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Antimycobacterial Activity and Cytotoxicity of Flavonoids from the Flowers of *Chromolaena odorata*

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From the flowers of *Chromolaena odorata* (*Eupatorium odoratum*) four flavanones, isosakuranetin (5,7-dihydroxy-4'-methoxyflavanone) (**1**), persicogenin (5,3'-dihydroxy-7,4'-dimethoxyflavanone) (**2**), 5,6,7,4'-tetramethoxyflavanone (**3**) and 4'-hydroxy-5,6,7-trimethoxyflavanone (**4**), two chalcones, 2'-hydroxy-4,4',5',6'-tetramethoxychalcone (**5**) and 4,2'-dihydroxy-4',5',6'-trimethoxychalcone (**6**), and two flavones, acacetin (5,7-dihydroxy-4'-methoxyflavone) (**7**) and luteolin (5,7,3',4'-tetrahydroxyflavone) (**8**) were isolated and identified. Compound **1** exhibited moderate antimycobacterial activity against *Mycobacterium tuberculosis* with the MIC value of 174.8 μM, whereas compounds **4**, **7**, and **8** exhibited weak activity with the MIC values of 606.0, 704.2 and 699.3 μM respectively. Compound **7** showed moderate cytotoxicity against human small cell lung cancer (NCI-H187) cells with the MIC value of 24.6 μM, whereas compound **8** exhibited moderate toxicity against NCI-H187 cells and week toxicity against human breast cancer (BC) cells with the MIC values of 19.2 and 38.4 μM respectively.

Key words: *Chromolaena odorata*, Asteraceae, Flavonoids, Antimycobacterial activity, Cytotoxicity, Struture-activity relationship

INTRODUCTION

Chromolaena odorata (L.) R. M. King & H. Robinson (synonym: *Eupatorium odoratum* L.) is a perennial scandent or semi-woody shrub belonging to the Asteraceae family. This plant species is native to central and south America and it is now distributed throughout Africa and tropical Asia (Muniappan and Marutani, 1991). In traditional medicine, a decoction of the leaf is used as a cough remedy and as an ingredient with lemon grass and guava leaves for the treatment of malaria. The juice pressed out of the crushed leaves is applied to cuts to stop bleeding. Other medicinal uses include antidiarrheal, astringent, antispasmodic, antihypertensive, antiinflammatory and diuretic (Iwu, 1993). In Thailand, leave juice is used as a haemostatic on wounds and antiinflammatory. A decoction of flowers is used as tonic, antipyretic and heart tonic (Bunyapraphatsara and Chokechaijaroenporn, 2000). Previous investigations of the leaves and stems of *C. odorata* revealed the presence of essential oils (Inya-Agha *et al.*, 1987; Lamaty *et al.*, 1992; Bamba *et al.*, 1993; Chowdhury, 2002), steroids (Ahmad *et al.*, 1967), triterpenes (Tarapatra *et al.*, 1974; Tarapatra *et al.*, 1977), flavonoids (Bose *et al.*, 1973; Tarapatra *et al.*, 1974; Bose *et al.*, 1974; Tarapatra *et al.*, 1977; Arene *et al.*, 1978; Barua *et al.*, 1978; Metwally *et al.*, 1981; Hai *et al.*, 1991; Triratana *et al.*, 1991; Hai *et al.*, 1995; Wollenweber *et al.*, 1995; Wollenweber and Roitman, 1996). Flowers of this plant species have been subjected to investigation for essential oils (Baruah and Leclercq, 1993), fats (Baruah and Pathak, 1993) and alkaloids (Biller *et al.*, 1994). We now report on the flavonoid constituents of the flowers of *C. odorata* and some biological evaluations of the isolated flavonoids.

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MATERIALS AND METHODS

General experimental procedures

Melting points were determined on an Electrothermal apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer FT-IR Spectrum BX spectrophotometer. ^{1}H-NMR spectra were recorded on a Bruker AVANCE 400 spectrometer. The residual nondeuterated solvent, $CHCl_3$, at 7.24 ppm was used as a reference. Electron impact (EI) mass spectra were measured with a Thermo Finnigan Polaris Q spectrometer. Unless indicated otherwise, Merck silica gel 60 (finer than 0.063 mm) was used for column chromatography. TLC was conducted on plates precoated with Merck silica gel 60 F_{254}. Spots on TLC were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating.

Plant material

The flowers of *C. odorata* were collected from Mai-khao, Talang district, Phuket province, in February, 1999. A voucher specimen of this plant, No. 0033 (RU), is deposited at the Faculty of Science, Ramkhamhaeng University.

Extraction and isolation

The dried flowers of *C. odorata* (1.3 kg) was pulverized and extracted successively with hot *n*-hexane, $CHCl_3$ and MeOH (3 L×4 for each extract) to give the hexane (27.54 g), $CHCl_3$ (28.79 g) and MeOH (46.89 g) extracts, respectively. The $CHCl_3$ extract (25.00 g) was chromatographed over silica gel (0.063-0.200 mm, 300 g), eluting with $CHCl_3$, $CHCl_3$-EtOAc, EtOAc, EtOAc-MeOH, with gradually increasing quantity of the more polar solvent. The eluates were examined by TLC and 18 groups of eluting fractions (C1-C18) were obtained. Fraction C6 (601 mg) was chromatographed (silica gel, 25 g) using *n*-hexane-EtOAc as eleunt, with increasing percentage of the more polar solvent, to give 13 subfractions. The 10th subfraction was chromatographed, eluted by *n*-hexane-EtOAc with increasing quantity of the more polar solvent, to yield compound **2** (1 mg). Fraction C7 (762 mg) was chromatographed (silica gel, 30 g) using *n*-hexane-$CHCl_3$, $CHCl_3$ and $CHCl_3$-MeOH as eluting solvents, to give 9 subfractions. The 6th subfraction was rechromatographed, with *n*-hexane-EtOAc and EtOAc as eluting solvents, to yield compound **5** (2 mg). The 7th subfraction was subjected to column chromatography in similar manner to that of the previous subfraction to give compound **3** (2 mg). The 8th subfraction was similarly rechromatographed to afford compound **1** (16 mg) and compound **6** (2 mg).

The MeOH extract (25.00 g) was chromatographed (silica gel, 0.063-0.200 mm, 300 g), eluting with EtOAc-MeOH under gradient condition to give 18 groups of eluting fractions (M1-M18). Fraction M7 (167 mg) was chromatographed (silica gel, 15 g), eluting with EtOAc and EtOAc-MeOH, to yield 8 subfractions. The 6th subfraction was rechromatographed twice to give compound **7** (2 mg). Fraction M9 (312 mg) was similarly subjected to two repeated column chromatographies to yield compound **4** (2.5 mg). Fraction M12 (1.03 g) was rechromatographed twice, using EtOAc and EtOAc-MeOH as eluents, to give compound **8** (8 mg).

Isosakuranetin (1)

White needles, mp: 178-179°C; IR ν_{max} cm^{-1}: 3158, 2956, 1636, 1598, 1518, 1496, 1302, 1253, 1164, 833; EIMS *m/z* (% rel. intensity): 286 $[M]^+$ (62), 152 (95), 134 (100), 124 (10); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.77 (1H, dd, *J* = 17.1, 3.0 Hz, H-3a), 3.09 (1H, dd, *J* = 17.1, 13.0 Hz, H-3b), 3.81 (3H, s, 4'-OMe), 5.33 (1H, dd, *J* = 13.0, 3.0 Hz, H-2), 5.84 (1H, br s, 7-OH), 5.95 (1H, d, *J* = 2.2 Hz, H-6), 5.97 (1H, d, *J* = 2.2 Hz, H-8), 6.93 (2×1H, d, *J* = 8.7 Hz, H-3', H-5'), 7.35 (2×1H, d, *J* = 8.7 Hz, H-2', H-6').

Persicogenin (2)

IR ν_{max} cm^{-1}: 3450, 2916, 2848, 1636, 1515, 1458, 1273, 1205, 1156; EIMS *m/z* (% rel. intensity): 316 $[M]^+$ (100), 286 (15), 167 (72), 150 (30), 149 (11), 137 (65); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.77 (1H, dd, *J* = 17.1, 3.0 Hz, H-3a), 3.06 (1H, dd, *J* = 17.1, 12.9 Hz, H-3b), 3.78 (3H, s, 7-OMe), 3.90 (3H, s, 4'-OMe), 5.31 (1H, dd, *J* = 12.9, 3.0 Hz, H-2), 5.66 (1H, br s, 3'-OH), 6.03 (1H, d, *J* = 2.2 Hz, H-6), 6.05 (1H, d, *J* = 2.2 Hz, H-8), 6.86 (1H, d, *J* = 8.3 Hz, H-5'), 6.91 (1H, dd, *J* = 8.3, 2.0 Hz, H-6'), 7.03 (1H, d, *J* = 2.0 Hz, H-2'), 11.95 (1H, s, 5-OH).

5,6,7,4'-Tetramethoxyflavanone (3)

IR ν_{max} cm^{-1}: 2934, 2847, 1677, 1600, 1516, 1484, 1457, 1261, 1200, 1106, 1020; EIMS *m/z* (% rel. intensity): 344 $[M]^+$ (72), 210 (100), 195 (78), 167 (33), 134 (2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.74 (1H, dd, *J* = 16.6, 2.2 Hz, H-3a), 3.01 (1H, dd, *J* = 16.6, 13.3 Hz, H-3b), 3.80, 3.81, 3.84, 3.92 (each 3H, each s, 5-OMe, 6-OMe, 7-OMe, 4'-OMe), 5.33 (1H, dd, *J* = 13.3, 2.2 Hz, H-2), 6.32 (1H, s, H-8), 6.93 (2×1H, d, *J* = 8.5 Hz, H-3', H-5'), 7.36 (2×1H, d, *J* = 8.5 Hz, H-2', H-6').

4'-Hydroxy-5,6,7-trimethoxyflavanone (4)

EIMS *m/z* (% rel. intensity): 330 $[M]^+$ (98), 210 (87), 195 (100), 167 (65), 120 (10); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.74 (1H, dd, *J* = 16.6, 2.8 Hz, H-3a), 2.99 (1H, dd, *J* = 16.6, 13.3 Hz, H-3b), 3.80, 3.85, 3.92 (each 3H, each s, 5-OMe, 6-OMe, 7-OMe), 5.31 (1H, dd, *J* = 13.3, 2.8 Hz, H-2), 6.32 (1H, s, H-8), 6.86 (2×1H, d, *J* = 8.5 Hz, H-3', H-5'), 7.31 (2×1H, d, *J* = 8.5 Hz, H-2', H-6').

2'-Hydroxy-4,4',5',6'-tetramethoxychalcone (5)
EIMS *m/z* (% rel. intensity): 344 $[M]^+$ (35), 210 (100), 195 (71), 167 (27); ^{1}H-NMR (400 MHz, $CDCl_3$) 3.81, 3.84, 3.88, 3.90 (each 3H, each s, 4-OMe, 4'-OMe, 5'-OMe, 6'-OMe), 6.27 (1H, s, H-3'), 6.92 (2×1H, d, J = 8.8 Hz, H-3, H-5), 7.58 (2×1H, d, J = 8.8 Hz, H-2, H-6), 7.82, 7.83 (each 1H, each br s, H-α, H-β), 13.74 (1H, s, 2'-OH).

4,2'-Dihydroxy-4',5',6'-trimethoxychalcone (6)
EIMS *m/z* (% rel. intensity): 330 $[M]^+$ (100), 210 (91), 195 (80), 167 (34); ^{1}H-NMR (400 MHz, $CDCl_3$) 3.81, 3.88, 3.91 (each 3H, each s, 4'-OMe, 5'-OMe, 6'-OMe,), 5.14 (1H, br s, 4-OH), 6.27 (1H, s, H-3'), 6.85 (2×1H, d, J = 8.6 Hz, H-3, H-5), 7.54 (2×1H, d, J = 8.6 Hz, H-2, H-6), 7.80, 7.81 (each 1H, each br s, H-α, H-β), 13.72 (1H, s, 2'-OH).

Acacetin (7)
IR ν_{max} cm^{-1}: 3450, 2927, 1654, 1560, 1508, 1241, 1190, 1165; EIMS *m/z* (% rel. intensity): 284 $[M]^+$ (100), 283 (7), 256 (2), 152 (5), 132 (20), 124 (4), 117 (4), 89 (4); ^{1}H-NMR (400 MHz, $CDCl_3$+CD_3OD, 15:1) δ: 3.84 (3H, s, 4'-OMe), 6.22 (1H, d, J = 1.9 Hz, H-6), 6.38 (1H, d, J = 1.9 Hz, H-8), 6.51 (1H, br s, H-3), 6.96 (2×1H, d, J = 8.8 Hz, H-3', H-5'), 7.79 (2×1H, d, J = 8.8 Hz, H-2', H-6').

Luteolin (8)
mp: 330-332°C; IR ν_{max} cm^{-1}: 3423, 2920, 1654, 1611, 1500, 1367, 1267, 1164, 1032, 839; EIMS *m/z* (% rel. intensity): 286 $[M]^+$ (100), 258 (26), 153 (14), 134 (7); ^{1}H-NMR (400 MHz, $CDCl_3$+CD_3OD, 10:1) δ: 6.21 (1H, d, J = 2.0 Hz, H-6), 6.37 (1H, d, J = 2.0 Hz, H-8), 6.45 (1H, s, H-3), 6.88 (1H, d, J = 8.3 Hz, H-5'), 7.31 (1H, dd, J = 8.3, 2.1 Hz, H-6'), 7.33 (1H, d, J = 2.1 Hz, H-2').

Antimycobacterial assay

The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). Antimycobacterial evaluations of the flavonoids **1** and **3-8** are shown in Table I.

Cytotoxicity assays

The cytotoxicity assays against human epidermoid carcinoma of the mouth (KB), human breast cancer (BC) and human small cell lung cancer (NCI-H187) cells were performed employing colorimetric method (Skehan *et al.*, 1990). The cytotoxicity evaluations of the flavonoids **1** and **3-8** are shown in Table II.

RESULTS AND DISCUSSION

Identification of flavonoids

Four flavanones, **1**, **2**, **3**, and **4**, were isolated from the flowers of *C. odorata*. The ^{1}H-NMR and EI mass spectral data of compound **1** was consistent with 5,7-dihydroxy-4'-methoxyflavanone (isosakuranetin) isolated previously from the leaves of *C. odorata* (Bose *et al.*, 1973). NOE experiment confirmed that the methoxyl group was located at the 4'-position.

Compound **2** was a minor flavanone isolated from this plant species. The ^{1}H-NMR features of **2** were similar to those of compound **1**, except for those of the B-ring which showed a 1,3,4-trisubstituted pattern. The methoxyl group was proven to be at the 4'-position, as judged from the NOE enhancement of H-5' resonance upon irradiation of the methoxyl resonance at δ 3.90. From the spectroscopic (^{1}H-NMR and EI mass spectra) evidence, compound **2** was concluded to be 5,3'-dihydroxy-7,4'-dimethoxyflavanone (persicogenin) isolated previously from *E. sternburgianum* (Gonzalez *et al.*, 1982).

Compound **3** exhibited similar ^{1}H-NMR spectral pattern to that of compound **1**, except for that of the A-ring which

Table I. Antimycobacterial activity of flavonoids

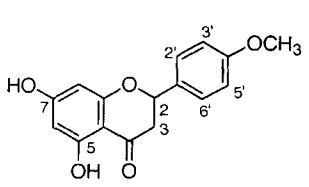
Compound	MIC (μM)
1	174.8
3	inactive[a]
4	606.0
5	inactive[a]
6	inactive[a]
7	704.2
8	699.3
Kanamycin sulfate[b]	4.29
Isoniazid[b]	0.44

[a]Inactive at > 200 μg/mL (> 581.4 μM for compounds **3** and **5**, and > 606.0 μM for compound **6**)
[b]Positive control

Table II. Cytotoxicity of flavonoids

Compound	IC_{50} (μM)		
	KB	BC	NCI-H187
1	inactive[a]	inactive[a]	inactive[a]
3	inactive[a]	inactive[a]	inactive[a]
4	inactive[a]	inactive[a]	inactive[a]
5	inactive[a]	inactive[a]	inactive[a]
6	inactive[a]	inactive[a]	inactive[a]
7	inactive[a]	inactive[a]	24.6
8	inactive[a]	38.4	19.2
Ellipticine[b]	5.40	5.93	1.58

[a]Inactive at > 20 μg/mL (> 69.9 μM for compounds **1** and **8**, > 58.1 μM for compounds **3** and **5**, > 60.6 μM for compounds **4** and **6**, and > 70.4 μM for compound **7**)
[b]Positive control



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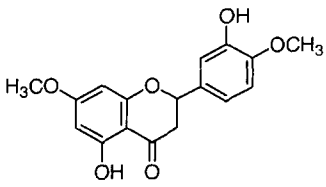

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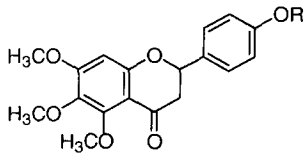

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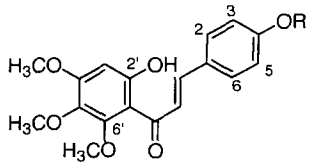
4 R = H



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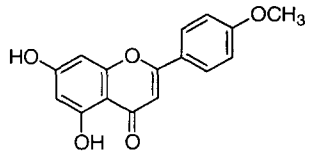
6 R = H



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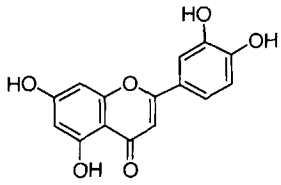

8

showed only one proton as a singlet signal of H-8 at δ 6.32. The ^{1}H-NMR spectral features of compounds **3** and **4** are very similar, except for the presence of an additional methoxyl group in the former. The presence of the fragment ion peak at *m/z* 134 in the EI mass spectrum of **3**, as compared to *m/z* 120 in that of **4** indicated that compound **4** was the 4'-demethoxyl analog of compound **3**. Compound **3** and **4**, therefore, were 5,6,7,4'-tetramethoxyflavanone and 4'-hydroxy-5,6,7-trimethoxyflavanone respectively. These compounds were isolated previously from the aerial parts of *C. odorata* (Barua *et al.*, 1978).

The ^{1}H-NMR spectroscopic data of compounds **5** and **6** showed characteristics of chalcones, the substitution pattern of the A-ring of which was the same as that of compounds **3** and **4**. The EI mass spectral fragmentation confirmed the identity of compounds **5** and **6** with 2'-hydroxy-4,4',5',6'-tetramethoxychalcone and 4,2'-dihydroxy-4',5',6'-trimethoxychalcone isolated respectively from the leaves (Bose *et al.*, 1973) and aerial parts (Barua *et al.*, 1978) of *C. odorata*.

The third group of flavonoids are the flavones **7** and **8**. The ^{1}H-NMR and mass spectral data of compound **7** were consistent with those of acacetin isolated previously from the leaves of this plant (Bose *et al.*, 1974). From the spectroscopic (IR, ^{1}H-NMR and EI mass spectra) data, compound **8** was concluded to be luteolin (Pollock and Stevens, 1965; Mabry *et al.*, 1970), a common flavone isolated from this plant species for the first time.

Biological activities of the isolated flavonoids

Antimycobacterial activity

Compounds **1** and **3-8** were subjected to antimycobacterial evaluations (Table I). The quantity of compound **2** was not sufficient for biological evaluations. The flavanone **1** exhibited moderate antimycobacterial activity against *Mycobacterium tuberculosis*, with the MIC value of 174.8 μM, whereas the flavanone **4**, and the flavones **7** and **8** exhibited weak activity with the MIC values of 606.0, 704.2 and 699.3 μM respectively. Since the flavanones **1** and **4**, and the flavones **7** and **8** were active in the test, it was likely that a double bond at the C-ring was not essential for antimycobacterial activity. Both the chalcones **5** and **6** were inactive in the test, it followed that one of the structural requirements for a flavonoid to exhibit antimycobacterial activity was the presence of ring C, either saturated or unsaturated. The inactivity of the flavanone **3** and the positive bioassay results of compounds **1** and **4** needed special attention. Comparison of the structure of compound **3** with that of compound **4**, it could be seen that methylation at the 4'-hydroxyl group of **4** to yield the corresponding methyl ether **3** resulted in loss of activity. Free 5- and 7-hydroxyl groups as well as the lack of a 6-methoxyl group seemed to increase biological activity. However, the existing data did not permit conclusion of the relatively high antimycobacterial activity of compound **1**

Cytotoxicity

Compounds **1** and **3-8** were evaluated for cytotoxicity against KB, BC and NCI-H187 cells (Table II). All the tested compounds were inactive against the KB cells. Only compound **8** was active against the BC cells with the weak MIC value of 38.4 μM. Compounds **7** and **8** exhibited moderate activity against the NCI-H187 cells with the MIC values of 24.6 and 19.2 μM, respectively. It was noteworthy that among the three classes of flavonoids tested only the flavones were active against some cancer cell lines.

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