Arch Pharm Res Vol 26, No 10, 816-820, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Antimycobacterial and Antioxidant Flavones from *Limnophila geoffrayi*

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(Received August 4, 2003)

The chloroform extract of the aerial part of *Limnophila geoffrayi* showed antimycobacterial and antioxidant activities. Bioassay-guided fractionation has led to the isolation of the flavones nevadensin (5,7-dihydroxy-6,8,4'-trimethoxyflavone, **1**) and isothymusin (6,7-dimethoxy-5,8,4'-trihydroxyflavone, **2**). Both compounds **1** and **2** exhibited inhibition activity against *Mycobacterium tuberculosis*, with equal MIC value of 200 μg/mL. Only compound **2** exhibited antioxidant activity against the radical scavenging ability of DPPH, with the IC_{50} value of 7.7 μg/mL. The crude hexane, chloroform and methanol extracts as well as the pure compounds **1** and **2** did not exhibit mutagenic activity in the *Bacillus subtilis* rec-assay.

Key words: *Limnophila geoffrayi*, Scrophulariaceae, Flavone, Nevadensin, Isothymusin, Antimycobacterial activity, Antioxidant activity, Mutagenic activity

INTRODUCTION

Limnophila geoffragi Bon. (Scrophulariaceae) is used as a vegetative ingredient in a traditional curry in northeastern Thailand. This plant species is used traditionally as an antipyretic, expectorant and lactogogue (Bunyapraphatsara and Chokechaijaroenporn, 2000). According to the northeastern Thai folkloric medicine, the dried whole plants that have been kept for one year is used to prepare a decoction as antidote for detoxification of poisons (Saralamp and Chuakul, 1999). No phytochemical investigation of this plant species has been reported to date. This paper describes the isolation and structural characterization of two flavones, nevadensin (**1**) and isothymusin (**2**), from the aerial part of this plant. Evaluations of antimycobacterial, antioxidant and mutagenic activities of the crude extracts and of compounds **1** and **2** have also been carried out.

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MATERIALS AND METHODS

General experimental procedures

Melting point were determined on an Electrothermal apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer FT-IR Spectrum BX spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 400 spectrometer operating at 400 and 100 MHz respectively. The residual nondeuterated solvent, DMSO-d_6, at 2.49 ppm and the solvent signal at 39.50 ppm were used as references for ^{1}H- and ^{13}C-NMR spectra, respectively. 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 aerial part of *L. geoffragi* was collected from Kamalasai district, Kalasin province, Thailand, in November 2001. A

voucher specimen (Boonmee Hansupo 003) is deposited at the CMU Herbarium, Faculty of Science, Chiang Mai University, Thailand.

Extraction and isolation

The pulverized, dried aerial plant material (615 g) was extracted successively with *n*-hexane, $CHCl_3$ and MeOH in a Soxhlet apparatus to give the hexane (7.52 g), $CHCl_3$ (7.79 g) and MeOH (50.10 g) extracts, respectively. The $CHCl_3$ extract which gave positive results to antimycobacterial and antioxidant assays were chromatographed over silica gel (0.063-0.200 mm, 200 g), eluting with *n*-hexane-$CHCl_3$, $CHCl_3$ and $CHCl_3$-MeOH, with gradually increasing quantity of the more polar solvent. The eluates were examined by TLC and 15 groups of eluting fractions (C1-C15) were obtained. Fraction C11 (4.99 g) was chromatographed (silica gel, 0.063-0.200 mm, 50 g) using $CHCl_3$ and $CHCl_3$-MeOH as eluent, with increasing percentage of the more polar solvent, to give 13 subfractions. The 10th subfraction (110 mg) was further chromatographed to yield nevadensin (**1**) (Farkas *et al.*, 1966) (15 mg). Fraction C13 (537 mg) was similarly chromatographed twice and isothymusin (**2**) (Wang *et al.*, 1999; Kelm *et al.*, 2000) (12 mg) was obtained.

Compound 1 (nevadensin): mp: 199-200°C (pale yellow needles from $CHCl_3$-MeOH); UV λ_{max} (MeOH) nm (log ε): 285 (3.85), 330 (3.83); IR ν_{max} cm^{-1}: 3100, 2965, 2935, 2832, 1659, 1594, 1565, 1500, 1425, 1402, 1356, 1240, 1183, 1125, 1072, 1030, 885, 795; EIMS *m/z* (% rel. intensity): 344 $[M]^+$ (78), 329 (100), 326 (7), 311 (10), 301 (36), 197 (9), 169 (21); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.76 (3H, s, 6-OMe), 3.853 and 3.857 (2×3H, each s, 4'-OMe and 8-OMe), 6.87 (1H, s, H-3), 7.14 (2×1H, d, *J* 8.9 Hz, H-3', H-5'), 8.02 (2×1H, d, *J* 8.9 Hz, H-2', H-6'), 12.75 (1H, s, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 55.5 (4'-OMe), 60.1 (6-OMe), 61.1 (8-OMe), 102.8 (C-10), 103.0 (C-3), 114.7 (C-3', C-5'), 122.9 (C-1'), 128.0 (C-8), 128.1 (C-2', C-6'), 131.6 (C-6), 145.4 (C-9), 148.3 (C-5), 151.1 (C-7), 162.3 (C-4'), 163.0 (C-2), 182.2 (C-4).

Compound 2 (isothymusin): mp: 214-215°C (pale reddish orange prisms from MeOH-EtOAc); UV λ_{max} (MeOH) nm (log ε): 306 (4.07), 330 (3.98); IR ν_{max} cm^{-1}: 3418, 3225, 2952, 2851, 1659, 1605, 1581, 1486, 1444, 1384, 1295, 1227, 1172, 1097, 1065, 1007, 956, 836; EIMS *m/z* (% rel. intensity): 330 $[M]^+$ (100), 329 (6), 315 (86), 312 (19), 301 (4), 297 (29), 287 (10), 197 (32), 169 (20); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.80 (3H, s, 6-OMe), 3.92 (3H, s, 7-OMe), 6.82 (1H, s, H-3), 6.92 (2×1H, d, *J* 8.7 Hz, H-3', H-5'), 8.01 (2×1H, d, *J* 8.7 Hz, H-2', H-6'), 12.43 (1H, s, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 60.4 (6-OMe), 61.1 (8-OMe), 102.4 (C-3), 106.3 (C-10), 115.9 (C-3', C-5'), 121.2 (C-1'), 128.7 (C-2', C-6'), 130.6 (C-8), 136.1 (C-6), 141.3 (C-9), 144.6 (C-5), 148.0 (C-7), 161.3 (C-4'), 164.2 (C-2), 182.7 (C-4).

Antimycobacterial assay

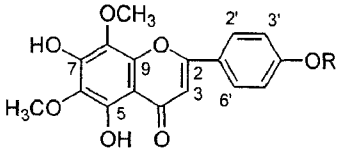
The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). The initial candidate compound dilutions were prepared in DMSO, and subsequent twofold dilutions were performed in 0.1 mL of 7H9GC medium in the microculture plates. 100 μL of 5×10^4 CFU/mL of *M. tuberculosis* in 7H9GC-Tween was added to each well of 96-well microculture plates containing of test compound. Plates were incubated at 37 °C for 7 d. To three control wells which contained drug and medium, bacteria and medium, and medium only, the Alamar Blue dye solution (20 μL of Alamar Blue solution and 12.5 μL of 20% Tween) was added daily until a color change from blue to pink occurred, at which time the dye was added to all remaining wells. Plates were incubated at 37°C, and results were recorded at 24 h post-dye addition. Fluorescence was measured with excitation at 530 nm and emission at 590 nm. Percent inhibition was defined (1 test well FU/mean FU of triplicate control wells)×100. The lowest drug concentration effecting an inhibition of ≥90% was considered the MIC. Experiments were usually completed within 10 d. Standard drugs rifampicin, isoniazid and kanamycin sulfate showed MIC of 0.003-0.0047, 0.025-0.05 and 1.25-2.5 μg/mL, respectively. Compounds **1** and **2** exhibited activity against *M. tuberculosis* with equal MIC value of 200 μg/mL.

Antioxidant assay

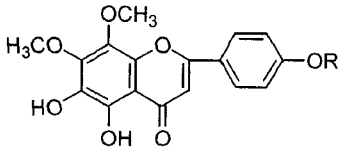
The antioxidant activity was determined on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical by a previously described method (Brand-Williams *et al.*, 1995) with a slight modification. 100 μL of the extract or compound in DMSO was added to 2.9 mL of a DPPH solution (4.5 mg DPPH in 100 mL EtOH) and the mixture was allowed to stand at 30°C for 30 min. The absorbance at 517 nm was then measured. The scavenging effect was determined by comparing the absorbance of the solution containing the test sample to that of control solution without the test sample taking the corresponding blanks. The percentage of the remaining DPPH against the standard concentration was plotted to obtain the amount of antioxidant necessary to decrease by 50% the initial DPPH concentration (IC_{50}). The standard antioxidant 2,6-di-(*tert*-butyl)-4-methylphenol (BHT, IC_{50} value of 5.7 μg/mL) was used as a positive control. Only compound **2** showed antioxidant activity, with the IC_{50} value of 7.7 μg/mL.

Mutagenecity assay

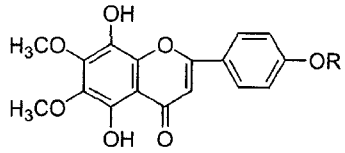
Mutagenecity activity of the crude extracts and of compounds **1** and **2** were tested using *B. subtilis* rec-assay in well diffusion method (Kada *et al.*, 1972). Rec-assay was performed using a DNA repair deficient strain (M45, Rec$^-$) and a DNA repair proficient atrain (H17, Rec$^+$) of *B. subtilis*. M45 and H17 strains were cultured in tryptic soy broth (TSB) overnight at 37°C and the cultures were diluted to 10^6 cells/mL compared with McFarland No. 2. Each *B. subtilis* culture was transferred (0.45 µL) in 15 mL of molten trypsic soy agar (TSA) and poured into sterile level petri dish (90 mm diameter) and well solidified. After boring 5 holes each of 6 mm diameter in each plate, 25 µL of each test sample including water (negative control), mitomycin C (50 µg/mL, positive control) was pipetted into holes. After 24 h of incubation at 37°C, the diameter of the inhibitory zones were measured in mm and expressed as M45/H17 ratio. Active compounds produce a M45/H17 value higher than 1.2. Each assay was repeated three times. It was found that none of the tested extracts and pure compounds showed mutagenic activity in the *B. subtilis* rec-assay.



1 R = CH_3
3 R = H



4 R = H
5 R = CH_3



2 R = H
6 R = CH_3

RESULTS AND DISCUSSION

Identification of the flavonoids

The UV and IR spectra of **1** suggested a flavone class of compound. The ^{1}H-NMR spectrum of **1** exhibited characteristic *p*-disubstituted B-ring as two doublets (J = 8.9 Hz) at δ 7.14 and 8.02, corresponding respectively to H-3'&H-5' and H-2'&H-6'. The singlet signal at δ 6.87 was assigned to H-3. As no other aromatic proton signal was present, it was suggested that the A-ring was fully substituted. The ^{1}H-NMR spectrum revealed the presence of three methoxyl signals at δ 3.76, 3.853 and 3.857. The EI-mass spectrum showed a molecular ion peak at *m/z* 344, corresponding to a penta-oxygenated flavone. One of the methoxyl groups was located at the 4'-position as evident from the NOE enhancement of a methoxyl resonance upon irradiation at the H-3'&H-5' frequency. The hydroxyl group at C-5 was free, as judged from the presence of the proton signal of a chelated hydroxyl group. The remaining two methoxyl groups should, therefore, either be at the 6- and 8-, 6- and 7-, or 7- and 8-positions. ^{13}C-NMR assignments of the carbon resonances of the B- and C-rings were confirmed by 2D experiments. However, the same techniques could not be applied to the fully-substituted A-ring. Comparison of the ^{13}C-NMR data of compound **1** with those of the established data of synthetic 5,7-dihydroxy-6,8-dimethoxy-, 5,8-dihydroxy-6,7-dimethoxy- and 5,6-dihydroxy-7,8-dimethoxyflavones (Horie *et al.*, 1998), it was concluded to be a flavone with a 5,7-dihydroxy-6,8-dimethoxyl substituents. The ^{1}H-NMR and ^{13}C-NMR data of compound **1** were consistent with those of the reported values of nevadensin (Horie *et al.*, 1995; Horie *et al.*, 1998).

Compound **2** showed similar ^{1}H-NMR spectral pattern to that of compound **1**. The only significant difference was the absence of a methoxyl signal in ^{1}H-NMR spectrum of the former. The missing methoxyl resonance was due to the 4'-hydroxyl group was free and this was confirmed by NOE experiment. Thus upon irradiation at the H-3'&H-5' frequency did not cause enhancement of any of the two methoxyl resonances. From the above evidence, it was logical to assume that this compound was the 4'-demethoxy analogue of compound **1** (i.e. compound **3** or demethoxysudachitin (Horie *et al.*, 1962; Greenham *et al.*, 2001). The co-occurrence of compound **1** and compound **3** in *Limnophila rugosa* (Liu *et al.*, 1991) also supported this assumption. However, careful examination of the ^{13}C-NMR of this compound revealed some differences with those of compound **1**. It was expected that the ^{13}C-NMR resonances of the A-ring carbons of **1** and its 4'-demethoxy analogue **3** to be the same or very similar (Horie *et al.*, 1998). In our case, it was found that the C-5 to C-10 resonances of **1** and this compound were different. It was thus possible that the structure of this compound was not **3**. This left the alternative possible structure to be either **2** or **4** (Ferreres *et al.*, 1985). Comparison of the ^{13}C-NMR data of this compound with those of the 4'-methyl ether analogue **5**, some notable differences in chemical shift values were observed, especially those of C-5, C-6, C-8 and C-9. On the other hand, the ^{13}C-NMR data of com-

pounds **2** and **6** were very similar, except for those of the C-4' and the presence of an additional carbon resonance of a methoxyl group. Moreover, the UV absorption bands of **2** was consistent with the reported values (Horie *et al.*, 1995 Barberan *et al.*, 1985), but it was different from those of compound **4** (Horie *et al.*, 1995) and compound **3** (Greenham *et al.*, 2001).

It should be noted that EI-mass spectral data of **2** were consistent with those of the reported values, both the fragmentation patterns and the relative intensity (Barberan *et al.*, 1986). The structure of compound **2** was thus unambiguously concluded to be 6,7-dimethoxy-5,8,4'-trihydroxyflavone (isothymusin).

Biological activities of compounds 1 and 2

Compounds **1** and **2** exhibited antimycobacterial activity with equal MIC value of 200 µg/mL. Since both flavones possess the same oxygenation pattern, this possibly led to a conclusion that different methyl substituents at the 7-, 8- and 4'-hydroxyl groups did not effect antimycobacterial activity of 5,6,7,8,4'-pentaoxygenated flavones.

When subjected to antioxidant evaluation using the scavenging ability of DPPH, only compound **2** was active to the test, with the IC_{50} value of 7.7 µg/mL. The contrast antioxidant activity between these two flavones needed special attention. It was obvious that the free 4'-hydroxyl group contributed to the antioxidant activity of compound **2** by delocalization of the electron at the 4'-hydroxyl group to the 4-keto group after the 4'-hydrogen being abstracted. The *p*-hydroquinone nature of the A-ring possibly also contributed to the relatively high antioxidant activity of **2**. It should also be noted that the free 7-hydroxyl group of compound **1** did not exert any radical scavenging activity by similar mechanism to that of the free 4'-hydroxyl group of compound **2**. One possible explanation was that, regardless of the radical scavenging ability of BHT, it was too sterically hindered. The antioxidant activity of compound **2** has previously been evaluated by a different system (Wang *et al.*, 1999; Kelm *et al.*, 2000).

Since *L. geoffragi* is used as a food ingredient, the crude extracts of the aerial part of this plant and the pure compounds **1** and **2** were evaluated for mutagenic activity. The results indicated that none of the tested extracts and compounds exhibited this activity. It was therefore beneficial that the tests were negative.

ACKNOWLEDGEMENTS

We thank the Biodiversity Research and Training Program (BRT) for financial support. Bioassay Research Facility of National Center for Genetic Engineering and Biotechnology (BIOTEC) is gratefully acknowledged for antimycobacterial assay. We are grateful to James F. Maxwell, Faculty of Science, Chiang Mai University, for identifying the plant specimen.

REFERENCES

Barberan, F. A. T., Ferreres, F., and Tomas, F., TLC, UV and acidic treatment in the differentiation of 5,6- and 5,8-dihydroxyflavones, 3-methoxyflavones and flavonols. *Tetrahedron*, 41, 5733-5740 (1985).

Barberan, F. A. T., Ferreres, F., Tomas, F., and Guirado, A., Electron impact mass spectrometric differentiation of 5,6-dihydroxy-7,8-dimethoxy- and 5,8-dihydroxy-6,7-dimethoxyflavones. *Phytochemistry*, 25, 923-925 (1986).

Brand-Williams, W., Cuvelier, M. E., and Berset, C., Use of a free radical method to evaluate antioxidant activity. *Food Sci. Technol.*, 28, 25-30 (1995).

Bunyapraphatsara, N. and Chokechaijaroenporn, O., Faculty of Pharmacy, Mahidol Univercity and National Center for Genetic and Engineering and Biotechnology. *Thai Medicinal Plants*, 3, 53 (2000).

Collins, L. A. and Franzblau, S. G., Microplate Alamar Blue Assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob. Agents Chemother.*, 41, 1004-1009 (1997).

Farkas, L., Nogradi, M., Sudarsanam, V., and Herz, W., Constituents of *Iva* species. V. Isolation, structure, and synthesis of nevadensin, a new flavone from *Iva nevadensis* M. E. Jones and *Iva acerosa* (Nutt.) Jackson. *J. Org. Chem.*, 31, 3228-3231 (1966).

Ferreres, F., Barberan, F. A. T., and Tomas, F., 5,6,4'-Trihydroxy-7,8-dimethoxyflavone from *Thymus membranaceus*. *Phytochemistry*, 24, 1869-1871 (1985).

Greenham, J., Vassiliades, D. D., Harborne, J. B., Williams, C. A., Eagles, J., Grayer, R. J., and Veitch, N. C., A distinctive flavonoid chemistry for the anomalous genus *Biebersteinia*. *Phytochemistry*, 56, 87-91 (2001).

Horie, T., Kawamura, Y., Yamamoto, H., Kitou, T., and Yamashita, K., Synthesis of 5,8-dihydroxy-6,7-dimethoxyflavones and revised structures for some natural flavones. *Phytochemistry*, 39, 1201-1210 (1995).

Horie, T., Ohtsuru, Y., Shibata, K., Yamashita, K., Tsukayama, M., and Kawamura, Y., ^{13}C-NMR spectral assignment of the A-ring of polyoxygenated flavones. *Phytochemistry*, 47, 865-874 (1998).

Horie, T., Shimoo, H., Masumura, M., and Okumura, S., Demethoxysudachitin. *Nippon Kagaku Zasshi*, 83, 602-604 (1962).

Kada, T., Tutikawa, K., and Sadaie, Y., *In vitro* and host-mediated "rec-assay" procedures for screening chemical mutagens, and phloxine, a mutagenic red dye detected. *Mutation Res.*, 16, 165-174 (1972).

Kelm, M. A., Nair, M. G., Strasburg, G. M., and DeWitt, D. L.,

Antioxidant and cyclooxygenase inhibitory phenolic compounds from *Ocimum sanctum* Linn. *Phytomedicine*, 7, 7-13 (2000).

Liu, M. C., Chen, Z. S., Chung, L. C., Yang, M. S., Ho, S. T., and Chen, M. T., Studies on hypotensive constituents of *Limnophila rugosa*. *Chung-hua Yao Hsueh Tsa Chih*, 43, 35-40 (1991).

Saralamp, P. and Chuakul W., editors. *Encyclopaedia of Medicinal Plants, Vol. 4: Kok Ya E-sarn (Medicinal Plants of the Northeast)*. Mahidol University Foundation, Bangkok, p. 159, (1999).

Wang, H., Nair, M. G., Strasburg, G. M., Booren, A. M., and Gray, J. I., Antioxidant polyphenols from tart cherries (*Prunus cerasus*). *J. Agri. Food Chem.*, 47, 840-844 (1999).