*: 345.1333 (M^++H); (Cald for $C_{19}H_{21}O_6$: 345.1347). EI-MS *m/z*: 344 (M^+, 26%), 313 (10), 195 (82), 168 (48), 149 (10), 148 (100), 109 (8), 91 (29), 65 (18). ^{1}H- and ^{13}C-NMR : Tables 1 and 2.

2,4,6,β-Tetramethoxy-3′,4′-methylenedioxychalcone (**7**): Pale yellow needles, mp 185—188 °C. UV λ_{max} (MeOH) nm (log ε): 220 (3.02), 233 (3.05), 277 (2.81), 381 (2.90). IR ν_{max} (Nujol) cm^{-1}: 2924, 1663, 1577, 1507, 1458, 1282, 1206, 1132, 1091, 919, 813. EI-MS *m/z*: 372 (M^+, 3%), 342 (99), 341 (100), 149 (27), 121 (20). *Anal.* Calcd for $C_{20}H_{20}O_7\cdot1/4H_2O$: C, 63.74; H, 5.48. Found: C, 64.04; H, 5.48. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

2′,4′,6′-Trimethoxy-3,4-methylenedioxychalcone[15] (**10**): Pale yellow needles, mp 143—144 °C. UV λ_{max} (MeOH) nm (log ε): 207 (4.68), 256 (4.05), 301 (4.07), 346 (4.38). IR ν_{max} (Nujol) cm^{-1}: 2922, 1638, 1597, 1490, 1450, 1264, 1207, 1158, 1033, 926, 825. EI-MS *m/z*: 342 (M^+, 46%), 327 (11), 315 (81), 314 (100), 283 (10). *Anal.* Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 66.49; H, 5.24. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Conversion of Compound 10 to Compound 4 To a solution of **10** (19.7 mg, 0.058 mmol) in CH_2Cl_2 (0.5 ml) were added Et_3SiH (0.028 ml, 0.18 mmol) and CF_3CO_2H (0.018 ml, 0.23 mmol). The whole mixture was stirred at room temperature for 18 h. Additional reagents [Et_3SiH (0.028 ml, 0.18 mmol) and CF_3CO_2H (0.015 ml, 0.20 mmol)] were added and the reaction mixture was stirred at room temperature for further 12 h. Excess amount of $NaHCO_3$ (200 mg) was added to the reaction mixture for neutralization, followed by filtration of insoluble material and evaporation to afford an orange oil (21.8 mg). The crude product was purified with preparative TLC (Merck, Art. 5715, hexane–EtOAc=3 : 1) to give three fractions.

Fraction 1 [1-(2,4,6-trimethoxyphenyl)-3-(3,4-methylenedioxyphenyl)-propane (**13**)]: Colorless needles, mp 68—69 °C (7.9 mg, 41.6%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.69—1.77 (2H, m, H-2), 2.53—2.61 (4H, m, 2×H-1, 2×H-3), 3.78 (6H, s, 2×OMe), 3.80 (3H, s, OMe), 5.90 (2H, s, OCH_2O), 6.12 (2H, s, H-3′, H-5′), 6.64 (1H, dd, J=7.8, 1.5 Hz, H-6″), 6.70 (1H, d, J=7.8 Hz, H-5″), 6.71 (1H, d, J=1.5 Hz, H-2″); FAB-MS *m/z*: 330 (M^+).

Fraction 2: A colorless oil (4.4 mg, 22.2%). This compound was identical with **4**.

Fraction 3: Pale yellow solid (2.7 mg, 13.7% as starting **10** recovery).

***In Vitro* Cytotoxic Assay** The cytotoxicity of the prepared constituents of *Millettia leucantha* against human lung cancer cell line, NCI-H460, was assessed by methyleneblue staining method. Briefly, 1.5×10^3 cells in RPMI1640 medium supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate were incubated in 96-well plates in the presence of serially diluted samples. After a 3-d culture, cells were stained with 0.05% methyleneblue dye for 30 min, and then thoroughly washed with distilled water. The stained dye was extracted with 3% HCl and OD660 was measured with microplate reader (Dynatech MR600) to determine cell growth inhibition.

Plaque Reduction Assay for Anti-viral Activity Plaque reduction assay was performed according to the reported method.[19] Briefly, HSV-1 (KOS) or HSV-2 (186) (30 plaque forming unit (PFU)/25 μl) was mixed with 25 μl of complete medium containing various concentrations of the test compound and incubated at 37 °C for 1 h. After incubation, the mixtures were added into Vero cells (6×10^5 cells/ml, 50 μl/well) in 96-well microtiter plates and incubated at 37 °C for 2 h. The overlay medium containing various concentrations of the test compound (100 μl/well) was added to the Vero cells and incubated at 37 °C in humidified CO_2 incubator for 2 d. The plaques were counted under an inverted microscope. The cells were stained with 1% crystal violet in 10% formalin for 1 h. The percent plaque inhibition was determined.

Materials and Methods for Anti-inflammatory Effects i) Cell Culture and Treatments: Mouse COX-1 and COX-2 null cell lines were provided by Dr. Leslie Ballou, University of Tennessee, Memphis, U.S.A. Cell culture components were purchased from Gibco BRL. All chemicals and NSAIDs were from Sigma Chemicals. ^{3}H-PGE_2 was from Amersham. Anti-PGE_2 antibody was purchased from Upstate Biotechnology. Samples were tested for COX inhibitory effects as reported previously.[20]

ii) Preparation of Test Compounds: Test compounds including **1**, **2**, **5**, **7**, **8**, **10** and **11** were first dissolved in DMSO at 1×10^{-1} g/ml. Each compound was subsequently diluted to 1×10^{-2} in 10% DMSO and serially mixed with culture media to yield the final concentrations of 1×10^{-5}, 3.3×10^{-6}, 1×10^{-6}, 3.3×10^{-7}, 1×10^{-7}, 3.3×10^{-8}, 1×10^{-8}, and 10×10^{-9} g/ml in 0.1% DMSO.

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