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In vitro Anti-Cancer Effect of Wellness-Compound (Ochnaflavone)

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In vitro 웰니스 화합물 (Ochnaflavone)에 의한 암세포 성장 저해

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Abstract Medicinal plants containing wellness-fusion-complex compound are increasingly being pursued as suitable alternative sources of various biological properties. In this study, inhibitory effect of *Quintinia acutifolia*, which is a New Zealand plant, on P388 murine lymphocytic leukemia cells using MTT [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl-tetrazolium bromide] assay. Based on ^{1}H-NMR, ^{13}C-NMR spectral data and other spectral analysis, 2,3,2",3"-tetrahydroochanaflavone (1) and 2",3"-dihydroochana-flavone (3) inhibited the leukemia cells were purified from the plants. 2,3,2",3"-tetrahydroochanaflavone (1) and 2",3"-dihydroochana-flavone (3) are biflavonoids possessing two basic flavonoids and actively inhibited growth of P388 murine lymphocytic leukemia cells with a 50% inhibitory concentration (IC_{50}) of 8.2 μ g/mL and 3.1 μ g/mL, respectively. Specially, 2",3"-dihydroochana-flavone (3) possessed unconjugated flavonone system, which isn't consist of a pair with B ring of 2,3,2",3"-tetrahydroochanaflavone (1). Therefore, the two compounds could be considered as a candidate for development of anticancer drugs and need to much studies in the future.

Key Words : Wellness-fusion-complex, Anti-cancer, Funtional materials, Biflavonoid, New Zealand plant, P388 murine lymphocytic leukemia cells

요 약 많은 식물들은 부작용이 적고, 가격이 저렴하며, 다양한 웰니스 융복합 화합물들을 함유하고 있기 때문에 다양한 제재에 이용되고 있다. 이 연구에서 뉴질랜드 식물인 *Quintinia acutifolia (Q. acutifolia)*로부터 쥐 백혈병 세포(P388 murine lymphocytic leukemia cells)의 성장을 저해하는 활성을 MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay에 의해 평가하였다. P388 murine lymphocytic leukemia 세포의 성장을 저해하는 2,3,2'',3''-tetrahydroochanaflavone (1)과 2'',3''-dihydroochana-flavone (3)을 1D/2D-NMR와 다른 분광학적 분석법에 의해 분리하였고, 구조를 규명하였다. 이 두 화합물은 두 개의 플라보노이드 기본구조를 갖는 바이플라보노이드(biflavonoid)로써 2,3,2'',3''-Tetrahydroochnaflavone (1)과 2'',3''-dihydroochana-flavone (3) 화합물은 P388 murine lymphocytic leukemia세포에 대해 50%의 성장저해를 나타내는 농도가 각각 8.2 μ g/mL와 3.1 μ g/mL로 나타났다. 특히 2'',3''-dihydroochana-flavone (3) 화합물은 2,3,2'',3''-tetrahydroochanaflavone (1)의 B 링(ring)에 쌍으로 결합되지 않은 플라본 구조 (unconjugated flavonone system)를 갖는 것으로 나타났다. 그럼으로 두 화합물은 향후 항암 치료제 개발에 이용될 수 있으며, 더 많은 연구가 요구된다.

주제어 : 웰니스 융복합, 항암, 생리활성, Biflavonoid, 뉴질랜드 식물, P388 murine lymphocytic leukemia cells

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1. Introduction

The chemistry of *Quintinia* has been explored very little. In 1918, *Q. serrata* bark extracts were examined in a survey of tannin contents of barks of some New Zealand flora [1]. The first report of individual chemical constitutents indentified, by paper chromatography, a flavonoid glycoside, astilbin (dihydroquercetin-3-rhamnoside), and its aglycone, dihydroquercetin, in a methanol extract.

More recently, flavones and flavonols and hydrocinnamates were noted during an investigation of the functional role of anthocyannins in the leaves of the same species [2,3,4,5,6,7,8]. The flavonoids identified were glycosides of quercetin and its 3′-methyl derivatives, isorhamnetin. Jayaprakasam et.al. investigated the leaves of *O. beddomei* and reported 7-o-methyltetrahydroochnaflavone, a new biflavanone, together with nine known flavonoids. The structures of ten compounds were elucidated by comparison of their physical and spectral data with the published data and spectroscopic methods [9,10,11,12]. Rao et al. reported that the isolation and characterization of two new biflavonoids. The acetone extract of *O. obtusata* was chromtographed over a Si gel column to yield 7-o-methylether and 2,3-dihydro- ochnaflavone by spectral and chemical transformation studies [4]. In this study, the cytotoxic activity of 2,3,2′′,3′′-tetrahydroochanaflavone (**1**) which isolated from *Q. acutifolia* was examined. The structure of cytotoxic compound (**1**) was determined by ^{1}D/^{2}D-NMR and other spectroscopic methods.

2. Materials and Methods

2.1 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 ^{1}H 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).

2.2 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.

2.3 Extraction and isolation

Isolation and identification from *Q. acutifolia* leaves was conducted by a previous study [5]. Briefly, 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. 2,3,2′′,3′′- Tetrahydroochanaflavone (**1**) was able to be obtained by semi- preparative HPLC using the method outlined below. (**1**, 1.0 mg, Rt 10.77 min) [4]: pale brown powder; optical rotation (c, 0.05, MeOH) $[\alpha]^{21.3}_{589}$ +451°, $[\alpha]^{21.9}_{577}$ +403°, $[\alpha]^{23.1}_{546}$ +323°, $[\alpha]^{22.8}_{435}$ -140°, $[\alpha]^{23.4}_{405}$ -236°; UV (MeOH) λ_{max} (log ε) 207 (4.67), 288 (4.38), 329 (3.91), (MeOH + NaOH), 209 (4.84), 323 (4.59), 396 (3.17) nm; IR (KBr) ν_{max} 3414, 1641, 1508, 1465, 1157 cm^{-1}. ^{1}H-NMR (Me_2CO-D_6) δ (1H, 5.57 dd, J= 3.5, 12.5 Hz, 2-H), (1H, 3.30, trans, dd, J= 5.0, 1.0 Hz, 3-H), (1H, 2.89, cis, t, J= 2.0 Hz, 3-H), (1H, 6.04 dd, J= 2.3, 1.0 Hz 6-H),[a] (1H, 6.07 t, J= 2.0 Hz 8-H),[b] (1H, 7.38 dd, J= 2.5, 1.0 Hz, 2′-H), (1H, 7.20 d, J= 8.5 Hz, 5′-H), (1H, 7.39 d, J= 2.5 Hz, 6′-H), (1H, 5.62, dd, J=3.0, 12.8 Hz, 2′′-H), (1H, 3.27, dd, trands, J= 1.0, 4.5 Hz, 3′′-H), ((1H, 2.86, t, J= 2.5 Hz, 3′′-H), (1H, 6.05, t, J=2.0 Hz, 6′′-H),[a] (1H, 6.05, t, J= 2.0 Hz, 8′′-H), (1H, 7.61, dd, J=8.5, 2.0 Hz, 2′′′-H), (1H, 7.07, dd, J=6.8, 2.06 Hz, 3′′′-H), (1H, 7.07, dd, J=6.8, 2.0 Hz, 5′′′-H), (1H, 7.61, d, J=8.5, 2.0 Hz, 6′′′-H), (OH, 12.14, s, 5-OH),[c] (OH, 9.12, brs, 7-OH), (OH, 10.38, brs, 7′′-OH), (OH, 12.15, s, 5′′-OH)[c];[a] Recorded in acetone-d_6 at 500 MHz and 25°C, referenced to solvent (δ 2.05). [b,c]Values with the same label within a column may be interchanged. ^{13}C-NMR ($CDCl_3$) δ 80.06 (C-2), 44.16 (C-3), 197.53 (C-4), 103.63 (C-4a), 165.56 (C-5), 96.55 (C-6), 168.25 (C-7), 96.55 (C-8), 164.79 (C-8a), 132.56 (C-1′), 12174 (C-2′), 144.12 (C-3′), 150.65 (C-4′), 118.37 (C-5′), 125.50 (C-6′), 80.06 (C-2′′′), 44.08 (C-3′′), 197.53 (C-4′′), 103.69 (C-4′′a), 165.85 (C-5′′), 97.03 (C-6′′), 166.96 (C-7′′), 97.45 (C-8′′), 164.75 (C-8′′a), 134.39 (C-1′′′), 129.59 (C-2′′′), 117.93 (C-3′′′), 159.91 (C-4′′′), 117.93 (C-5′′′), 129.59 (C-6′′′). HRESIMS m/z 541.1140 (100), $[M-H]^-$ (calcd for $C_{30}H_{21}O_{10}$, 541.1138), 270 $[M-H^2]^{2-}$, 577 $[M_2-H]^-$; TLC R_f (Si-gel, hexane/EtOAc; 1 : 1) 0.36 (vis. vanillin/H_2SO_4).

2.4 HPLC Method for analysis of 2,3,2'',3''-tetrahydroochanaflavone (1)

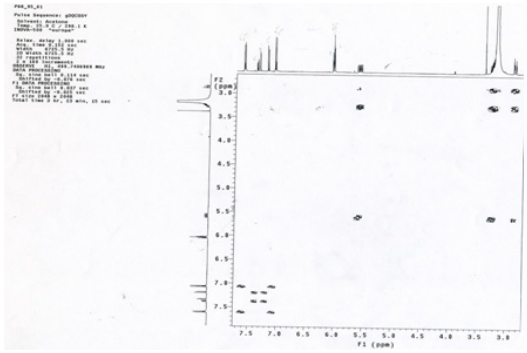
Analysis was carried at 25℃ on a C_{18} column (Phenomenex Prodigy ODS (**3**), 5 μm, 100 A, 250 x 4.6 mm) with a 2 x 4 mm C_{18} guard column. The following gradient program was used (time {min}, ratio of CH_3CN - H_2O each containing 0.1% trifluoroacetic acid). The flow rate was 4.5 mL/min, with an injection volume of 5 μL. Detection was at 280 nm, and the retention time was 10.77 min (**1**).

2.5 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 multi well 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) [13,14].

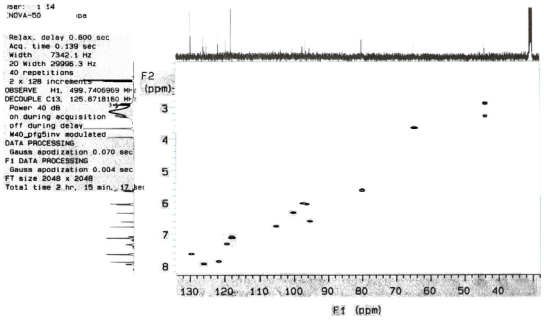
3. Results and Discussion

2,3,2′′,3′′-Tetrahydroochanaflavone (**1**) exhibited a broad OH absorption band at 3414/cm in the IR spectrum. There were two distinct carbonyl absorption bands at 1641/cm (consistent with a flavanone). **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 12.14, and 12.15). A flavanone structure was further implicated by the presence of only one ABX system characteristic of a flavanone (δ 5.57, dd, J=12.5, 3.5 Hz; 3.30, dd, J=5.0, 1.0 Hz, 2.89, t, J=2.0 Hz). These showed correlations in the COSY spectrum [Fig. 1] to two patterns of two overlapping double doublets (centered at δ 2.86 and 3.27, H-3/H-3′′) associated with methylene carbon signals (δ 44.16, 44.08), suggesting that both of the flavonoid units were flavanones.



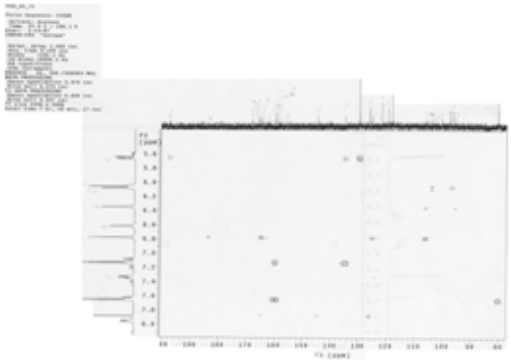
[Fig. 1] COSY Spectrum of 2,3,2'',3'' -tetrahydroochanaflavone (1)

These observations accounted for nine of oxygen atoms, suggesting that the remaining one was involved in the linkage between the two units. One bond ^{1}H-^{13}C-NMR correlations were determined by HSQC experiments [Fig. 2].



[Fig. 2] HSQC Spectrum of 2,3,2'',3'' -tetrahydroochanaflavone (1)

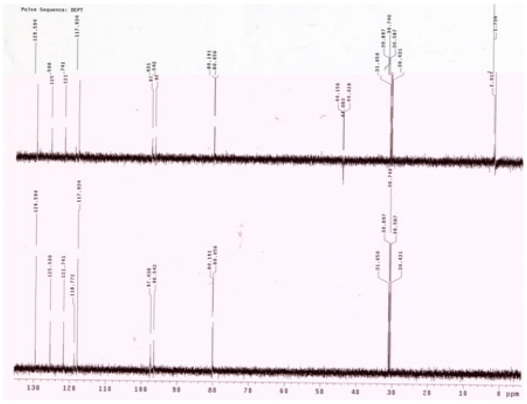
A feature of the ^{1}H-NMR spectrum was a coupled pair of two protons, doublets of multiples (δ 7.07, 7.61, H-3′′′/H-5′′′ and H-2′′′/H-6′′′) consistent with a *p*-disubstituted benzene ring. The former of these showed a correlation in the CIGAR spectrum [Fig. 3] to an aromatic carbon signal at 134.4 (C-1′′′) which also was correlated to the H-2′′ signal (δ 5.62). The latter showed further ocrrelation to the C-2′′′/C-6′′′ carbon signal (δ 129.6). This established the linkage between the B and C rings of one flavanone unit. Both the H-3′′′/H-5′′′ and the H-2′′′/H-6′′′ proton signals (δ 7.07, 7.61) showed correlations in the CIGAR spectrum to an oxygenated aromatic carbon signal at δ 159.9 (C-4′′′), thereby establishing structure (**1**).



[Fig. 3] CIGAR Spectrum of 2,3,2'',3'' -tetrahydroochanaflavone (1)

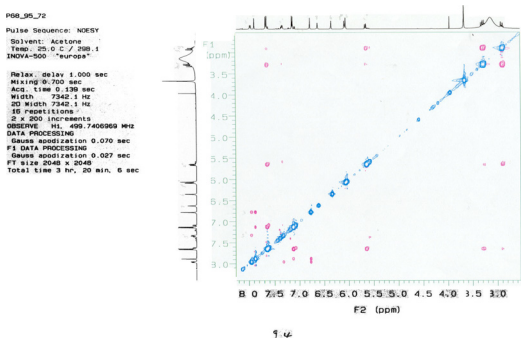
The decoupled and DEPT ^{13}C-NMR spectra displayed signals for all the 15 carbons, including two flavanone

carbonyls and nine oxygen-bearing quaternary carbons, supporting the biflavanone structure **1** [Fig. 4].



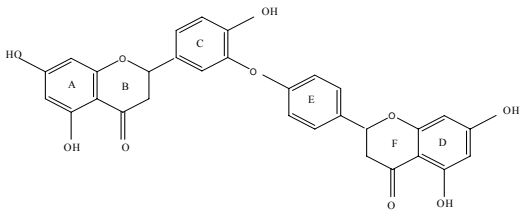
[Fig. 4] DEPT Spectrum of 2,3,2",3" -tetrahydroochanaflavone (1)

The remaining unfilled bonds could now be joined to connect the two flavanone units by an ether linkage connecting C-3′ to C-4′′′. Support for this comes from the observation of NOESY correlations between H-2′ and both H-3′′′ and H-5′′′ [Fig. 5].

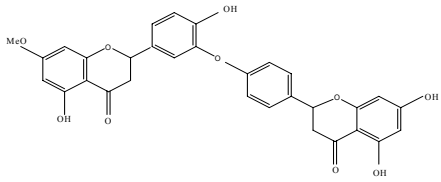


[Fig. 5] NOESY Spectrum of 2,3,2",3" -tetrahydroochanaflavone (1)

This compound was identified as 2,3,2′′,3′-tetrahydro-ochanaflavone (**1**) [3]. This compound (**1**) has the similarity with 7-*O*-methyl-tetrahydroochnaflavone (**2**) using spectroscopic methods. The ^{1}H-NMR spectrum of 7-*O*-methyltetrahydroochnaflavone (**2**) showed one methoxyl proton signals (δ 3.83) [Fig. 6] [9].



1



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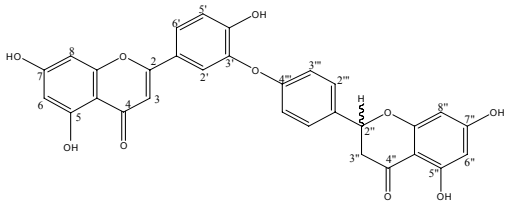
[Fig. 6] The structures of 2,3,2",3" -tetrahydroochanaflavone (1) and 7-O-methyl-tetrahydroochnaflavone (2)

2′′,3′′-Dihydroochanaflavone (**3**) exhibited a broad OH absorption band at 3411 cm^{-1} in the IR spectrum. There were two distinct carbonyl absorption bands at 1640/cm (consistent with a flavanone) and 1611/cm (more typical of a flavone). Like the previous three compound, **3** 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′′-dihydroochana-flavone (**3**) [15,16]. Although there was some similarities between the ^{1}H and ^{13}C-NMR spectra of **1** and **3**, there was significant differences in the chemical shifts of the protons in the 1,3,4-trisubstituted benzene rings. Especially some chemical shifts showed between the ^{1}H and ^{13}C-NMR spectra of **1** and **3** in flavanone structure (B ring). The negative ion HRESIMS of compound (**1**) had [M-H]$^{-}$ at 541.1135, [M-H^{2}]$^{2-}$ at 270.6, [M+Cl]- at 577.1,

$[M_2-H]^-$ at 1083.3, which were all consistent with the molecular formula $C_{30}H_{21}O_{10}$, Again the UV spectrum in methanol exhibited absorption maximum typical of flavanone (λ_{max} 207, 288, 329 nm). Crude extract ethanol leaf extracts were active against *B. subtilis* and *T. mentagrophytes* at a concentration of 150 μg/disk [17]. Biflavonoids **1** and **3** were subjected to some antimicrobial activities at this same concentration. Compounds **1** and **3** showed slight p388 activity with IC_{50} values of 8.2 and 3.1 μg/mL respectively. This cytotoxic of **3**, because the B ring of **1** accompanied with unconjugated flavanone system [Figs. 6 and 7].



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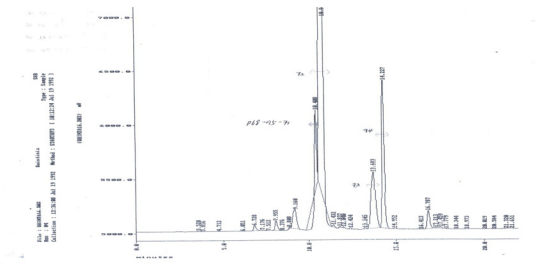
[Fig. 7] The structure of 2'',3'' –dihydroochanaflavone (3)

A quantitative HPLC method for analysis of 2,3,2′′,3′′-tetrahydro- ochanaflavone (**1**) was developed [Fig.. 8]. 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 C_{18} guard column. Examination of 40-45% ethyl acetate/hexane fractions from *Q. acutifolia* leaves ethanol extracts showed that compound (**1**) was present and the retention time was 10.77 min (**1**).



[Fig. 8] HPLC Chromatogram of 2,3,2'',3'' –tetrahydroochanaflavone (1)

2,3,2′′,3′′-Tetrahydro- ochanaflavone (**1**) showed an antimicrobial activity at a concentration of 150 μg/disk, and then this compound (**1**) showed some cytotoxic activities with IC_{50} 8.2 μg/mL against P388 murine lymphocytic leukemia cells <Table 1>.

〈Table 1〉 Cytotoxic activity of 2,3,2'',3'' –tetrahydroochanaflavone (1) and 2'',3''–dihydroochanaflavone (3) from Quintinia acutifoli

P388 Assay	Tested material		
	1	3	Mitomycin C
IC_{50} (μg/mL)	8.2	3.2	0.06[a]

[a]Toxicity 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.

4. Conclusion

2,3,2′′,3′′-tetrahydroochanaflavone (**1**) showed some antimicrobial activity at a concentration of 150 μg/disk. Biflavonoids **1** and **3** were subjected to some antimicrobial activities at this same concentration. Compounds **1** and **3** showed slight p388 activity with IC_{50} values of 8.2 and 3.1 μg/mL respectively. This cytotoxic activity of compounds **1** had lower than that of **3** because of the B ring of unconjugatd flavanone system.

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