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Flavonoids from the Fresh Leaves of *Kalanchoe tomentosa* (Crassulaceae)

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Abstract: Flavonoids compounds such as kaempferol (**1**), kaempferol-3-*O*-β-D-glucopyranoside or astragalin (**2**) and kaempferol-3-*O*-α-L-rhamnoside or afzelin (**3**) have been isolated from the fresh leaves of *Kalanchoe tomentosa* (Crassulaceae). The chemical structure of isolated compounds **1-3** were identified by spectroscopic evidences and comparison with those compound previously reported. Compounds **1-3** showed cytotoxic activity against P-388 murine leukimia cells with IC_{50} values of 51.8, > 100 and 3.32 μg/mL, respectively.

Keywords: Crassulaceae, cytotoxic activity, flavonoids, *Kalanchoe tomentosa*, P-388 murine leukimia cells.

INTRODUCTION

Kalanchoe tomentosa (Crassulaceae) is a perennial, succulent medicinal herb which grown in high terrain and indigenous to low altitude of Indonesia [1]. The leaves of *K. tomentosa* are used in Indonesian folk medicine for the treatment of fever, infections, rheumatism and skin diseases [2]. The genus of *Kalanchoe* reported to contain bufadienolides [3-5], triterpenoids [6] and flavonoids [7-9] which possess multiplebiological activities such as blocking human lymphocyte proliferation [6,10], cytotoxic agents [11], insecticidal properties [5, 12] and inhibiting cancer cell growth [13, 14]. However, information about phytochemical constituents of *K. tomentosa* is unavailable. Our preliminary screening for novel cytotoxic agents from Indonesian *Kalanchoe* plants, we found that the methanolic extract of *K. tomentosa* exhibited significant cytotoxic effect against P-388 murine leukimia cells. In this communication, the isolation and structure identification of flavonoids (**1-3**) along with their cytotoxic activity against P-388 murine leukimia cells will be described.

EXPERIMENTAL

General Experimental Procedures

Ultra-Violet spectra were 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} mass spectrometer. NMR spectra were recorded with a JEOL JNM A-500 spectrometer using tetra methyl silane (TMS) as an internal standard. Chromatographic separation was done 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) and detection was achieved with 10% H_2SO_4 in ethanol followed by heating.

Plant Material

The fresh leaves of *K. tomentosa* were collected from Lembang Discrict, West Bandung, Indonesia in May, 2011. The plant was identified in Bogoriense Herbarium, Bogor, Indonesia and a voucher specimen (No. B0-129211) was deposited at the herbarium.

Extraction and Isolation

Fresh grounded leaves (20 Kg) of *K. tomentosa* were extracted with MeOH at room temperature. The MeOH extract was evaporated under reduced pressure to yield a dark brown residue (360 g). The MeOH extract was dissolved in water and partitioned succesively with *n*-hexane, EtOAc and *n*-butanol. Evaporation of each solvents resulted in the crude extract of *n*-hexane (30.5 g), EtOAc (64.5 g) and *n*-butanol (43.5 g), respectively. The *n*-hexane, ethyl acetate and *n*-butanol extracts exhibited a cytotoxic activity against P-388 murine leukimia cells with IC_{50} values of 56.5, 24.4 and 45.2 μg/mL, respectively. A portion of the EtOAc extract (50 g) was subjected to vacuum liquid chromatography on silica gel G60 using gradient elution of *n*-hexane-EtOAc-MeOH to afford 15 fractions (A01-A015). Fraction A05 (3.5 g) was further subjected to column chromatography on silica gel (70-230 mesh) using mixture of *n*-hexane-EtOAc (10:0-5:1) as eluting solvents to afford 10 fractions (B01-B10). Fraction B04 (230 mg) was subjected to flash column chromatography on silica gel (230-400 mesh), eluted with $CHCl_3$-MeOH (9:1), to give **1** (12.4 mg). Fraction A06-A07 was combined (4.2 g) and subjected

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to silica gel column chromatography on silica gel (70-230 mesh) using mixture of *n*-hexane-acetone (10:0-1:1) as eluting solvents to give 10 fractions (C01-C10). Fractions C06 (185 g) were subjected to column chromatography on silica gel (230-400 mesh) using mixture of $CHCl_3$-MeOH (10:0-1:1) as eluting solvents to afford 6 fractions (D01-D6). Fraction D03 (73 mg) was subjected to preparative TLC on silica gel GF_{254}, eluted with $CHCl_3$:MeOH (9.5:0.5) to give **2** (12.5) and **3** (7.8 mg).

Determination of Cytotoxic Activity

P-388 cells were seeded into 96-well plates at an initial cell density of approximately 3 x 10^4 cells cm^{-3}. After 24 hours of incubation for cell attachment and growth, concentrations of samples were added. The samples first were dissolved in DMSO at the required concentration. Subsequent six concentrations were prepared using PBS (phosphoric buffer solution, pH = 7.30 - 7.65). Control wells only DMSO. The assay was stopped after a 48 hours incubation periode by adding MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetra-zolium bromide and the incubation was continued for another 4 hours, in which the MTT-stop solution containing SDS (sodium dodecyl sulphate) was added and another 24 hours 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

Fresh leaves of *K. tomentosa* were grounded and extracted with methanol at room temperature and concentrated. The methanol extract was suspended in water and successively partitioned with *n*-hexane, ethyl acetate and *n*-butanol. The extracts were tested for their cytotoxic activity against P-388 murine leukimia cells and the ethyl acetate extract showed strongest cytotoxic activity. Subsequent phytochemical analysis was therefore focused on the ethyl acetate extract. The ethyl acetate extract was chromatographed over a vacuum liquid cromatography (VLC) column packed with silica gel 60 by gradient elution. The VLC fractions were repeatedly subjected to silica gel column chromatography and preparative TLC on silica gel GF_{254} to afford three flavonoid compounds **1-3** (Fig. **1**).

Compund **1** was isolated as a yellowish amorphous. Its UV spectra showed maximum absorption at 272 and 364 nm in MeOH. Its UV spectra showed bathoromic shifts at 280 and 376 nm with NaOH and $AlCl_3$. IR λ_{max} (KBr): 3420, 1690, 1605, 1260, and 720 cm^{-1}. ^{1}H-NMR (CD_3OD, 500 MHz): δ 6.28 (1H, d, *J*=1.95 Hz, H-6), 5.95 (1H, d, *J*=1.95 Hz, H-8), 7.15 (2H, d, *J*=6.90 Hz, H-2', H-6'), 7.01 (2H, d, *J*=6.80 Hz, H-3', H-5'). ^{13}C-NMR (CD_3OD, 125 MHz): δ 157.7 (C-2), 136.6 (C-3), 176.6 (C-4), 162.3 (C-5), 99.2 (C-6), 164.9 (C-7), 99.1 (C-8), 104.5 (C-4a), 156.9 (C-8a), 123.3 (C-1'), 128.4 (C-2'), 114.5 (C-3'), 159.4 (C-4'), 114.5 (C-5'), 128.4 (C-6'). HR-TOFMS (positive ion mode) *m/z* 287.0486 $[M+H]^+$, (calcd. for $C_{15}H_{10}O_6$), *m/z* 286.0477).

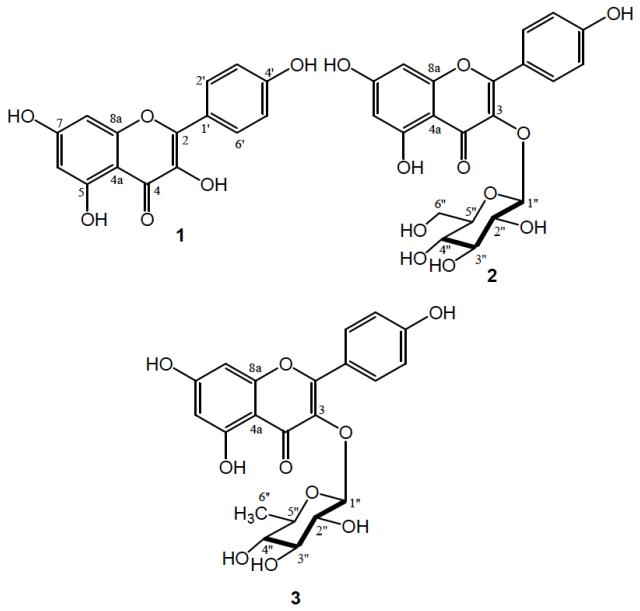

Fig. (1). Structures of isolated compounds **1-3**.

Compound **1** has a molecular formula of $C_{15}H_{10}O_6$ from its HR-TOFMS spectral data (*m/z* 287.0486 $[M+H]^+$) and NMR data, thus requiring eleven double bond equivalents. 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 having free 4'- and 5- hydroxyl groups in **1**. The IR spectrum of **1** showed the absorption band correspond to hydroxyl (3420 cm^{-1}), conjugated carbonyl (1690 cm^{-1}) and conjugated double bond (1605 cm^{-1}). The ^{1}H-NMR spectrum of **1** showed the presence of two *meta*-coupled aromatics protons at δ 6.28 and 5.95 corresponds to H-6 and H-8 with coupling constant 1.95 Hz. The ^{1}H-NMR spectrum of **1** also showed the presence of two doublet signals at δ 7.15 (2H, d, *J*=6.80 Hz, H-2' and H-6') and 7.01 (2H, d, *J*=6.80 Hz, H-3' and H-5') corresponds to the four aromatic protons in ring B, characteristics for the 1',4'-disubstituted flavone. 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 176.6. The double bond equivalent was accounted for one out of the total eleven double bond equivalents. The remaining ten double bond equivalents were consistent to flavonol structure [14]. A comparison of the NMR data of **1** with those of kaempferol [14, 15], revealed that the structures of the two compounds are very similar, therefore, compound **1** was identified as kaempferol, which was reported for the first time from *K. tomentosa*.

Compound **2** was isolated as a yellowish solid. UV spectrum λ_{max} (MeOH) nm: 267, 350; IR ν_{max} (KBr) cm^{-1}: 3400, 1730, 1607, 1078. ^{1}H-NMR (CD_3OD, 500 MHz): δ 6.18 (1H, d, *J*=1.90 Hz, H-6), 6.36 (1H, d, *J*=1.90 Hz, H-8), 7.04 (2H, d, *J*=6.95 Hz, H-2', H-6'), 6.85 (2H, d, *J*=6.95 Hz, H-3', H-5'), 5.20 (1H, d, *J*=7.5 Hz, H-1''), 3.79 (1H, m, H-2''), 3.42 (1H, m, H-3''), 3.42 (1H, m, H-4''), 3.72 (1H, m, H-5''), 3.52 (1H, dd, *J*=12.0, 4.5 Hz, H_a-6''), 3.68 (1H, dd, *J*=12.0, 2.0 Hz, H_b-6''). ^{13}C-NMR (CD_3OD, 125 MHz): δ 154.6 (C-2), 135.6 (C-3), 179.4 (C-4), 106.6 (C-4a), 162.6

(C-5), 100.2 (C-6), 165.8 (C-7), 98.4 (C-8), 159.6 (C-8a), 122.6 (C-1'), 128.4 (C-2'), 116.3 (C-3'), 160.8 (C-4'), 116.3 (C-5'), 128.4 (C-6'), 97.1 (C-1''), 79.7 (C-2''), 78.1 (C-3''), 74.3 (C-4''), 78.4 (C-5''), 66.6 (C-6''). HR-TOFMS (positive ion mode) *m/z* 449.3567 $[M+H]^+$, (calcd. for $C_{21}H_{20}O_{11}$), *m/z* 448.3769).

Compound **2** has a molecular formula of $C_{21}H_{20}O_{11}$ from its HR-TOFMS spectral data (*m/z* 449.3567 $[M+H]^+$) and NMR data, indicating the presence of twelve double bond equivalents. UV spectrum of **1** displayed λ_{max} at 267 and 350 nm indicated the presence of a flavonoid skeleton [14]. IR spectrum of **1** indicated the presence of hydroxyl (3400 cm^{-1}), carbonyl (1710 cm^{-1}), conjugated double bond (1607 cm^{-1}) and ether groups (1078 cm^{-1}). ^{1}H-NMR spectrum of **2** displayed that protons in B ring gave a doublet (2H, d, *J*=6.95 Hz) at δ 7.04 and 6.85, suggested the presence *p*-disubstituted benzene ring. Protons in ring A were observed at δ 6.18 (1H, d, *J*=1.90 Hz) and 6.36 (1H, d, *J*=1.90 Hz), respectively, suggested the presence of *meta*-aromatic proton at H-8 and H-6. Based on these observation and comparison of NMR data previously reported [15], indicated that **2** had a kaempferol as a glycone. The ^{1}H-NMR spectra of **2** also showed the presence of an anomeric proton as a doublet at δ5.20 suggesting a sugar residue was identified as β-glucopyranose on the basis of coupling constant ($J_{1'',2''}$=7.5 Hz) [15, 16]. 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 [15]. Based on the above evidences and biogenetic point of view occurance the flavonoid structure in *Kalanchoe* genus, suggested that compound **2** having flavonoid structure [14-16] consists of kaempferol as an aglycone moiety and β-D-glucoside as a sugar residue. The position of the β-D-glucosyl moiety in **2** was identified at C-3 position on the basis of COSY and HMBC correlations as shown in Fig. (**2**). Consequently, structure **2** was identified as kaempferol-3-*O*-β-D-glucoside or known as astragalin consistent to the reported literature values [16, 17], which was reported for the first time from *K. tomentosa*.

Compund **3** was isolated as a yellow solid. Its UV spectra showed maximum absorption at 276 and 370 nm in MeOH. IR ν_{max} (KBr): 3360, 1670, 1607, 1508, 1452, 1024 and 820 cm^{-1}. ^{1}H-NMR (CD_3OD, 500 MHz): δ12.55 (1H, s, 5–OH), 7.20 (2H, d, *J*=7.20 Hz, H-2', H-6'), 6.90 (2H, d, *J*=7.20 Hz, H-3', H-5'), 6.10 (1H, d, *J*=2.0 Hz, H-8), 5.95 (1H, d, *J*=2.0 Hz, H-6), 5.49 (1H, d, *J*=1.9 Hz, H-1''), 3.80 (1H, dd, *J*=8.0, 7.1 Hz, H-2''), 3.45 (1H, dd, *J*=10.0, 4.30 Hz, H-3''), 3.42 (1H, t, *J*=10.0 Hz, H-4''), 3.74 (1H, dd, *J*= 10.0, 6.1 Hz, H-5''), 0.94 (3H, d, *J*= 6.0 Hz, H-6''); ^{13}C-NMR (CD_3OD, 125 MHz): δ 153.6 (C-2), 134.6 (C-3), 179.7 (C-4), 104.5 (C-4a), 163.3 (C-5), 98.6 (C-6), 165.5 (C-7), 99.4 (C-8), 155.0 (C-8a), 124.0 (C-1'), 128.4 (C-2'), 114.6 (C-3'), 158.0 (C-4'), 114.6 (C-3'), 128.4 (C-2'), 97.0 (C-1''), 74.8 (C-2''), 77.5 (C-3''), 78.6 (C-4''), 75.6 (C-5''), 20.8 (C-6''). HR-TOFMS (positive ion mode) *m/z* 433.1104 $[M+H]^+$, (calcd. for $C_{21}H_{20}O_{10}$), *m/z* 432.1056).

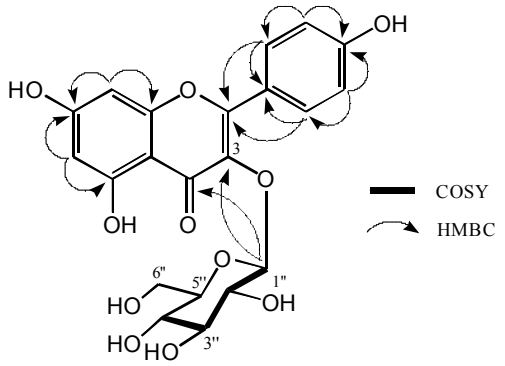

Fig. (2). Selected COSY and HMBC Correlations of **2**.

Compound **3** has a molecular formula of $C_{21}H_{20}O_{10}$ from its HR-TOFMS spectral data (*m/z* 433.1102 $[M+H]^+$) and NMR data, thus requiring twelve degrees of unsaturation. The UV spectrum of **3** showed λ_{max} at 276 and 370 nm indicated a flavonoid structure [14,15]. The IR spectrum of **3** showed the presence of hydroxyl (3360 cm^{-1}), conjugated carbonyl (1670 cm^{-1}), conjugated double bond (1607 cm^{-1}) and ether group (1024 cm^{-1}). ^{1}H- and ^{13}C NMR signals of **3** were similar to those of **2**, the main differences are the presence of a secondary methyl group at as a doublet at δ 0.94 (3H, d, *J*=5.7 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 **3** was identified as kaempferol-3-*O*-α-L-rhamnoside consistent with previously reported [14,18], which was reported for the first time from *K. tomentosa*.

The cytotoxic effects of the three isolated compounds **1**-**3** against the P-388 murine leukimia cells were conducted according to the method described in previous paper [19, 20] and were used an artonin E (IC_{50} 0.3 μg/mL) as a positive control [21]. Compounds **1**-**3** showed cytotoxicity against P-388 murine leukimia cells with IC_{50} values of 51.8, > 100 and 3.32 μg/mL, respectively, suggested that the presence of rhamnoside sugar unit in flavonoid structure can increase cytotoxic activity whereas the presence of glucoside unit can decrease cytotoxic activity.

CONCLUSION

The ethyl acetate extract of *K. tomentosa* fresh leaves showed the presence of flavonoid structure, kaempferol (**1**), kaempferol-3-*O*-β-D-glucoside or astragalin (**2**) and kaempferol-3-*O*-α-L-rhamnoside or afzelin (**3**). The presence of rhamnoside sugar unit in flavonoid structure can increase the cytotoxic activity against P-388 murine leukimia cells.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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