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Flavonoid Compounds from the Leaves of *Kalanchoe prolifera* and Their Cytotoxic Activity against P-388 Murine Leukimia Cells

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Abstract – Seven flavonoid compounds, kaempferol (**1**), quercetin (**2**), quercetin-3-*O*-β-D-glucopyranoside (**3**), kaempferol-3-*O*-β-D-glucopyranoside (**4**), kaempferol-3-*O*-α-L-rhamnoside (**5**), quercetin-3-*O*-sophoroside (**6**) and quercetin-3-*O*-rutinoside (**7**), were isolated from the methanolic extract of leaves of *Kalanchoe prolifera*. Compounds **1-7** were isolated for first time from this plant. These compounds were evaluated their cytotoxic activity against P-388 murine leukimia cells *in vitro*. Among those compounds kaempferol (**1**) and quercetin (**2**) showed strongest cytotoxic activity with IC_{50} values of 4.45 ± 0.05 and 6.28 ± 0.02 μg/mL, respectively.
Keywords – Flavonoids, *Kalanchoe prolifera*, Crassulaceae, P-388 murine leukimia cell

Introduction

Kalanchoe prolifera belong to Crassulaceae family is a perennial herb and succulent leaves which grown in high terrain and indigenous to low altitude of Indonesia[1]. The leaves of *K. prolifera* are used in Indonesian traditional medicine for the treatment of fever, infections, rheumatism and skin diseases[1,2]. The genus of *Kalanchoe* reported to contain bufadienolides[3-5], triterpenoids[6] and flavonoids[7-9] and to possess biological activities such as blocking human lymphocyte proliferation[6,10], cytotoxic[11], insecticidal[5,12] and inhibiting cancer cell growth[13,14]. However, thus so far there is no phytochemistry reported from *K. prolifera*. Our preliminary screening in search for novel cytotoxic agents from Indonesian *Kalanchoe* plants, we found that the methanolic extract of *K. prolifera* exhibited significant cytotoxic against P-388 murine leukimia cells *in vitro*. Repeated column chromatography of the methanolic extract of fresh leaves of *K. prolifera* by bioassay-guided isolation resulted in the isolation of seven flavonoids (Fig. 1). The isolation, structure identification, and cytotoxic evaluation against P-388 murine leukimia cells of these isolated compounds are described herein.

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Experimental

General experimental procedures – Ultra-violet spectra was recorded in methanol on Jasco UV-1575 spectrophotometer. The IR spectra were measured on a Perkin Elmer spectrum-100 FT-IR in KBr. Mass spectra were obtained with a Water Qtof HR-MS XEV^{otm} and Water TQD MS/MS mass spectrometers. NMR spectra were recorded with a JEOL JNM A-500 spectrometer using tetra methyl silane (TMS) as an internal standard. Chromatographic separation were carried out on silica gel 60 (Merck). PTLC glass plates were precoated with silica gel GF_{254} (Merck, 0.25 mm). TLC plates were precoated with silica gel GF_{254} (Merck, 0.25 mm), detection was achieved with 10% H_2SO_4 in ethanol, followed by heating and under ultra-violet at λ 254 and 367 nm.

Plant materials – The fresh leaves of *K. prolifera* were collected from Manaco Botanical Garden, Lembang, Bandung, West Java Province, Indonesia in May, 2014. The plant was identified by the staff of the Bogoriense Herbarium, Bogor, Indonesia and a voucher specimen

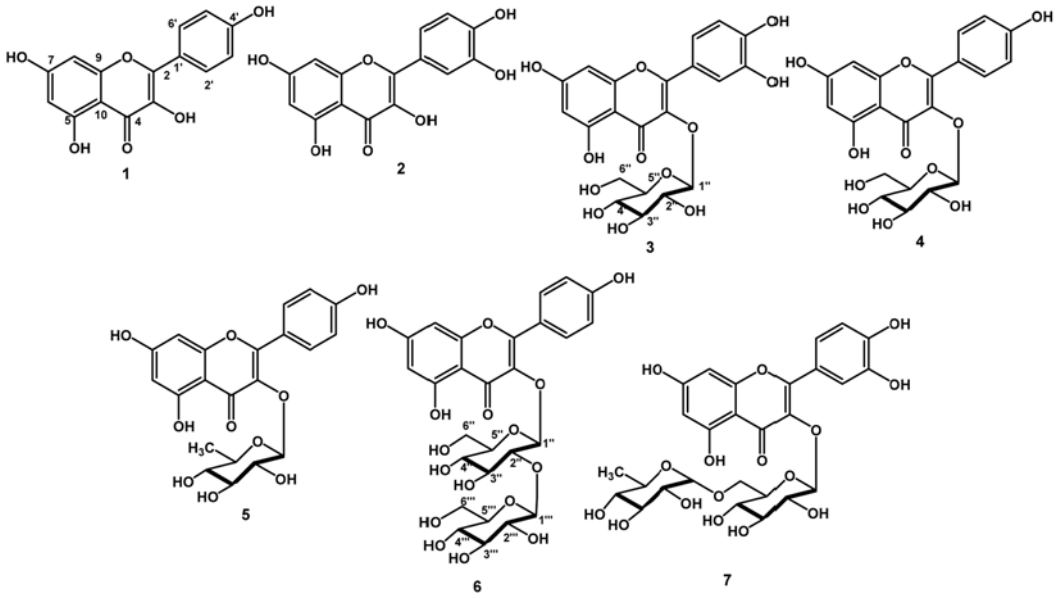

Fig. 1. The structure of compounds **1 - 7** isolated from *K. prolifera*.

(No. B0-129211) was deposited at the herbarium.

Extraction and isolation – The fresh ground leaves (20 kg) of *K. prolifera* was extracted with methanol exhaustively (50 L) at room temperature for 3 days. After removal of the solvent under vacuum, the viscous concentrated of MeOH extract (320 g) was first suspended in H_2O and then partitioned with *n*-hexane, EtOAc and *n*-butanol, successively. Evaporation resulted in the crude extract of *n*-hexane (40.2 g), EtOAc (84.5 g) and *n*-butanol (53.5 g), respectively. The ethyl acetate extract showed strongest cytotoxic activity against P-388 murine leukimia cells with IC_{50} value of 24.4 μg/mL. The EtOAc extract was subjected to vacuum liquid chromatography using gradient elution of *n*-hexane-EtOAc-MeOH to afford 15 fractions (A-O). Fraction C (4.5 g) was further subjected to column chromatography using mixture of *n*-hexane-EtOAc (10:0-6:1) as eluting solvents to afford 8 subfractions (C1-C8). Fraction C4 (340 mg) was subjected to flash column chromatography on silica gel, eluted with $CHCl_3$-MeOH (9:1), to give **1** (15.4 mg). Fractions C6-C7 were combined (4.4 g) and subjected to silica gel column chromatography using mixture of *n*-hexane-acetone (10:0-1:1) as eluting solvents to give 10 subfractions (C6.1-C6.10). Subfractions C6.6 (285 mg) was preparative TLC on silica gel GF_{254}, eluted with $CHCl_3$:MeOH (9.5:0.5) to give **2** (42.5 mg) and **3** (12.8 mg). Fractions D and E were combined (5.2 g) was further subjected to column chromatography using mixture of *n*-hexane-EtOAc (10:0-5:1) as eluting solvents to afford 6 subfractions (D1-D6). Subfraction D5 (340 mg) was subjected to flash column chromatography on silica gel, eluted with $CHCl_3$-MeOH (9.5:0.5), to give **4** (12.4 mg). Fractions D4-6 were combined (3.4 g) and subjected to silica gel column chromatography using mixture of *n*-hexane/acetone (10:0-1:1) as eluting solvents to give 7 subfractions (D4.1-D4.7). Subfractions D4.2 and D4.3 were combined (485 mg) was preparative TLC on silica gel GF_{254}, eluted with $CHCl_3$:MeOH (9.75: 0.25) to give **5** (42.5). Fractions F-H were combined (6.2 g) was further subjected to column chromatography using mixture of *n*-hexane-EtOAc-MeOH as eluting solvents to afford 10 subfractions (F1-F10). Fraction F6 (240 mg) was subjected to flash column chromatography on silica gel, eluted with *n*-hexane:acetone (7:3) and $CHCl_3$-MeOH (9.5:0.5), to give **6** (12.4 mg). Fractions F3-6 were combined (4.2 g) and subjected to silica gel column chromatography using mixture of *n*-hexane/acetone (10:0-1:1) as eluting solvents to give 6 subfractions (F3.1-F3.6). Fractions F3.2-F3.4 were combined (385 mg) was preparative TLC on silica gel GF_{254}, eluted with *n*-hexane: acetone (3:2) and $CHCl_3$:MeOH (9.75:0.25) to give **7** (12.5 mg).

Kaempferol (1) – Yellow amorphous powder; UV (MeOH): λ_{max} (log ε) 272 (4.0), 364 (3.7) nm; IR (KBr) ν_{max} cm^{-1}: 3420, 1690, 1605, 1260, 720; ^{1}H-NMR (CD_3OD, 500 MHz): δ_H 8.04 (2H, dd, J = 11.5, 2.8 Hz, H-2′, H-6′), 6.95 (2H, dd, J = 9.8, 2.7 Hz, H-3′, H-5′), 6.52 (1H, d, J = 2.0, H-8), 6.28 (1H, d, J = 2.0, H-6); ^{13}C-NMR (CD_3OD, 125 MHz): Table 1; ESI-TOFMS (*m/z* 285.2263 $[M-H]^+$), calcd. for $C_{15}H_{10}O_6$ *m/z* 286.2270.

Quercetin (2) – Yellow amorphous powder; UV (MeOH): λ_{max} (log ε) 274 (3.8), 360 (3.6) nm; IR (KBr) ν_{max} cm^{-1}:

Table 1. ^{13}C-NMR data for compounds **1** - **7** (125 MHz in CD_3OD)

Position C	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	δ_C (mult.)	δ_C (mult.)	δ_C (mult.)	δ_C (mult.)	δ_C (mult.)	δ_C (mult.)	δ_C (mult.)
2	146.8 (s)	147.7 (s)	158.4 (s)	156.4 (s)	153.6 (s)	156.7 (s)	159.0 (s)
3	136.6 (s)	135.7 (s)	135.6 (s)	133.3 (s)	134.6 (s)	133.5 (s)	135.6 (s)
4	176.6 (s)	176.8 (s)	179.1 (s)	177.4 (s)	179.7 (s)	177.9 (s)	179.4 (s)
5	162.3 (s)	160.7 (s)	163.0 (s)	161.3 (s)	163.3 (s)	161.7 (s)	163.5 (s)
6	99.2 (d)	98.2 (d)	98.0 (d)	99.1 (d)	98.6 (d)	98.4 (d)	99.8 (d)
7	164.9 (s)	163.9 (s)	168.4 (s)	164.2 (s)	165.5 (s)	164.5 (s)	167.1 (s)
8	94.4 (d)	94.5 (d)	95.6 (d)	93.8 (d)	99.4 (d)	93.9 (d)	95.2 (d)
9	157.7 (s)	156.1 (s)	160.0 (s)	156.5 (s)	155.0 (s)	156.0 (s)	158.5 (s)
10	104.1 (s)	103.0 (s)	105.7 (s)	104.1 (s)	104.5 (s)	104.8 (s)	106.2 (s)
1′	123.3 (d)	121.9 (d)	121.2 (s)	121.0 (s)	124.0 (s)	121.6 (s)	123.2 (s)
2′	125.9 (d)	115.0 (d)	115.9 (d)	131.0 (d)	128.4 (d)	115.8 (d)	117.7 (d)
3′	116.3 (d)	145.0 (d)	146.5 (s)	115.2 (d)	114.6 (d)	145.3 (s)	146.0 (s)
4′	160.1 (s)	145.8 (s)	151.4 (s)	160.0 (s)	158.0 (s)	148.9 (s)	150.1 (s)
5′	116.3 (d)	115.6 (d)	116.9 (d)	115.2 (d)	114.6 (d)	116.5 (d)	116.1 (d)
6′	125.9 (d)	124.5 (d)	121.3 (d)	131.0 (d)	128.4 (d)	122.3 (d)	116.1 (d)
1″			104.4 (d)	101.2 (d)	97.0 (d)	99.1 (d)	104.7 (d)
2″			75.7 (d)	74.3 (d)	74.8 (d)	83.2 (d)	75.7 (d)
3″			78.1 (d)	76.5 (d)	77.5 (d)	70.1 (d)	78.2 (d)
4″			71.2 (d)	69.9 (d)	78.6 (d)	76.5 (d)	71.4 (d)
5″			78.4 (d)	77.6 (d)	75.6 (d)	77.2 (d)	77.2 (d)
6″			62.6 (t)	60.9 (t)	20.8 (q)	61.4 (t)	61.5 (t)
1‴						104.5 (d)	102.4 (d)
2‴						74.9 (d)	71.4 (d)
3‴						77.0 (d)	72.1 (d)
4‴						69.6 (d)	73.9 (d)
5‴						78.0 (d)	69.7 (d)
6‴						61.2 (t)	17.8 (q)

3430, 1680, 1610, 1250; ^{1}H-NMR (CD_3OD, 500 MHz): δ_H 7.65 (1H, d, J = 2.1 Hz, H-2′), 7.50 (1H, dd, J = 8.4, 2.1 Hz, H-6′), 6.85 (1H, d, J = 8.4 Hz, H-5′), 6.40 (1H, d, J = 2.0 Hz, H-8), 6.20 (1H, d, J = 2.0 Hz, H-6); ^{13}C-NMR (CD_3OD, 125 MHz): Table 1; ESI-TOFMS spectral data (*m/z* 285.2263 $[M-H]^+$, calcd. for $C_{15}H_{10}O_7$ *m/z* 302.40).

Quercetin-3-*O*-β-D-glucopyranoside (**3**) – Yellow powder; UV (MeOH): λ_{max} (log ε) 264 (3.85), 350 (3.58) nm; IR (KBr) ν_{max} cm^{-1}: 3310, 1662, 1602, 1040; ^{1}H-NMR (CD_3OD, 500 MHz): δ_H 7.64 (1H, dd, J = 8.4, 1.9 Hz, H-6′), 7.47 (1H, d, J = 1.9 Hz, H-2′), 6.79 (1H, d, J = 8.4 Hz, H-5′), 6.38 (1H, d, J = 2.0 Hz, H-8), 6.16 (1H, d, J = 2.0 Hz, H-6); (^{13}C-NMR (CD_3OD, 125 MHz): Table 1.

Kaempferol-3-*O*-β-D-glucopyranoside (**4**) – Yellow amorphous powder; UV (MeOH): λ_{max} (log ε) 266 (3.9), 356 (3.7) nm; IR (KBr) ν_{max} cm^{-1}: 3251, 1710, 1607, 1045; ^{1}H-NMR (CD_3OD, 500 MHz): δ_H 8.05 (2H, dd, J = 11.4, 2.8 Hz, H-2′, H-6′), 6.95 (2H, dd, J = 9.7, 2.7 Hz, H-3′, H-5′), 6.50 (1H, d, J = 2.0, H-8), 6.30 (1H, d, J = 2.0, H-6), 5.48 (1H, d, J = 7.8 Hz, H-1″), 3.72 (1H, dd, J = 12.0, 4.2 Hz, H-6″b), 3.58 (1H, dd, J = 12.0, 4.5 Hz, H-6″a), 3.55 (1H, d, J = 11.4, H-3″), 3.32 (1H, d, J = 11.4 Hz, H-2″), 3.21 (1H, d, J = 7.8 Hz, H-5″), 3.20 (1H, m, H-4″); ^{13}C-NMR (CD_3OD, 125 MHz): Table 1; TQD MS ES$^-$ *m/z* $[M-H]^-$ 447.34, calcd. for $C_{21}H_{20}O_{11}$ *m/z* 448.35].

Kaempferol-3-*O*-α-L-rhamnoside (**5**) – Yellow amorphous powder; UV (MeOH): λ_{max} (log ε) 266 (3.8), 354 (3.6) nm; IR (KBr) ν_{max} cm^{-1}: 3278, 1655, 1607, 1062; ^{1}H-NMR (CD_3OD, 500 MHz): δ_H 7.20 (2H, d, J = 7.18 Hz, H-2′, H-6′), 6.90 (2H, dd, J = 7.20 Hz, H-3′, H-5′), 6.10 (1H, d, J = 2.0, H-8), 5.95 (1H, d, J = 2.0, H-6), 5.49 (1H, d, J = 1.9 Hz, H-1″), 3.80 (1H, dd, J = 8.0, 7.10 Hz, H-2″), 3.74 (1H, dd, J = 10.0, 6.10 Hz, H-5″), 3.45 (1H,

Table 2. Cytotoxicity activity of compounds **1** – **7** against P-388 murine leukemia cells

Compounds	IC_{50} (μg/mL)
Kaempferol (**1**)	4.45 ± 0.05
Quercetin (**2**)	6.28 ± 0.02
Quercetin-3-*O*-glucoside (**3**)	29.15 ± 0.05
Kaempferol-3-*O*-glucoside (**4**)	35.92 ± 0.02
Kaempferol-3-*O*-rhamnoside (**5**)	32.15 ± 0.04
Quercetin-3-*O*-sophoriside (**6**)	50.44 ± 0.02
Quercetin-3-*O*-rutinoside (**7**)	58.54 ± 0.03

dd, *J* = 10.0, 4.30 Hz, H-3″), 3.42 (1H, t, *J* = 10.0 Hz, H-4″), 0.94 (3H, d, *J* = 6.0 Hz, H-6″); ^{13}C-NMR (CD_3OD, 125 MHz): Table 2; ESI-TOFMS $[M–H]^-$ *m/z* 431.11, calcd. for $C_{21}H_{20}O_{11}$ *m/z* 432.10.

Quercetin-3-*O*-sophoriside (6) – Yellow powder; UV (MeOH): λ_{max} (log ε) 262 (3.83), 352 (3.50) nm; IR (KBr) ν_{max} cm^{-1}: 3320, 1672, 1620, 1030; ^{1}H-NMR (CD_3OD, 500 MHz): δ_H 7.60 (1H, dd, *J* = 8.5, 2.2 Hz, H-6′), 7.55 (1H, d, *J* = 2.2 Hz, H-2′), 6.87 (1H, d, *J* = 8.5 Hz, H-5′), 6.40 (1H, d, *J* = 2.1 Hz, H-8), 6.19 (1H, d, *J* = 2.1 Hz, H-6), 5.70 (1H, d, *J* = 7.3 Hz, H-1″); 4.60 (1H, d, *J* = 7.8 Hz, H-1″), 3.71 (1H, m, H-6″a), 3.70 (1H, m, H-6‴a), 3.56 (1H, m, H-6″b), 3.54 (1H, m, H-3″), 3.50 (1H, m, H-2″), 3.48 (1H, m, H-6‴b), 3.47 (1H, m, H-3‴), 3.46 (1H, m, H-2‴), 3.45 (1H, m, H-4″), 3.38 (1H, m, H-5″), 3.36 (1H, m, H-4‴), 3.34 (1H, m, H-5‴); ^{13}C-NMR (CD_3OD, 125 MHz): Table 2; TQD MS-ES$^+$ $[M+H]^+$ *m/z* 625.59.

Quercetin-3-*O*-rutinoside (7) – Yellow powder; UV (MeOH): λ_{max} (log ε) 268 (3.80), 356 (3.58) nm; IR (KBr) ν_{max} cm^{-1}: 3350, 1670, 1615, 1020; ^{1}H-NMR (CD_3OD, 500 MHz): δ_H 7.68 (1H, dd, *J* = 8.5, 2.2 Hz, H-6′), 7.70 (1H, d, *J* = 2.2 Hz, H-2′), 6.90 (1H, d, *J* = 8.4 Hz, H-5′), 6.45 (1H, d, *J* = 2.1 Hz, H-8), 6.25 (1H, d, *J* = 2.1 Hz, H-6), 5.17 (1H, d, *J* = 7.8 Hz, H-1″), 4.56 (1H, d, *J* = 1.6 Hz, H-1‴), 3.85 (1H, *J* = 11.0, 1.6 Hz, H-6″a), 3.67 (1H, dd, *J* = 3.5, 1.6 Hz, H-2‴), 3.57 (1H, dd, *J* = 9.4, 3.4 Hz, H-3‴), 3.53 (1H, m, 5‴), 3.52 (1H, m, H-6″b), 3.44 (1H, d, H-3″), 3.42 (1H, m, H-2″), 3.41 (1H, m, H-2″), 3.34 (1H, m, H-5″), 3.29 (1H, m, H-4‴), 1.16 (3H, d, *J* = 6.2 Hz, H-6‴); ^{13}C-NMR (CD_3OD, 125 MHz): Table 2; TQD MS ES$^-$ $[M–H]^-$ *m/z* 611.51.

Cytotoxicity assay – The P-388 cells were seeded into 96-well plates at an initial cell density of approximately 3 × 10^4 cells cm^{-3}. After 24 h of incubation for cell attachment and growth, varying concentrations of samples were added. The compounds added were first dissolved in DMSO at the required concentration. Subsequent six desirable concentrations were prepared using PBS (phosphoric buffer solution, pH = 7.30 – 7.65). Control wells received only DMSO. The assay was terminated after a 48 h incubation period by adding MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; also named as thiazol blue] and the incubation was continued for another 4 h, in which the MTT-stop solution containing SDS (sodium dodecyl sulphate) was added and another 24 h incubation was conducted. Optical density was read by using a micro plate reader at 550 nm. IC_{50} values were taken from the plotted graph of percentage live cells compared to control (%), receiving only PBS and DMSO, versus the tested concentration of compounds (μg/mL). The IC_{50} value is the concentration required for 50% growth inhibition. Each assay and analysis was run in triplicate and averaged.

Results and Discussion

The methanol extract of the fresh leaves of *K. prolifera* was succesively partitioned with *n*-hexane, EtOAc and *n*-BuOH. Repeated column chromatography using silica gel of the EtOAc soluble fractions led to the isolation of seven flavonoid compounds (Fig. 1). The structures of the isolated compounds were determined by spectroscopic methods including 1D, 2D NMR and ESI-TOFMS and TQD MS/MS. To the best our knowledge, compounds **1** - **7** were isolated from *K. prolifera* for the first time.

Compound **1** was obtained as yellow amorphous powder. The molecular formula was established to be $C_{15}H_{10}O_6$ from its ESI-TOFMS spectral data (*m/z* 285.2263 $[M-H]^+$), and NMR spectra (Table 1), thus requiring eleven degrees of unsaturations. The UV spectrum of **1** showed λ_{max} at 272 and 364 nm and exhibited a bathochromic shift by added NaoH and $AlCl_3$ suggested the presence of flavonoid structure with 4′ and 5-hydroxyl groups[15]. The IR spectrum of **1** showed the absorption band correspond to hydroxyl (3420 cm^{-1}), carbonyl (1690 cm^{-1}) and double bond (1605 cm^{-1}) groups. The ^{1}H-NMR spectrum of **1** showed the presence of *meta*-coupled of aromatic protons at δ_H 6.28 (1H, d, *J* = 2.0 Hz) and 6.52 (1H, d, *J* = 2.0 Hz) corresponds to H-6 and H-8, respectively. The ^{1}H-NMR spectrum of **1** also showed the presence of two doublet signals at δ_H 8.04 (2H, d, *J* = 2.8, 11.5 Hz, H-2′ and H-6′) and 6.95 (2H, d, *J* = 2.8, 9.7 Hz, H-3′ and H-5′) corresponds to four aromatic protons in ring B, characteristics for the 1′,4′-disubstituted flavone[16]. A total fifteen carbon signals were observed in the ^{13}C-NMR spectrum. These were assigned by DEPT and HMQC experiments to fourteen sp^2 carbons and a carbonyl signal at δ_C 176.6. The degree

of unsaturations was accounted for eight out of the total eleven double bond equivalents. The remaining three degree of unsaturation were consistent to flavonol structure[17]. A comparison of the NMR data of **1** with those of kaempferol,[18-20] revealed that the structures of the two compounds are very similar, therefore, compound **1** was identified as kaempferol.

Compound **2** was obtained as a yellow amorphous powder. The ESI-TOFMS of **1** gave a ion peak $[M-H]^+$ at *m/z* 301.40, compatible with the molecular formula $C_{15}H_{10}O_7$. Its UV absorptions in MeOH were consistent with the presence of a 3, 5, 7, 3′, 4′- pentahydroxyflavone structure[15]. The ^{1}H- and ^{13}C-NMR spectra of **1** exhibited resonances due to aromatic systems. The ^{13}C-NMR signals of **1** were assigned with the help of an HMQC experiment. In the ^{1}H-NMR spectrum of **1**, the aromatic region exhibited an ABX system at δ_H 7.65 (1H, d, J = 2.1 Hz, H-2′), 7.50 (1H, dd, J = 2.1, 8.4 Hz, H-6′), and 6.87 (1H, d, J = 8.4 Hz, H-5′) due to a 3′, 4′ disubstitution of ring B and a typical *meta*-coupled pattern for H-6 and H-8 protons (δ_C 6.20 and 6.40, d, J = 2.0 Hz). The ^{13}C-NMR spectrum of **1** showed the presence of 15 aromatic carbon signals. Based on the NMR data and comparison of the data given in the literature previously[18-19], the structure of compound **2** was identified as quercetin.

Compound **3** was isolated as yellow amorphous powder. In the UV spectral analysis of **3** gave a typical MeOH spectrum of quercetin derivatives[15,18]. The NMR spectra showed the presence of a quercetin moiety and sugar residue whose aglycone parts were the same as those of compound **2**. An anomeric proton signal of **3** appeared at δ_H 5.23 (1H, d, J = 7.6 Hz, H-1″), and the resonances at δ_H 3.71 (1H, dd, J = 2.5, 12.0 Hz, H-6b″), 3.56 (1H, dd, J = 5.0 12.0, Hz, H-6a″), 3.48 (1H, t, J = 9.2 Hz, H-2″), 3.44 (1H, t, J = 8.8 Hz, H-3″), 3.42 (1H, m, H-4″), 3.39 (1H, m, H-5″); together with the corresponding carbon resonances inferred from the HMQC spectrum suggested the presence of β-glucopyranose units. In the HMBC spectrum, a crosspeak between C-3 and H-1″ established the linkage point quercetin and sugar moieties. Consequently, the structure of **3** was identified as quercetin-3-*O*-β-D-glucopyranoside[16,21].

Compound **4** was isolated as yellow amorphous powder and has a molecular formula of $C_{21}H_{20}O_{11}$ from its LC-MS spectral (*m/z* 448.35) and NMR data (Table 1). In the UV and IR spectral analysis of **4** gave a typical spectra of kaempferol derivatives[15,17]. The NMR spectra of **3** was very similar to those of **1**, except the presence of an anomeric proton as a doublet at δ_H 5.48 corresponds to sugar residue and was identified as β-glucopyranose on

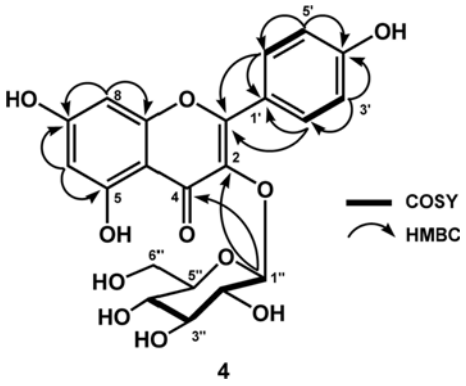

Fig. 2. Key HMBC (→) and COSY (—) correlations of **3**.

the basis of coupling constant ($J_{1'',2''}$ = 7.8 Hz)[19,22]. The ^{13}C-NMR spectrum showed 21 carbon signals, which were classified by their chemical shifts and the DEPT spectra as one oxygenated sp^3 methylene, four oxygenated sp^3 methines, one anomeric carbon, six sp^2 methines, eight sp^2 quartenary carbon and one carbonyl. The double bond equivalen was accounted for eight out of the total twelve double bond equivalents. The remaining four double bond equivalens were consistent with the flavone skeleton with a β-glucose residue[19,22]. Based on the above evidences and biogenetic point of view occurance the flavonoid structure in *Kalanchoe* genus, suggested that compound **4** having flavonoid structure consists of kaempferol as an aglycone moiety and β-D-glucoside as a sugar residue. The position of the β-D-glucosyl moiety in **4** was identified at C-3 position on the basis of COSY and HMBC correlations as shown in Fig. 2. Consequently, structure **4** was identified as kaempferol-3-*O*-β-D-glucopyranoside or astragalin consistent to the reported literature values[19,20,22,23].

Compound **5** was isolated as yellow amorphous powder and has a molecular formula of $C_{21}H_{20}O_{11}$ from its LC-TOFMS spectral (*m/z* 432.11) and NMR data (Table 2), thus requiring twelve degrees of unsaturation. In the UV and IR spectral analysis of **5** gave a typical spectra of kaempferol derivatives[15,17]. NMR signals of **5** were similar to those of **3**, the main differences are the presence of a secondary methyl group at as a doublet at δ_H 0.94 (3H, d, J = 6.0 Hz) and absence of oxymethylene group at C-5″ position of the sugar unit, suggested that compound **3** is consist a flavonoid skeleton of kaempferol as an aglycone moiety and rhamnoside as a sugar unit. The anomeric proton had a coupling constant of 1.9 Hz, conforming the α-orientation of L-rhamnosyl moiety. Based on the above spectral data, structure **4** was identified as kaempferol-3-*O*-α-L-rhamnoside or afzelin consistent with previously reported[17,20].

Compound **6** was isolated as yellow amorphous powder. The TQD MS-ES$^+$ $[M+H]^+$ *m/z* 625.59, compatible with the molecular formula of $C_{27}H_{30}O_{17}$, thus requiring thirteen degree of unsaturations. In the UV spectral analysis of **6** gave a typical spectra of quercetin derivatives[15,18]. The ^{1}H-NMR spectra of **6** was very similar to those of **5**, except the presence of a newly anomeric proton at δ_H 4.60 (1H, d, J = 7.8 Hz, H-1‴), and oxygenated methylene protons at δ_H 3.48 (1H, m, H-6a‴) and 3.70 (1H, m H-6b‴), suggested that compound **6** as a quercetin-*O*-diglicoside. The ^{13}C-NMR of **6** showed 27 carbon signals, which were classified by their chemical shifts and DEPT spectra as one conjugated carbonyl at δ_C 177.9, two oxygenated sp^3 methylene at δ_C 61.2 (C-6‴) and 61.4 (C-6″), eight oxygenated sp^3 methine at δ_C 83.2, 78.0, 77.2, 77.0, 76.5, 74.9, 70.1 and 69.6, two anomeric carbons at δ_C 104.5 and 99.1, five sp^2 methine and nine sp^2 quartenary carbons. The degree of unsaturation was accounted for eight out of the total thirteen degree of unsaturations. The remaining five degree of unsaturations were consistent with the flavone skeleton with di-glucose residue[23-25]. The stereochemistry of two glucose was ditermined, based on vicinal coupling constant at δ_H 5.70 (1H, d, $J_{1'',2''}$ = 7.3 Hz, H-1″) and 4.60 (1H, d, $J_{1''',2'''}$ = 7.8 Hz, H-1‴), indicated β-oriented of two glucose unit. A comparison of the NMR data of **6** with those of quercetin-3-*O*-sophoroside[24,25], revealed that the structures of two compounds are very similar, therefore, compound **6** was identified as quercetin-3-*O*-sophoroside.

Compound **7** was isolated as yellow amorphous powder. The molecular formula of **7** was established to be $C_{27}H_{30}O_{17}$, based on TQD MS ES$^-$ $[M-H]^-$ *m/z* 611.51 and NMR spectra, thus requiring thirteen degree of unsaturations. The UV and IR spectra of **7** were similar to those of **6** gave a typical of quercetin derivatives[15,18]. The NMR of **7** was similar to those of **6**, except the absence one of the oxygenated methylene at [δ_C 61.2, δ_H 3.48 (1H, m, H-6a‴), 3.70 (1H, m H-6b‴),] in sugar units and the presence of newly methyl signal at [δ_C 17.8, δ_H 1.16 (3H, d, J = 6.2 Hz) in sugar unit, suggested that the presence of a rhamnosyl sugar unit in **7**. The stereochemistry of glucose at C-1″ and rhamnose unit at C-1‴ were determined based on of vicinal coupling constant at $J_{1'',2''}$ = 7.8 Hz and $J_{1''',2'''}$ = 1.6 Hz), indicated α-oriented between two sugar units[19,22]. A comparison of the NMR data of **7** with those of quercetin-3-*O*-rutinoside[26,27], revealed that the structures of two compounds are very similar, therefore, compound **7** was identified as quercetin-3-*O*-rutinoside or rutin.

The cytotoxicity effects of the seven isolated compounds **1** - **7** against the P-388 murine leukemia cells were conducted according to the method described in previous paper[28-30] and were used an Artonin E (IC_{50} 0.3 μg/mL) as a positive control[31]. The cytotoxicity activities of isolated compounds **1** - **7** are shown in Table 3. Among those flavonoid compounds, flavonoid without sugar unit such as kaempferol (**1**) and quercetin (**2**) showed strongest cytotoxic activity, while flavonoid with one or two sugars unit showed weak or no cytotoxic activity. These results suggested that the presence of sugar unit in flavonoid structure can decrease cytotoxic activity against P-388 murine leukimia cells.

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