In vitro Cytotoxic Activity of Biflavonoid against P388 Murine Lymphocytic Leukemia Cells

Jae Sook Lee, Seung Hwa Baek[1]*

Department of Beauty Coordiation, Cheongyang College,
1: Department of Herbal Resources, Professional Graduate School of Oriental Medicine and Institute of Basic Natural Sciences, Wonkwang University

Biflavonoid (1) showed no antimicrobial activity at a concentration of 150 μg/disc. However, the crude extract of *Quintinia acutifolia* Kirk inhibited the growth of *Bacillus subtilis* and the dermatophytic fungus *Trichophyton mentagrophytes*. 2",3"-Dihydroochanaflavone (1) showed some cytotoxicity with IC_{50} value of 3.1 μg/mL against P388 murine lymphocytic leukemia cells (positive control: mitomycin C IC50 0.06 μg/mL). The structure was determined by spectroscopic methods.

Key words : Biflavone (1), cytotoxic activity, P388 murine lymphocytic leukemia cells, spectroscopic methods

Introduction

Q. acutifolia grows in lowland and higher mountain forests and in found in the North Island and northern South Island[1]. The *Quintinia* chemistry is of several flavonols, iridoids, proanthocyanidines and alkaliods from the plants of Escalloniaceae[2], The crude extract of *Quintinia acutifolia* Kirk inhibited the growth of the Gram positive bacterium *Bacillus subtilis* ATCC 19659, (3 mm inhibition zone) and the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (3 mm inhibition zone) at 150 μg/disc, and cytotoxic to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC50 50,000 μg/mL) at 150 μg/disc. However, *Candida albicans* (ATCC 14053) did not observed the antimicrobial activity and the cytotoxic activity to BSC monkey kidney cells (@ 5 mg/mL) at 150 μg/disc[3]. In this study, the cytotoxic activity of 2",3"-dihydroochana- flavone (1) which isolated from *Q. acutifolia* was examined.

Materials and Methods

1. General experimental procedures.

Solvents for extraction and chromatography were distilled prior to use. Preparative silica gel TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254}, visualized with an UV lamp then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) and heating. UV spectrum was recorded with a Jasco V-550 UV spectrophotometer. IR spectrum was obtained with a Perkin-Elmer 1600 FTIR as a film on a NaCl disk. NMR spectra were recorded at 25℃ on a Varian INOVA 500 NMR spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C, using solvent signals as references ($CHCl_3$ at 7.25 ppm, $CDCl_3$ at 77.0 ppm). DEPT, HSQC, CIGAR and NOESY experiments were run at 25℃. EIMS was obtained on a VG70-250S double-focusing magnetic sector mass spectrometer. Column chromatography used octadecyl-functionalized sillica gel (Aldrich C_{18}) and 40-63 μm silica gel 60 (Merck). TLC was carried out on silica gel F_{254} plates (Merck), with the solvent system hexane - ethyl acetate(9 : 1).

2. Plant materials

Leaves of *Quintinia acutifolia* (*Q. acutifolia*) were collected in June 2001 from the Botanic Gardens, Dunedin, New Zealand, and were identified by A. Evans. Voucher specimens (010615-01) have been deposited in the Plant Extracts Unit Herbarium, Chemistry Department, University of Otago, Dunedin, New Zealand.

3. Preparation of the extract

Air-dried *Q. acutifolia* leaves (100.1 g) were ground in a in a Waring Blender, with ethanol (3 x 500 ml) and chloroform (500 ml). The combined extracts were filtrated, and the solvent was evaporated in vacuo. This afforded a brown-green solid

* To whom correspondence should be addressed at : Seung Hwa Baek, Department of Herbal Resources, Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan 570-749, Korea.
· E-mail : shbaek@wonkwang.ac.kr, · Tel : 063-850-6225
· Received : 2006/07/15 · Revised : 2006/08/16 · Accepted : 2006/09/15

mass (20.56 g) which was stored at 4℃ until tested.

4. Extraction and isolation.

Air dried *Q. acutifolia* leaves (100.1 g) was collected, extracted with ethanol (3 x 500 mL), $CHCl_3$ (1 x 500 mL), and concentrated under vacuum to give a deep green powder (20.55 g). The subsample (10.0 g) of the crude extract was fractionated by RP chromatography over C_{18} Si gel [10.0 g precoated on C_{18} Si gel (20.0 g), loaded onto a C_{18} column (90 g)]. The column was eluted with solvent mixtures of H_2O - CH_3CN - $CHCl_3$ - EtOH - hexane in decreasing polarity to give eleven fractions (H_2O - CH_3CN - $CHCl_3$ - EtOH - hexane ratio, solvent volume, mass): A, 1:0:0:0:0, 180 mL, 188 mg; B, 9;1:0:0:0, 180 mL, 185 mg; C. 3:1:0:0:0, 180 mL, 430 mg; D, 1:1:0:0:0, 180 mL, 300 mg; E, 1:3:0:0:0, 180 mL, 900 mg; F, 1:9:0:0:0, 180 mL, 1,300 mg; G, 0:1:0:0:0, 180 mL, 1,134 mg; H, 0:1:1:0:0, 180 mL, 2,800 mg; I, 0:0:1:0:0, 180 mL, 700 mg; J, 0:0:0:1:0, 180 mL, 188 mg; K, 0:0:0:0:1, 180 mL, 308 mg. P388 and *B. subtilis* activity was noted in fractions 6 and 7, and these were chosen for further investigation. The separation was repeated to gain additional material. These fractions (2.40 g) was precoated onto Si gel (4.80 g) and further fractionated on Si gel (150 g). This column was eluted with ethyl acetate - hexane (15% 340 mL; 20-25% 400 mL; 25% 620 mL; 25-30% 520 mL; 30-35% 490 mL; 40-45% 585 mL; 50-80% 1,040 mL; 100%, 50% ethyl acetate - methanol 850 mL). Altogather, 8 fractions of progressively increasing polarity were subsequently collected (mass): Fr. 1, 7.5 mg; Fr. 2, 170 mg; Fr. 3, 19.4 mg; Fr. 4, 43 mg; Fr. 5, 68 mg; Fr. 6, 150 mg; Fr. 7, 135 mg; Fr. 8, 1,752 mg. The pure compound (1) was able to be obtained by semipreparative HPLC using the method outlined below. (1, 6.2 mg, Rt 10.576 min)[4]: cream-colored powder; $[\alpha]^{24.0}_{589}$ +426°, $[\alpha]^{24.2}_{577}$ +362°, $[\alpha]^{24.4}_{546}$ +294°, $[\alpha]^{24.7}_{435}$ -150°, $[\alpha]^{25.1}_{405}$ -297° (c 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 290 (4.344), 329 (4.173), 323 (4.484, NaOH), 3.97 (4.266, NaOH) nm; IR (film) ν_{max} 3411, 3250, 2954, 1702, 1640, 1611, 1507, 1465, 1438, 1353, 1305, 1261, 1258, 1224, 1162, 1126, 1067, 1030, 1014, 999, 977, 947, 889, 874, 834, 770, 744, 694, 641, 597, 561, 511 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ (1H, 6.78 s, 3-H), (1H, 6.35 s, 6-H), (1H, 6.62 s, 8-H), (1H, 7.89 d, J= 2.0 Hz, 2'-H), (1H, 7.33 d, J= 8.5 Hz, 5'-H), (1H, 7.96 dd, J= 7.3, 1.5 Hz, 6'-H), (1H, 5.66 dd, J=13.0, 3.0 Hz, 2''-H), (1H, 3.30, dd, J= 17.3, 13.0 Hz, α2.91, dd, J=17.0, 3.0 Hz, β 3''-H), (1H, 6.06 d, J=1.0 Hz, 6'''-H), (1H, 6.08 s, 8''-H), (1H, 7.65, d, J=8.5 Hz, 2'''-H), (1H, 7.13, d, J=8.5 Hz, 3'''-H), (1H, 7.13, d, J=8.5 Hz, 5'''-H), (1H, 7.65, d, J=8.5 Hz, 6'''-H), (OH, 13.04, s, 5-OH), (OH, 12.25, s, 5''-OH); ^{13}C-NMR ($CDCl_3$) δ 161.8 (C-2), 105.5 (C-3), 184.8 (C-4), 103.9 (C-4α), 164.8 (C-5), 100.4 (C-6), 166.0 (C-7), 95.5 (C-8), 159.8 (C-8α), 125.0 (C-1'), 121.8 (C-2'), 144.7 (C-3'), 155.7 (C-4'), 119.4 (C-5'), 126.0 (C-6'), 80.2 (C-2''), 44.1 (C-3''), 198.0 (C-4''), 102.4 (C-4''α), 165.7 (C-5''), 97.6 (C-6''), 168.1 (C-7''), 96.6 (C-8''), 161.8 (C-8''α), 134.8 (C-1'''), 129.7 (C-2'''), 118.0 (C-3'''), 159.4 (C-4'''), 118.0 (C-5'''), 129.7 (C-6''').

5. HPLC Method for analysis of 2'',3''-dihydroochanaflavone (1)

Analysis was carried at 25℃ on a C_{18} column (Phenomenex Prodigy ODS (3), 5 μm, 100 A, 250 x 4.6 mm) with a 2 x 4 mm C18 guard column. The following gradient program was used (time {min}, ratio of CH_3CN-H_2O each containing 0.1% trifluoroacetic acid). The flow rate was 4.5 mL/min, with an injection volume of 5 μL. Detection was at 280 nm, and the retention time was 10.576 min (1).

6. Screening for antiviral activity

The extract was applied (30 μL of a 5 mg/mL solution) to a small filter-paper disc, dried, and assayed for antiviral activity using Schroeder et al.s methods[5]. The results were observed either cell death (cytotoxicity), inhibition of virus replication, no effect (i.e., all of the cells show viral infection), or a combination of all three. The results were noted as the approximate size of the circular zone, radiating from the extract sample, from 1+ to 4+ representing 25% through to whole well sized zones. The notation used is inhibition/antiviral activity. The type of antiviral effect, indicated by a number after the size of the zone, was also considered important and may give some indication as to the mode of cytotoxic action.

7. Screening for antibacterial and antiyeast activities

Activity against the following bacterial strains and yeast was tested: multiresistant *Bacillus subtilis* (ATCC 19659), *Staphylococcus aureus* (ATCC 6538P) *Escherichia coli* (ATCC 25922), and *Candida albicans* (ATCC 14053). Extracts were dissolved and diluted in an appropriate solvent (usually ethanol : water) to a concentration of 5 mg/mL. Test plates are prepared from Mueller Hinton agar containing extract to give a final concentration of 100 μg extract/mL agar. Activity growing cultures of the test strains were diluted in saline so as to deliver 10^4 colony forming units onto the test, control (solvent), and blank (agar only) plates with a multipoint inoculator. Inoculated plates were incubated overnight at 37℃. Growth on the blank and control plates was checked and, if satisfactory, growth on the test plates was scored for each test strain as follows: (-) inhibition, no reduction in growth compared with the control, (+) inhibition, no growth. Solutions of compound for assay were dried onto 6 mm filter paper disks, which were then placed onto seeded agar Petri dishes

and incubated. Activity was observed as a zone of inhibition around the disk, with its width recorded from the edge of the disk in mm. HM and SM refer to the observed margin surrounding the zone of inhibition. (H= hazy, S= sharp).

8. Screening for antifungal activity

Activity against the following fungal strain was tested: *Trichophyton mentagraphytes* (ATCC 28185) local strain]. Fungal spore suspensions of the test oraganisms were applied to dextrose agar plates. Aliquots of the extract solutions were applied to filter paper discs, at 30 μg extract/disc, and dired at 37℃ for two hours. These discs were applied to the agar plates, two per plate, and incubated at 28℃.

9. Screening for cytotoxic activity

This is a measure of the ability of a sample to inhibit the multiplication of murine leukaemia cells. The sample was dissolved in a suitable solvent, usually ethanol, at 5 mg/mL, and 30 μL of this solution was placed in the first well of a multiwell plate. Seven two-fold dilutions were made across the plate. After addition of the cell solution, the concentration range in the test wells was 25,000 down to 195 ng/mL. After incubation for three days, the plates were read using an ELISA palte reader at 540 nm wavelength. Automated reading of the plates was possible with the addition of a MTT tetrazolium salt (yellow color). Healthy cells reduce this salt to MTT formazan (purple color).

Results and Discussion

An crude extract of *Q. acutifolia* was prepared by grinding dried plant material and extracted with ethanol and chloroform. A crude extract was cytotoxic to P388 murine leukaemia cells ATCC CCL 46 P388D1, (IC_{50} 50 μg/mL) and not cytotoxic to BSC monkey kidney cells (@ 5 mg/mL at 150 μg/disc). The main cytotoxic components were biflavonoids. Table I does not show the antiviral activity against *Herpes simplex* Type I *virus* (ATCC VR 733) and *Polio* Type I *virus* (Pfizer vaccine strain) (@ 5 mg/mL at 150 μg/disc). The crude extract inhibited the growth of the Gram-positive bacterium and fungus of the extract prepared from *Q. acutifolia*. As indicated in Table 1, this crude extract inhibited the growth of the Gram-positive bacterium *Bacillus subtilis* ATCC 19659, (3 mm inhibition zone at 150 μg/disc) and the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (3 mm inhibition zone at 150 μg/disc). No activity was observed against the fungus *Candida albicans* (ATCC 14053) at 150 μg/disc. This extract showed weaker antimicrobial activity than chloramphenicol and nystatin(Table 1)[6].

Table 1. Biological activities of the crude extract from *Quintinia acutifolia*

Assay	Tested material			
	Crude extract	Chloramphenicol	Nystatin	Mitomycin C
Cytotoxicity[a]				
BSC-1 cells	-			
P388				
IC_{50}	50,000[b]			60.0[c]
Antiviral activity[d]				
Herpes simplex virus	-			
Polio virus	-			
Antimicrobial activity[e]				
B. subtilis	SM 3	SM 12	0	
C. albicans	-	0	SM 11	
T. mentagrophytes	SM 3	0	HM 8	

[a]% of well showing cytotoxic effects, with virus growing in cytotoxic zone. @ 5 mg/mL, 150 μg/disc: -: no activity. BSC-1 cells: African green monkey kidney cells. [b]Toxicity of sample to P388 murine leukaemia cells (ATCC CCL 46 P388D1) in ng/mL at 150 μg/disc. [c]Toxicity of sample to P388 murine leukaemia cells (ATCC CCL 46 P388D1) in ng/mL at 0.06 μg/disc. P388 : Concentration of the sample required to inhibit cell growth to 50% of a solvent control. [d]Antiviral assays. @ 5 mg/mL, 150 μg/disc: Zone of cytotoxic activity: -: no activity. [e]Width of zone of inhibition in mm: 150 μg/disc: -: no reduction in growth, 0: not determined. Chloramphenicol: 30 μg/disc, Nystatin: 100 unit/disk. SM: Sharp margin, HM: Hazy margin, numbers refer to zone of inhibition (mm)

2″,3″-Dihydroochanaflavone (1) exhibited a broad OH absorption band at 3411 cm^{-1} in the IR spectrum. There were two distinct carbonyl absorption bands at 1640 cm^{-1} (consistent with a flavanone) and 1611 cm^{-1} (more typical of a flavone). Like the previous three compound, 1 showed optical activity, constistent with at least one flavanone unit, The ^{1}H-NMR spectrum showed no methoxyl proton signals, two intramolecular hydrogen-bonded hydroxyl proton signals (δ 12.25, and 13.04). A flavone/flavanone structure was further implicated by the presence of only one ABX system characteristic of a flavanone (δ 5.66, dd, J=13.0, 3.0 Hz; 3.30, dd, J=17.3, 13.0 Hz, 2.91, dd, J= 17.0, 3.0 Hz), along with a singlet at δ 6.78, consistent with a flavone. This compound was identified as 2″,3″-dihydroochanaflavone (1). This compound has been reported twice previously, once from *Ochna interrima*[4] and once from *Luxemburgia nobilis*, both members of the Ochnaceae. Our ^{1}H-NMR data (in obtained in acetone-d[6] and ^{13}C-NMR data (in DMSO-d_6) matched those of the compound reported from Ochna interrima[4]. An alternative structure, 2″,3″-dihydroochanaflavone (1), has been reported from Ochna obtusata[7]. Although there were some similarities between the ^{1}H-NMR spectra of 1 and those reported for 2″,3″-dihydroochanaflavone, there were significant differences in the chemical shifts of the protons in the 1,3,4-trisubstituted benzene rings.

A quantitative HPLC method for analysis of 2″,3″-dihydroochanaflavone (1) was developed (Fig. 5). Examination of ethanol extracts of leaf samples from two specimens of *Q. acutifolia* (970128-14 and 010615-01) showed

that compound (1) was present in ratios of 1.40 : 3.48. 2'',3''-Dihydroochanaflavone (1) showed no antimicrobial activity at a concentration of 150 μg/disk, but this compound (1) showed some cytotoxic activity with IC_{50} 3.1 μg/mL against P388 murine lymphocytic leukemia cells.

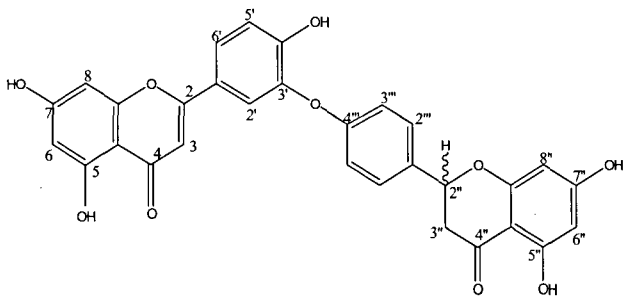

Fig. 1. The structure of 2'',3''-dihydroochanaflavone (1).

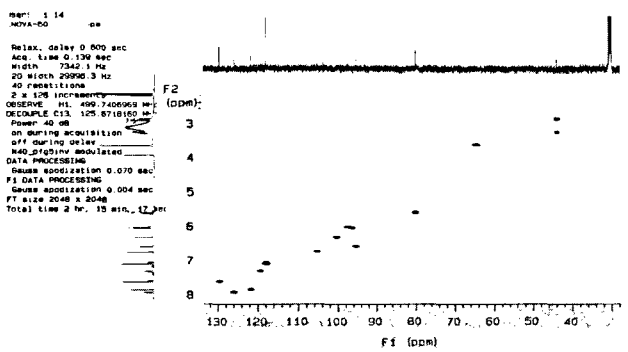

Fig. 2. HSQC Spectrum of 2'',3''-dihydroochanaflavone (1).

Fig. 3. CIGAR Spectrum of 2'',3''-dihydroochanaflavone (1).

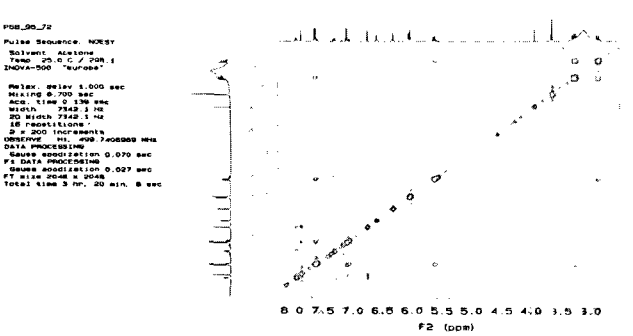

Fig. 4. NOESY Spectrum of 2'',3''-dihydroochanaflavone (1).

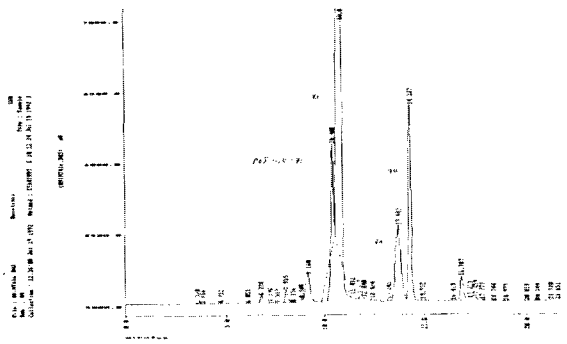

Fig. 5. HPLC Chromatogram of 2'',3''-dihydroochanaflavone (1)

Table 2. Cytotoxic activity of 2'',3''-dihydroochanaflavone (1).

Cytotoxicity	Tested material	
	1	Mitomycin C
P388 IC_{50}	3.1[a]	0.06[b]

[a]Cytotoxicity of sample to P388 murine leukaemia cells (ATCC CCL 46 P388D1) in μg/mL. [b]Cytotoxicity of sample to P388 murine leukaemia cells (ATCC CCL 46 P388D1) in μg/mL. P388 : Concentration of the sample required to inhibit cell growth to 50% of a solvent control.

In conclusion. the crude ethanol extract of *Q. acutifolia* leaves inhibited the growth of *Bacillus subtilis* ATCC 19659, and the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, and cytotoxic to P388 murine leukaemia cells ATCC CCL 46 P388D1, at 150 μg/disc. However, 2'',3''-dihydroochanaflavone (1) showed no antimicrobial activity at a concentration of 150 μg/disk, but this compound (1) showed some cytotoxic activity with IC_{50} 3.1 μg/mL against P388 murine lymphocytic leukemia cells.

Acknowledgements

We thank Dr. N.B. Perry at the Plant Extracts Research Unit, New Zealand Institute for Crop & Food Research Ltd, Department of Chemistry, University of Otago in New Zealand. This work was supported by Wonkwang University in 2006.

References

1. Fisher, M.E. Growing New Zealand Plants, Shrubs and Trees, David Batmen: Auckland, 1994.
2. http://www.biodiversity.uno.edu/delta/angio/ww/escallon.htm
3. Lee, J.S., Chung, J.G., Oh, H.J., Na, Y.S., Baek, S.H. Antifungal activity of the crude extract from Quintinia acutifolia on the dermatophytic fungus. Kor. J. Orien. Physiol. Pathol., 19:508-510, 2005.
4. Likhitwitayawuid, K., Rungserichai, R., Ruangrungsi, N., Phadungcharoen, T. Flavonoids from Ochna integerrima. Phytochem., 56:353-357, 2001.

5. Schroeder, A.C., Hughes, R.G. Jr, Bloch, A. Synthesis and biological effects of acyclic pyrimidine nucleoside analogues. J. Med. Chem., 24:1078-1083, 1981.
6. Baek, S.H., Lim, J.A., Kwag, J.S., Lee, H.O., Chun, H.J., Lee, J.H., Perry, N.B. Screening for biological activity of crude extract and bioactive fractions from Brochyglottis monroi. Kor J Orien Physiol & Pathol 17(3):826-828, 2003.
7. Rao, K.V., Sreeramulu, K., Rao, C.V., Gunasekar, D., Martin, M.T., Bodo, B. Two new biflavonoids from Ochna obtusata. J. Nat. Prod., 60:632-634, 1997.