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Anti-inflammatory flavonoids and pterocarpanoid from *Crotalaria pallida* and *C. assamica*

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Abstract—One new isoflavone, 5,7,4′-trihydroxy-2′-methoxyisoflavone (**3**) and seven, and four known compounds were isolated from the barks of *Crotalaria pallida* and the seeds of *C. assamica*, respectively. The known compounds, apigenin (**1**) and 2′-hydroxygenistein (**2**), isolated from *C. pallida*, showed significant concentration-dependent inhibitory effects on the release of β-glucuronidase and lysozyme from rat neutrophils in response to formyl-Met-Leu-Phe/cytochalasin B (fMLP/CB) with IC_{50} values of 2.8 ± 0.1 and 17.7 ± 1.9, and 5.9 ± 1.4 and 9.7 ± 3.5 μM, respectively. The known compounds, daidzein (**4**) and 2′-hydroxydaidzein (**6**), isolated from *C. pallida*, inhibited of the release of lysozyme and β-glucuronidase from rat neutrophils in response to fMLP/CB with IC_{50} values of 26.3 ± 5.5 and 13.7 ± 2.6 μM, respectively. Compounds **1** and **4** also showed significant concentration-dependent inhibitory effects on superoxide anion generation in rat neutrophils stimulated with fMLP/CB with IC_{50} values of 3.4 ± 0.3 and 25.1 ± 5.0 μM, respectively. Compounds **1** and **5**, previously isolated from *C. pallida*, showed the inhibition of NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages and LPS/interferon-γ (IFN-γ)-stimulated N9 microglial cells with IC_{50} values of 10.7 ± 0.1 and 13.9 ± 1.1 μM, respectively. Flavonoids, suppressed chemical mediators in inflammatory cells, may have value in treatment and prevention of central and peripheral inflammatory diseases associated with excess production of chemical mediators.

1. Introduction

The genus *Crotalaria* has 300 species world-wide with only about 19 species reported in Taiwan. The genus produces mainly pyrrolizidine alkaloids but some flavonoids have been reported by Wanjala et al.[1] The genus are known in homeopathy for their antirheumatic, antiphlogistic and expectorant activities.[2]

As a part of our ongoing search for anti-inflammatory constituents from natural sources, we have investigated and reported the anti-inflammatory constituents of *Crotalaria pallida* AIT. and *C. assamica* BENTH (Leguminosae).[3,4] It is conceivable that mast cells, neutrophils, and macrophages are the important players in inflammatory disorders. Activation of microglial cells also plays a crucial role in inflammatory diseases of CNS. Thus, inhibition of the activation of these inflammatory cells appears to be an important therapeutic target for small molecular drug for the treatment of inflammatory disease. Our recent report have demonstrated that the two pterocarpanoids, crotafurans A (**8**) and B (**9**), previously isolated from *C. pallida*, showed potent inhibitory effects on the NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages or in LPS/interferon-γ (IFN-γ)-stimulated N9 microglial cells. Crotafuran B also showed potent inhibitory effects on the release of β-glucuronidase and lysozyme from rat neutrophils in response to formyl-Met-Leu-Phe/cytochalasin B (fMLP/CB). In a continuing search for anti-inflammatory constituents from *C. pallida* and *C. assamica*, a new isoflavone, 5,7,4′-trihydroxy-2′-methoxyisoflavone (**3**) and seven known constituents, β-sitosterol, lupeol, morin, weighteone, 2′-hydroxygenistein (**2**), daidzein (**4**) and 2′-hydroxydaidzein (**6**), and four known constituents, taxifolin, naringenin, quercetin-7-*O*-β-D-glucopyranoside (**7**), and naringenin-7-*O*-β-D-glucopyranoside were further isolated from the barks of *C. pallida*

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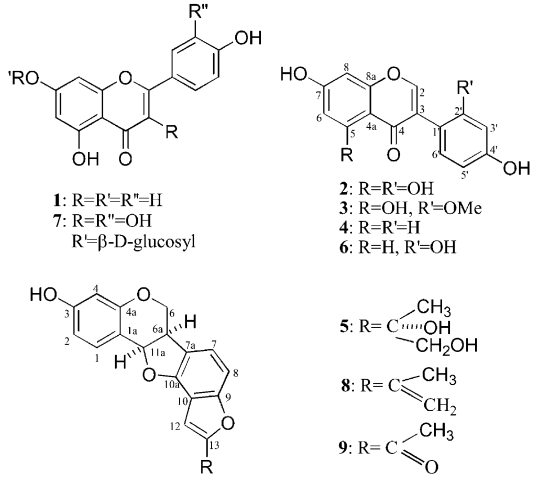
and the seeds of *C. assamica*, respectively. In the present paper, the structure elucidation of the new isoflavone, **3**, and anti-inflammatory activity of apigenin (**1**) and crotafuran E (**5**), previously isolated from *C. pallida*,[4] **2**, **3**, **4**, **6**, and **7**, are reported.



2. Structural Elucidation

The molecular formula of **3** was determined to be $C_{16}H_{12}O_6$ by HREIMS (*m/z* 300.0636 $[M]^+$, Δ +0.3 mmu). The UV absorption maxima resemble to those of 2′-hydroxygenistein (**2**).[5] The IR absorption of **3** implied the presence of OH (3410 cm^{-1}), conjugated ketone (1626 cm^{-1}), and aromatic ring (1561 cm^{-1}). The ^1H NMR spectrum of **3** showed proton signals for a methoxyl group at δ 3.89 (3H, s), six aromatic protons at δ 6.35 (1H, dd, *J* = 8.0, 2.0 Hz, H-5′), 6.38 (1H, d, *J* = 2.0 Hz, H-6), 6.44 (1H, d, *J* = 2.0 Hz, H-8), 6.46 (1H, d, *J* = 2.0 Hz, H-3′), 7.00 (1H, d, *J* = 8.0 Hz, H-6′), and 7.95 (1H, s, H-2).[6,7] The above evidence and the bathochromic shifts induced by adding of NaOAc, $AlCl_3$, and NaOMe in the UV spectrum of **3**, suggests that **3** is a 5,7,4′-trihydroxy-2′-methoxyisoflavone (**3**). The ^{13}C NMR spectrum (Experimental) of **3** was assigned by comparing the corresponding chemical shift values of 2′-hydroxygenistein, **7** genistein, **8** and data reported in the literature.[9] The ^{13}C NMR supports that the characterization of **3**.

3. Biological results and discussion

The anti-inflammatory activity of **1**–**7** was studied in vitro for their inhibitory effects on chemical mediators release from mast cells, neutrophils, macrophages, and microglial cells. Compounds **1**–**7** did not show significant inhibition of mast cells degranulation stimulated with compound 48/80 (10 μg/mL) (data not shown). FMLP (1 μM)/CB (5 μg/mL) stimulated the release of β-glucuronidase and lysozyme from rat neutrophils. Compounds **1** and **2** had potent and concentration dependent inhibitory effects on neutrophil degranulation. Compounds **4** and **6** showed significant inhibitory effects on lysozyme and β-glucuronidase release from rat neutrophils stimulated with fMLP/CB, respectively (Table 1). This clearly indicated that the hydroxylation at C-5 and C-2′ of **4** enhanced the inhibitory effects on neutrophil degranulation stimulated with fMLP/CB. The hydroxylation at C-2′ of **4** enhanced the inhibitory effect on the β-glucuronidase release, but did not enhance the inhibitory effect on lysozyme release from the fMLP/CB-stimulated rat neutrophils.

FMLP (0.3 μM)/CB (5 μg/mL) or phorbol 12-myristate 13-acetate (PMA) (3 nM) stimulated superoxide anion generation in rat neutrophils. As shown in Table 2, compounds **1** and **4** had potent inhibitory effects on fMLP/CB-induced superoxide anion generation, while had no significant inhibition of PMA-induced response. It is conceivable that fMLP/CB and PMA induce superoxide anion formation by activating the same oxidase in neutrophils but that they utilize different transduction mechanisms and are regulated differently.[10] The observations that **1**–**7** had no appreciable effect on PMA-induced response suggest the involvement of PMA-independent signaling pathway.

Treatment of RAW 264.7 macrophages with LPS (1 μg/mL) or N9 microglial cells with LPS (10 ng/mL)/IFN-γ (10 unit/mL) for 24 h induced NO production as assessed by measuring the accumulation of nitrite, a stable metabolite of NO, in the media based on Griess

Table 1. The inhibitory effects of compounds on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with fMLP/CB

Compounds	IC_{50} (μM)[a]	
	β-Glucuronidase	Lysozyme
1	2.8±0.1	17.7±1.9
2	5.9±1.4	9.7±3.5
3	>30 (46.6±5.4)	>30 (39.4±3.7)
4	>30 (41.2±9.2)	26.3±5.5
5	>30 (13.9±3.5)	>30 (1.8±10.4)
6	13.7±2.6	>30 (36.7±9.1)
7	>30 (46.7±0.9)	>30 (41.4±6.8)
Trifluoperazine	7.8±0.6	9.0±1.4

[a] When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. Data are presented as means±s.e.m. (*n* = 3–5). Trifluoperazine was used as a positive control.

Table 2. The inhibitory effects of compounds on superoxide anion generation in rat neutrophils stimulated with fMLP/CB or PMA

Compounds	IC_{50} (μM)[a]	
	FMLP/CB	PMA
1	3.4±0.3	>30 (22.1±11.6)
2	>30 (48.0±3.3)	>30 (6.3±11.4)
3	>30 (36.2±0.7)	>30 (33.1±1.1)
4	25.1±5.0	>30 (17.1±1.4)
5	>30 (−11.9±4.1)	>30 (34.9±3.0)
6	>30 (41.0±1.2)	>30 (9.6±5.3)
7	>30 (11.4±0.3)	>30 (−6.1±6.5)
Trifluoperazine	6.6±0.2	2.7±0.6

[a] When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. Data are presented as means±s.e.m. (*n* = 3–5). Trifluoperazine was used as a positive control.

Table 3. The inhibitory effects of compounds on the accumulation of NO_2^- in the culture media of RAW 264.7 cells in response to LPS and N9 cells in response to LPS/IFN-γ

Compounds	IC_{50} (μM)[a]	
	RAW 264.7 cells	N9 cells
1	10.7±0.1	>30 (20.5±2.0)
2	>30 (24.9±0.2)	>30 (32.3±1.4)
3	>30 (20.2±1.6)	>30 (15.7±1.5)
4	>30 (36.5±1.2)	>30 (48.1±0.5)
5	>30 (38.2±1.5)	13.9±1.1
6	>30 (25.2±2.5)	>30 (10.2±3.7)
7	>30 (18.6±1.0)	>30 (4.9±5.5)
1400W[b]	6.1±0.1	2.2±0.1

[a] When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. Data are presented as means±s.e.m. (n=3–5). Trifluoperazine was used as a positive control.

[b] *N*-(3-aminomethyl)benzylacetamidine (1400W) was used as a positive control.

reaction. As shown in Table 3, NO production in RAW 264.7 macrophages and N9 microglial cells was markedly suppressed by **1** and **5**, respectively. Compounds **8** and **9**, previously reported pterocarpanoid, showed significant inhibitory effects on the NO production in RAW 264.7 cells while **9** showed a potent inhibitory effect on the NO production in N9 cells. As shown in Table 3 and it clearly indicated that a lipophilic group substituted at the C-13 of a ptercarpanoid moiety enhanced the inhibitory effect on NO production in LPS-stimulated RAW 264[7] cells and LPS/IFN-γ-stimulated N9 cells. The result also indicated that the isoflavones shown in Table 3 had no significant inhibitory effect on NO production in RAW 264.7 cells and N9 cells. Compound **1**–**7** did not affect the tumor necrosis factor-α (TNF-α) formation from RAW 264.7 cells and N9 cells stimulated with LPS and LPS/IFN-α, respectively (data not shown). In previously reported by several groups,[11–13] the anti-inflammatory flavonoids were discussed. They make mention about the inhibition of lysozyme release and superoxide anion formation in rat neutrophils, and NO production in macrophages. The studying result of apigenin was reconcilable to our present study. This study verifies that the flavone derivative **1** and isoflavone derivatives **2**, **4**, and **6** exert potent inhibitory effects on the release of chemical mediator from inflammatory cells. NO plays a central role in macrophage-induced cytotoxicity and has been demonstrated to implicate in the pathology of central neurologic diseases and also in the peripheral tissue damage associated with acute chronic inflammation[14–16] and septic shock.[17] The present study suggests that the inhibition of NO production by **1** in macrophage and **5** in microglial cells may have value in the therapeutic treatment or prevention of certain central as well as peripheral inflammatory diseases associated with the increase of NO production.

4. Extraction and isolation

The barks of *C. pallida* (8 kg) and the seeds of *C. assamica* (125 g) were extracted with methanol at room temperature, respectively. The MeOH extract of *C. pallida* (85 g) was subjected to column chromatography over silica gel. Elution with cyclohexane (C_6H_{12}):acetone (9:1) yielded **1** (9 mg), β-sitosterol (30 mg), and lupeol (20 mg); C_6H_{12}:EtOAc (3:2) yielded **9** (5 mg); C_6H_{12}:acetone (1:1) yielded **2** (3 mg), **4** (5 mg), **5** (5 mg), and weighteone (6 mg); CH_2Cl_2:acetone (5:1) yielded **3** (4 mg) and **6** (6 mg). The MeOH extract of *C. assamica* (20 g) was eluted with C_6H_{12}:acetone (1:1) to yield taxifolin (7 mg); CH_2Cl_2:MeOH (15:1) to yield naringenin (6 mg); $CHCl_3$:MeOH (5:1) to yield **7** (14 mg) and naringenin-7-*O*-β-D-glucopyranoside (4 mg). The known compounds were characterized by comparing the various spectroscopic data with data reported in literature[7,8,18–20] or spectroscopic data of authentic samples.

5,7,4′ - Trihydroxy - 2′ - methoxyisoflavone (**3**). White powder, mp 282°; UV (MeOH) λ max (log ε) 210 (4.52), 257 (4.57), 292 (4.26) nm; IR (KBr) ν max 3140, 1626, 1561 cm^{-1}; HREI MS m/z 300.0636 $[M]^+$, $C_{16}H_{12}O_6$, calcd for 300.0633; ^1H NMR (400 MHz, methanol-d_4) see text; ^{13}C NMR (100 MHz, methanol-d_4, δ) 56.5 (C-9), 96.1 (C-8), 97.7 (C-6), 104.7 (C-5′), 108.3 (C-3′), 109.0 (C-4a), 112.5 (C-1′), 125.3 (C-3), 133.0 (C-6′), 154.5 (C-2), 158.1 (C-4′), 160.2 (C-2′), 161.5 (C-8a), 163.0 (C-5), 165.0 (C-7), 179.0 (C-4).

5. Bioassay procedures

The inhibitory assays for chemical mediator induced by various stimulants in mast cells, neutrophils, RAW 264.7 cells and N9 cells were performed by the methods described in ref 21.

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