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Flavonoids from Indonesian Silver Fern (*Pityrogramma calomelanos*) and Their Cytotoxicity Against Murine Leukemia P-388 Cells

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Abstract
Flavonoid compounds, namely 2',6'-dihydroxy-4'-methoxydihydrochalcone, kaempferol, and quercetine had been separated from the aerial part of the fern *Pityrogramma calomelanos.* Their structures were elucidated based on the spectroscopic evidence and by comparison with reported literature data. All isolates showed cytotoxicity against the murine leukemia P-388 cells.
Keywords: *Pityrogramma calomelanos,* flavonoid, murine leukemia P-388 cells

1. Introduction

Pityrogramma calomelanos was one of the ferns belonging the Polypodiaceae family widely distributed in tropical Asia, especially Indonesia. It usually grew in open region, near streams, slope of mountain, and old wall (Steenish & Holttum 1982). This fern was used as the ornamental plant and phytoremediation land polluted arsenic (As), zink (Zn), lead (Pb), and mercury (Hg) (Visoottiviseth et al, 2002). Therefore, the chemical constituents of *P. calomelanos* and its bioactivity had not been reported. In the course of our studies, three flavonoid namely 2',6'-dihydroxy-4'-methoxydihydrochalcone (**1**), kaempferol (**2**), and quercetine (**3**) had been isolated from the aerial part of *P. calomelanos.*. In this paper, we reported the isolation and structure determination of those isolates and evaluation of their cytotoxicity against murine leukemia P-388 cells.

2. Materials and Methods

2.1 General Experimental Procedures

Melting point was measured by Fisher John melting point apparatus and was uncorrected. UV spectra were recorded on Shimadzu Pharmaspec UV-1700 spectrophotometer. IR spectrum in KBr film was determined by Buck Scientific-500 spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured by JEOL JNM ECA-500 spectrometer [operating at 500 MHz (^{1}H) and 125.7 MHz (^{13}C)]. Mass spectrum (MS) was recorded on Shimadzu QP-5000 spectrometer using electron impact (EI) ion mode. Kieselgel 60 GF-254 (Merck) and silica gel G 60 63-200 μm (Merck) were used for vacuum liquid chromatography (VLC) and flash chromatography (FC), respectively. Precoated silica gel 60 F-254 (Merck) 0.25 mm, 20 x 20 cm was used for thin layer chromatography (TLC) and spots were detected by spraying with the sulphuric acid solution 5% (v/v) in ethanol followed by heating. Cytotoxicity of flavonoid isolates against murine leukemia P-388 cells were evaluated using MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] assay (Cos et al, 2001; Sutoyo et al, 2007).

2.2 Plant Materials

The aerial part of *P. calomelanos* was collected from Sawahan district, Nganjuk, East Java, Indonesia in March 2014. A voucher spesimen was identified and deposited at the herbarium of the Purwodadi Botanical Garden, East Java, Indonesia.

2.3 Extraction and Isolation

The aerial part dried powdered of *P. calomelanos* (640 g) was exhaustively extracted successively with *n*-hexane (2 L x 3), ethyl acetate (2 L x 3), and acetone (2 L x 3) at room temperature. The ethyl acetate extract, and acetone extract were evaporated in vacuo, revealed 25.3 g (blackish green), and 10.5 g (blackish brown) residue, respectively.

A portion of ethyl acetate extract (8.0 g) was chromatographed by VLC and eluted with solvents of increasing polarity (*n*-hexane, *n*-hexane-ethyl acetate, ethyl acetate) yielded 120 fractions (15 mL each). Removal of the solvent under reduced pressure of the combined fractions of 50-75 gave the brown solid (1,2 g). It was recrystalized in benzene yielded a flavonoid 2',6'-dihydroxy-4'-methoxydihydrochalcone (**1**) (239 mg).

While a portion of acetone extract (5.0 g) was chromatographed by VLC and eluted with solvents of increasing polarity (chloroform, chloroform-methanol, methanol) produced 127 fractions (15 mL each). Removal of the solvent under reduced pressure of the combined fractions of 60-100 gave the brown solid (1,2 g). It was rechromatographed by FC with chloroform-acetone (9:1) as eluen, obtained 60 fractions (10 mL each). The fractions 12-30 were collected, recrystalized in chloroform-methanol yielded a flavonoid 3, 5, 7, 4'-tetrahydroxy flavone (kaempferol) (**2**) (30 mg). While the combined fractions of 47-59 gave a flavonoid 3, 5, 7, 3', 4'-pentahydroxy flavone (quercetine) (**3**) (20 mg).

2',6'-dihydroxy-4'-methoxydihydrochalcone (**1**) was obtained as pale yellow crystal (benzene), mp. 169-171°C, which gave positive test with $FeCl_3$ (greenish yellow) and Shinoda test (Mg-HCl) (yellow). It showed one spot on TLC using three eluents system with Rf of 0.86 (chloroform-ethyl acetate = 9 : 1), 0.44 (*n*-hexane-ethyl acetate = 4 : 1), and 0.31 (*n*-hexane-ethyl aceate = 9 : 1) as well as one peak on chromatogram of gas chromatography at Rt = 26.497 min. UV (MeOH) λ_{max} (log ε) : 285 (4.70), 336 (sh) (3.88) nm; (MeOH + NaOH): 295 (4.75), 361 (sh) (4.34) nm; (MeOH+$AlCl_3$): 306 (4.73), 371 (sh) (3.76) nm; (MeOH+$AlCl_3$+HCl): 306 (4.81), 368 (sh) (3.96) nm; (MeOH+NaOAc): 287 (4.66) nm; (MeOH+NaOAc+H_3BO_3): 286(4.67) nm. IR (KBr) $\bar{\nu}_{max}$: 3253 (OH), 3014 (aromatic C-H), 2969, 2969, 2862 (alkyl C-H), 1646 (chelated C=O), 1593, 1527 (aromatic C=C), 1435, 1384, 1216, 1074 cm^{-1}. ^{1}H-NMR (500 MHz,$CDCl_3$) δ (ppm) : 3.02 (2H, *t*, *J* = 7.95 Hz, H-β), 3.40 (2H, *t*, *J* = 7.3 Hz, H-α), 3.79 (3H, *s*, 4'-OCH_3), 5.93 (2H, *s*, H-3'and H-5'), 7.25 (5H, *m*, H-2,3,4,5,6). ^{13}C-NMR (125,8 MHz,$CDCl_3$) δ (ppm) : 30.7 (C-β), 45.8 (C-α), 55.7 (4'-OCH_3), 94.6 (C-3',5'), 104.9 (C-1'), 126.1 (C-4), 128.6 (C-2,6), 128.7 (C-3,5), 141.8 (C-1), 165.7 (C-2',4',6'), 204.7 (C=O). EIMS, m/z (rel. int., %): 272 (25), 255 (6), 177 (3), 167 (100, base peak), 140 (38), 136 (3), 124 (3), 111 (6), 104 (6), 91 (22), 77 (6), 69 (6), 51 (6), 39 (6).

Kaempferol (**2**) was obtained as yellow needles crystal ($CHCl_3$-acetone), mp. 271-273°C, which gave positive test (green) with $FeCl_3$ and Shinoda test (Mg-HCl) (orange). It showed one spot on TLC using three eluents system with Rf of 0.36 (chloroform-acetone = 3 : 1), 0.44 (chloroform-ethyl acetate = 1 : 1), and 0.73 (chloroform-methanol = 5 : 1). UV (MeOH) λ_{max} (log ε) : 267, 367 nm; (MeOH + NaOH): 275, 322 (sh), 405 nm; (MeOH+$AlCl_3$): 270, 353 (sh), 421 nm; (MeOH+$AlCl_3$+HCl): 269, 351 (sh), 421 nm; (MeOH+NaOAc): 276, 343 (sh), 426 nm; (MeOH+NaOAc+H_3BO_3): 270, 343 (sh), 423 nm. IR (KBr) $\bar{\nu}_{max}$: 3414 (OH), 3036 (aromatic C-H), 1659 (chelated C=O), 1613, 1567, 1510 (aromatic C=C), 1381, 1308, 1249, 1178 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ (ppm) : 6.17 (1H, *d*, *J* = 1.9 Hz, H-6), 6.38 (1H, *d*, *J* = 1,9 Hz, H-8), 6.89 (2H, *d*, *J* = 9,2 Hz, H-3',5'), 8.07 (2H, *d*, *J* = 8,6 Hz, H-2',6'). ^{13}C-NMR (125,8 MHz, CD_3OD) δ (ppm) : 94.5 (C-8), 99.3 (C-6), 104.6 (C-10), 116.4 (C-3', C-5'), 123.8 (C-1'), 130.8 (C-2', C-6'), 137.2 (C-3), 148.1 (C-2), 158.3 (C-5), 160.6 (C-4'), 162.6 (C-9), 165.7 (C-7), 177.4 (C-4).

Quercetine (**3**) was obtained as yellow needles crystal ($CHCl_3$-acetone), mp. more than 300 °C, which gave positive test (green) with $FeCl_3$ and Shinoda test (Mg-HCl) (orange). It showed one spot on TLC using three eluents system with Rf of 0.25 (chloroform-acetone = 3 : 1), 0.62 (chloroform-methanol = 5 : 1), and 0.23 (chloroform-methanol = 9 : 1). UV (MeOH) λ_{max} (log ε): 255, 372 nm; (MeOH + NaOH): 272, 325 (sh), 411 nm; (MeOH+$AlCl_3$): 270, 445 nm; (MeOH+$AlCl_3$+HCl): 264, 354 (sh), 429 nm; (MeOH+NaOAc): 272, 332 (sh), 451 nm; (MeOH+NaOAc+H_3BO_3): 272, 332 (sh), 451 nm. IR (KBr) $\bar{\nu}_{max}$: 3411 (OH), 3012 (aromatic C-H), 1641 (chelated C=O), 1510 (aromatic C=C), 1014, 883, 819 cm^{-1}. ^{1}H-NMR (500 MHz,CD_3OD) δ (ppm) : 6.18 (1H, *d*, *J* = 1.9 Hz, H-6), 6.39 (1H, *d*, *J* = 1.8 Hz, H-8), 6.88 (1H, *d*, *J* = 8.6 Hz, H-5'), 7.64 (1H, *dd*, *J* = 1.9 Hz and 8,0 Hz, H-6'), 7.74 (1H, *d*, *J* = 1,9 Hz, H-2'). ^{13}C-NMR (125.8 MHz, CD_3OD) δ (ppm) : 94.5 (C-8), 99.3 (C-6), 104.6 (C-10), 116.0 (C-2'), 116.3 (C-5'), 121.7 (C-6'), 123.6 (C-1'), 137.4 (C-3), 146.3 (C-4'), 148.9 (C-3'), 158.4 (C-2, 9), 162.6 (C-5), 165.8 (C-7), 178.9 (C-4).

2.4 Cytotoxicity Assay against Murine Leukemia P-388 Cells

Murine leukemia P-388 cells [ex HSRRB lot number: 113098 seed (JCRB007)] were obtained from Tokyo University of Pharmacy and Life Sciences, Japan. Cells were seeded into culture flasks with growth medium RPMI 1640 (Nissui, Japan), supplemented with 5% fetal bovine serum (FBS) (Gibco) and 100 μg/ mL kanamycin sulfate (Meiji, Japan). Cells were seeded into 96-well plates at a density of 3.10^3 cells in 100 μL growth medium per well. The plate were incubated at 37 °C under a humidified atmosphere containing 5% CO_2. After 24 hours, the medium was discarded and 10 μL of test solution in DMSO (Merck) with the various concentrations were added. After 72 hours, medium was removed and 20 μL of MTT solution [5 mg MTT (Sigma) dissolved in 1 mL FBS] was added to each well. Four hours later, the formazan product was solubilized by the addition of 100 μL 10% (w/v) sodium dodecyl sulfate (Merck)-0.01 N HCl (Merck). The mixture of phosphoric acid buffer solution (Nissui, Japan), DMSO, and cells were used as a positive control. The optical density of each well was measured using an automatic microplate reader with a test wavelength of 550 nm and a reference wavelength of 700 nm. The absorbace is directly proportional to the number of living cells. The cytotoxicity of each test compound was expressed as an IC_{50} value (the concentration in μg/mL that inhibits cell growth by 50% compared with cell controls), and calculated by probit analysis (Cos et al, 2001; Sutoyo et al,

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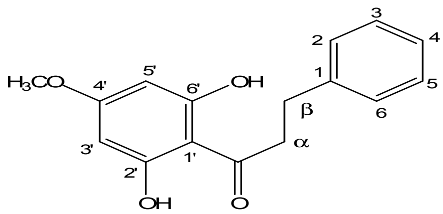
2007).

3. Results and Discussion

3.1 2',6'-dihydroxy-4'-methoxy-dihydrochalcone ***(1)***

Compound **1** showed the positive results on the test using $FeCl_3$ reagent (yellowish green) and Shinoda test (Mg + HC1) (yellow). It indicated that isolate was a flavonoid compound (Robinson, 1991). The absorption bands of IR spectrum at 3267 (OH), 3023 (aromatic C-H), 2968, 2938 (alkyl C-H), 1647 (chelated C=O), 1597, 1529 (aromatic C=C) supported that isolate was a flavonoid. The UV spectrum of 1 indicated absorbtion characteristic of dihydrochalcone-type compounds at 285 nm (band II) and 336 nm (sh) (band I) [7]. No bathochromic shift of band II on adding of NaOH and NaOAc reagents indicated that the isolates did not have a free OH group at C-4'. The bathochromic shift of band II on adding of $AlCl_3$ + HCl reagent supports the existence of an OH group free at C-6'. While the addition of NaOAc + H_3BO_3 did not cause the bathochromic shift of band II. This showed the absence of ortho-dihydroxy group at A ring in flavonoid isolate. Two triplet proton signal at δ_H 3.02 and 3.40 ppm due to H-α and H-β, respectively, supported that **1** had a basic skeleton of dihydrochalcone. While the presence of singlet proton signal at δ_H 3.79 ppm indicated that C-4' binded a methoxy group. Multiplet proton signal at δ_H 7.25 ppm showed that **1** had a structure similar to the B ring of pinocembrine (Bick et al, 1972) that was not substituted. The ^{13}C-NMR spectrum of **1** exhibited 11 carbon signals represented 16 carbon signals, consisted of alkyl carbon [δ_c 30.7 (C-β), 45.8 (C-α)], methoxy carbon [δ_c 55.7 (4'-OCH_3)], aryl carbon [δ_c 94.6 (C-3',5'), 104.9 (C-1'), 126.1 (C-4), 128.6 (C-2,6), 128.7 (C-3,5), 141.8 (C-1)], oxyaryl carbon [δ_c 165.7 (C-2',4',6')] and carbonyl carbon [δ_c 204.7 (C=O)]. The EIMS spectrum of **1** showed a molecular ion peak at m/z 272, corresponding a molecular formula $C_{16}H_{16}O_4$. From the above results, compound **1** was identified as 2',6'-dihydroxy-4'-methoxy-dihydrochalcone.



(1)

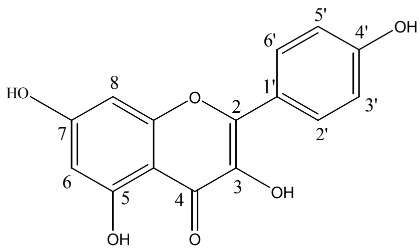
3.2 Kaempferol ***(2)***

The positive results on the test using $FeCl_3$ reagent (yellowish green) and Shinoda test (Mg + HC1) (yellow) of compound **2** indicated that it was a flavonoid compound (Robinson, 1991). The absorbtion maxima at 267 (band II) and 367 nm (band I) in the UV spectrum supported that **2** was a flavonol with a free 3-hydroxyl group (Markham 1982). The batochromic shift of band I on adding NaOH reagent (38 nm) and $AlCl_3$ + HCl reagent (54 nm) indicated the presence of a hydroxyl group at C-4' and C-5, respectively. The presence of a hydroxyl group at C-7 was exhibited by batochromic shift of band II (9 nm) on adding NaOAc reagent. No batochromic shift on adding NaOAc + H_3BO_3 reagent supported that **1** didn't have ortho-di hydroxyl group at A and B rings. The IR spectrum of **2** clearly disclosed absorbtion bands for OH group (3414 cm^{-1}), chelated carbonyl group (1659 cm^{-1}), and aromatic C=C (1613, 1567, 1510 cm^{-1}). The ^{1}H-NMR spectrum of **2** exhibited four doublet proton signals at δ_H 6.17, 6.38, 6.89 and 8.07 (Table 1). Two doublet proton signals at δ_H 6.17 (J =1.9 Hz) and 6.38 (J = 1.9 Hz) due to a pair of meta coupled protons H-6 and H-8 in the A-ring, respectively, supported the presence of a hydroxyl group at C-5 and C-7. While two doublet proton signals at δ_H 6.89 (J = 9.2 Hz, H-3',5') and 8.07 (J = 9.2, H-2',6') due to two pairs of ortho-cop;ed protons in the B-ring, confirmed the presence of a hydroxyl group at C-4'. The ^{13}C-NMR spectrum exhibited 15 carbon signals which corresponded to **2**, containing five oxy aryl carbons [δ_C 148.1 (C-2), 158.3 (C-5), 160.6 (C-4'), 162.6 (C-9), and 165.7 (C-7)], one oxyolefine carbon [δ_C 137.2 (C-3)], and one carbonyl carbon [δ_C 177.4 (C-4)] (Table 1). The correlation spectroscopy (HMQC and HMBC) spectral data supported complete assignment of all proton-bearing carbon signals of **2**.

Table 1. ^{1}H , ^{13}C, HMQC, HMBC NMR Data of **2** in CD_3OD and ^{1}H , ^{13}C NMR of Kaempferol in CD_3OD (Suyatno, 2008)

Position of C	Compound **2**			Kaempferol	
	δ_H (mult, *J* dalam Hz)	δ_C	^{1}H-^{13}C HMBC	δ_H (mult, *J* dalam Hz)	δ_C
1	-	-	-	-	-
2	-	148.1	-	-	147.0
3	-	137.2	-	-	136.4
4	-	177.4	-	-	176.4
5	-	158.3	-	-	157.6
6	6.17 (*d*, 1.85)	99.3	C-7,C-8,C-9,C-10	6.2 (*d*, 2)	99.0
7	-	165.7	-	-	165.1
8	6.38 (*d*, 1.85)	94.5	C-4,C-5,C-6,C-7,C-10	6.5 (*d,* 2)	94.3
9	-	162.6	-	-	161.8
10	-	104.6	-	-	103.9
1’	-	123.8	-	-	123.0
2’	8.07 (*d*, 8.55)	130.8	C-2,C-3’,C-4’,C-5’,C-6’	8.1 (*dd,* 9.3)	130.3
3’	6.89 (*d*, 9.15)	116.4	C-1’,C-4’,C-5’	7.0 (*dd,* 9.3)	116.2
4’	-	160.6	-	-	160.2
5’	6.89 (*d*, 9.15)	116.4	C-1’,C-3’,C-4’	7.0 (*dd,* 9.3)	116.2
6’	8.07 (*d*, 8.55)	130.8	C-2,C-2’,C-3’,C-4’,C-5’	8.1 (*dd,* 9.3)	130.3

Futher supporting evidence of structure **2** for kaemferol came from comparison of the ^{1}H-NMR and ^{13}C-NMR spectral data with those of reported data in literature (Markham & Geiger 1994, Li Bin & Luo Yongming, 2002; Suyatno, 2008). From the above results, compound **2** was proposed for the structure of kaempferol (3,5,7,4’-tetrahydroxy flavone).



(2)

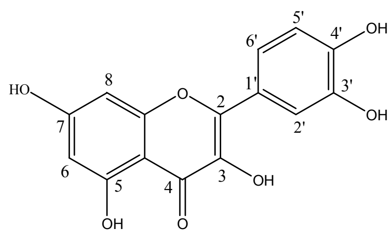
3.2 Quercetine ***(3)***

Compound **3** showed the positive results on the test using $FeCl_3$ reagent (yellowish green) and Shinoda test (Mg + HC1) (yellow). It indicated that **3** was a flavonoid compound (Robinson, 1991). The absorbtion maxima at 255 (band II) and 372 nm (band I) in the UV spectrum supported that **3** was a flavonol with a free 3-hydroxyl groups (Markham 1982). The batochromic shift of band I on adding NaOH reagent (39 nm) and $AlCl_3$ + HCl reagent (57 nm) indicated the presence of a hydroxyl group at C-4' and C-5, respectively. The presence of a hydroxyl group at C-7 was exhibited by batochromic shift of band II (17 nm) on adding NaOAc reagent. Batochromic shift on adding NaOAc + H_3BO_3 reagent (79 nm) supported that **3** had ortho-di hydroxyl group at A and B rings. The IR spectrum of **3** clearly disclosed absorbtion bands for OH group (3411 cm^{-1}), chelated carbonyl group (1641 cm^{-1}), and aromatic C=C (1510 cm^{-1}). The ^{1}H-NMR spectrum of **3** exhibited four doublet proton signals at δ_H 6.18, 6.39, 6.88 and 7.74 (Table 2). Two doublet proton signals at δ_H 6.18 (*J* =1.9 Hz) and 6.39 (*J* = 1.8 Hz) due to a pair of meta coupled protons H-6 and H-8 in the A-ring, respectively, supported the presence of a hydroxyl group at C-5 and C-7. While the presence two doublet proton signals at δ_H 6.88 (H-5’) and 7.74 ppm (H-2’) as well as a double douplet proton signal at δ_H 7.64 ppm (H-6’) supported the presence 3’,4’-dihydroxy group at B-ring. The existence ortho dihydroxy group caused H-2’ interacted with H-6’ at meta position with *J* = 1.9 Hz, H-5’ interacted with H-6’ at ortho position with J = 8,6 Hz, while H-6’ interacted with H-5’ at ortho position and H-2’ at meta position with *J* value of 1.9 Hz and 8 Hz, respectively. No singlet peak at δ_H 7 ppm caused by vinilic proton at C-3, corresponded to quercetine having hydroxyl group at C-3. The ^{13}C-NMR spectrum exhibited 15 carbon signal which corresponded to quercetine containing five oxyaryl carbons [δ_C 162.6 (C-5), 148.9 (C-3’), 146.3 (C-4’), 158,4 (C-9), and 165.8 (C-7)], two oxy olefine carbons [δ_C 158.4 (C-2), 37.4 (C-3)] and one carbonyl carbon [δ_C 178.9 (C-4)] (Table 2). The correlation spectroscopy (HMQC and HMBC) spectral data supported complete assignment of all proton-bearing carbon signals of quercetine.

Table 2. ^{1}H , ^{13}C, HMQC, HMBC NMR Data of **3** in CD_3OD and ^{1}H, ^{13}C NMR Data of Quercetine in CD_3OD (Suyatno, 2008)

Position of C	Compound **3**			Quercetine	
	δ_H (mult, *J* dalam Hz)	δ_C	^{1}H-^{13}C HMBC	δ_H (mult, *J* dalam Hz)	δ_C
1	-	-	-	-	-
2	-	158.4	-	-	156.9
3	-	137.4	-	-	134.6
4	-	178.9	-	-	178.2
5	-	162.6	-	-	161.8
6	6.18 (*d*, 1.85)	99.3	C-5,C-7,C-8,C-10	6.20 (*d*, 2.1)	99.3
7	-	165.8	-	-	165.1
8	6.39 (*d*, 1.89)	94.5	C-6,C-9,C-10,C-7	6.42 (*d*, 2.1)	94.1
9	-	158.4	-	-	157.7
10	-	104.6	-	-	104.4
1’	-	123.6	-	-	121.6
2’	7.74 (*d*, 1.85)	116.0	C-3’,C-4’,C-6’	7.69 (*d*, 2.1)	115.9
3’	-	146.3		-	145.7
4’	-	148.9	-	-	149.0
5’	6.88 (*d*, 8.6)	116.3	C-1’,C-3’,C-4’	6.90 (*d*, 8.5)	116.1
6’	7.64 (*dd*, 1.85 & 7.95)	121.7	C-2’,C-3’,C-4’,C-5’	7.55 (*dd*, 2.1 & 8.5)	121.2

Futher supporting evidence of structure **3** for quercetine came from comparison of the ^{1}H-NMR and ^{13}C-NMR spectral data with those of reported data in literature (Markham & Geiger 1994; Suyatno, 2008). From the above results, **3** was proposed for the structure of quecetine (3,5,7,3’,4’-pentahydroxy flavone).



(3)

3.3 Cytotoxicity Isolates against Murine Leukemia P-388 Cells

Based on the cytotoxicity test, found that compound **1, 2,** and **3** showed cytotoxic activity against murine leukemia P-388 cells in vitro with IC_{50} values of 1.6, 14.1 and 6.4 µg /mL, respectively. Thus compund **1** and **3** strongly inhibited murine leukemia P-388 cells (IC_{50} < 10 µg/ mL, while compund **2** showed moderate activity against murine leukemia P-388 cells ($10 \leq IC_{50} < 100$ µg/ mL) (Stevgny et al, 2005). Cytotoxicity of **1** on cancer cell lines have not been reported before, so this is the first report of its cytotoxicity against murine leukemia P-388 cells.

4. Conclusions

Three flavonoid compounds namely 2',6'-dihydroxy-4'-methoxy-dihydrochalcone, kaempferol, and quercetine were separated from the fern *Pityrogramma calomelanos.* All isolated showed cytoxicity against murine leukemia P-388 cells and they had potency to be developed as natural anticancer agent.

5. Acknowledgements

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