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# A new flavone and cytotoxic activity of flavonoid constituents isolated from *Miliusa balansae* (Annonaceae)

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A new flavone named miliufavol [8-(2-hydroxybenzyl)-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3,7-dimethoxy-4*H*-chromen-4-one or 8-C-(o)-hydroxybenzylpachypodol] from *Miliusa balansae* (Annonaceae) was isolated and structurally elucidated by spectroscopic means besides four known flavones: ombuine, chrysosplenol B, pachypodol and chrysosplenol C. These flavonoids exhibited interesting cytotoxic activity against three human cell lines (KB, Hep-G2, RD) with $IC_{50}$ values $< 5$ μg/ml.

## 1. Introduction

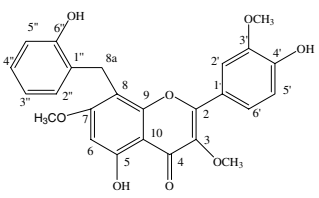
The plant *Miliusa balansae* Fin. & Gagn. is a shrub of the family Annonaceae (Ho 1999). In Chinese traditional medicine, this plant is used for gastropathy and glomerulonephropathy (Wu et al. 2001). Recently, two geranyl homogentisic acid derivatives named milusate and miliusol, two new styryl compounds, some flavanones, dihydrochalcones and flavones were isolated from this plant (Ho 1999; Kamperdick et al. 2002; Huong et al. 2004). In this paper we report on the isolation and the structure elucidation of a new flavone named miliufavol (**1**) as well as the cytotoxicity of four flavones isolated from the same plant.

## 2. Investigations, results and discussion

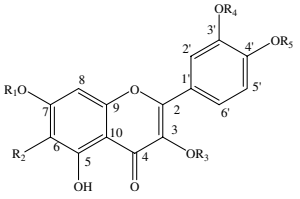
Compound **1** was obtained as yellow amorphous solid. Its IR spectrum showed the presence of hydroxyl groups (3532 and 3251 $cm^{-1}$) and carbonyl group (1659 $cm^{-1}$). The molecular formula of **1** was determined to be $C_{25}H_{22}O_8$ from $[M + Na]^+$ peak at m/z 473.12243 and $[M + H]^+$ peak at 451.14042 in the high resolution mass spectrum (HR-ESI-TOF-MS), which is supported by the EIMS with a molecular ion peak at m/z 450 $[M]^+$, as well as the fragments 344 $[M-HOC_6H_4CH_2 + H]^+$, 343 $[M-HOC_6H_4CH_2]^+$ and 221 $[344\text{-}HOC_6H_3OCH_3]^+$. In the negative HR-ESI-TOF-MS the peak at m/z 449.12344 corresponds the $[M-H]^+$ fragment. The Fig. shows mass spectral fragmentation of **1**. Full analysis of HH-COSY, HMQC and HMBC confirmed **1** to be a new flavone.

The $^1H$ and $^{13}C$ NMR spectra exhibited three methoxyl groups ($\delta_C$ 60.2, 56,8 and 56.1; $\delta_H$ 3.91 s, 3.99 s and 3.71 s), three hydroxyl groups ($\delta_H$ 12.93 s, 8.51 s and 8.39 s) together with other signals corresponding to this structure (Table 1).

The o-hydroxybenzyl moiety was determined by the analysis of $^1H$ NMR spectrum (4.21 s, 2H8a, 4 H in o-hydroxybenzyl ring are neighbouring each other to give the corresponding special pattern and coupling constants: 6.74 dd (7.4; 1.8, H2″), 6.67 td (7.4; 1.2, H3″), 7.01 td (7.4; 1.8, H4″), 6.90 dd (8.0; 1.2, H5″). On the other hand this structural feature was confirmed by H−H COSY correlations: H2″/H3″ (st), H3″/H4″ (st), H4″/H5″ (st). The C−H long range correlations from the $^1H$-$^{13}C$ HMBC experiment and NOESY enhancements confirmed the location of the methoxy and hydroxyl groups as shown in structure **1**. The linkage of the hydroxy benzyl group at C8 was proven by the correlations: H8a/C8, C7, C9 and 7-O$\underline{C}H_3$ (Galal et al. 1997). The correlations H6 (6.59 ppm)/C8, C10, C5, C7, 7-OC$\underline{H_3}$ and 5-O$\underline{H}$ have also been found. From this spectral data we can conclude that



1. Miliufavol



2. Ombuine: $R_1=R_5=CH_3$, $R_2=R_3=R_4=H$
3. Chrysosplenol B: $R_1=R_3=R_4=CH_3$, $R_2=OCH_3$, $R_5=H$
4. Pachypodol: $R_1=R_3=R_4=CH_3$, $R_2=R_5=H$
5. Chrysosplenol C: $R_1=R_3=R_4=CH_3$, $R_2=OH$, $R_5=H$

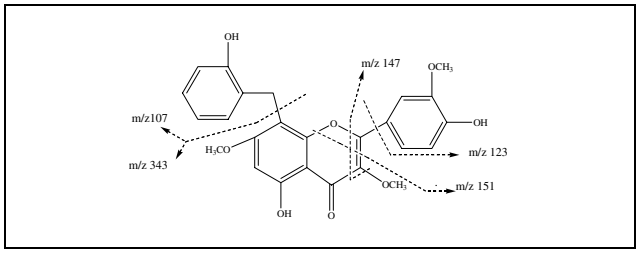



Fig.: Proposed mass spectral fragmentation of compound **1**

compound **1** is a C8-o-benzyl derivative of pachypodol, which has also been isolated from this plant. This conclusion was further supported by comparison of a part of $^1$H and $^{13}$C NMR data (ppm) of **1** and pachypodol **(4)** (see Table 2). The new compound **1** has been named miliufavol.

Besides compound **1**, ombuine (**2**) (Itokawa et al. 1981) and chrysosplennol B (**3**) (De Pascual Teresa et al. 1980; Roesler et al. 1984) were isolated. Pachypodol (**4**) (Itokawa et al. 1981) and chrysosplenol C (**5**) (Yoshinori et al. 1985) from this plant. The cytotoxicity of compounds **2**–**5** against the cancer cell lines KB (human epidemoid carcinoma), Hep-G2 (Hepatoma-G2) and RD (Rhabdosarcoma) was investigated. These assays are based on the method of Likhiwitayawuid et al. (1993) and Skehan et al. (1990). The $IC_{50}$ values are shown in Table 3. The results showed that all four compounds were found active against three tested cell lines. Among them, pachypodol (**4**) has strong activities against two cell lines (KB: 0.7 μg/ml, Hep-G2: 0.55 μg/ml). It seems to be interesting to do further studies on the cytotoxic activity of pachypodol.

## 3. Experimental

Mps: HMK-Boetius, FTIR: Nicolet Impact 410, NMR: Bruker Avance 500 MHz, EIMS: HP 5989B, 70 eV, HR-ESITOF-MS: QStar Pulsar (Applied Biosystems), C.C: silicagel (230–400 mesh): Merck.

### *3.1. Plant material*

Leaves and branches *of Miliusa balansae* Fin. & Gagn. were collected near Hoang Hoa Tham village, Chi Linh district, Hai Duong province, North Viet Nam, in Oct. 2000 and identified by Dr. Ngo Van Trai, Institute of Materia Medica, Ha noi. A voucher specimen (No. TC023) is deposited at this Institute.

### *3.2. Extraction and isolation*

Air dried ground leaves and branches (2.3 kg) of *M. balansae* were extracted several times with MeOH–$H_2O$ (95-5) at room temperature to give 200 g MeOH extract after evaporating the solvents. 160 g of this extract were redissolved in water and subjected to a liquid-liquid partition with EtOAc and n-BuOH, succesively, giving 110 and 40 g extract, respectively. The EtOAc extract was separated by CC on silica gel (230–400 mesh) using solvent mixtures with increasing polarity (n-hexane, EtOAc and MeOH) giving 10 fractions. Fraction 4 was crystallized from $CH_2Cl_2$ to give 1.2 g of compound **4**. Fraction 5 contained compound **2** which was purified by CC on silica gel using n-hexane-EtOAc mixture with increasing polarity to give 200 mg of compound **2**.

Compound **1** and compound **3** were isolated again by CC of fraction 5 with solvents $CHCl_3$-(5 → 20% EtOAc) to give compound **1** (5 mg) and compound **3** (7 mg). Fraction 6 was crystallized from $CH_2Cl_2$–MeOH and yielded 2.5 g of compound **5**.

Miliufavol (**1**): m.p 250–252 °C ($CHCl_3$), IR (KBr), $\nu^*_{max}$ 3532, 3251, 2963, 2856, 1659, 1605, 1566, 1452, 1338, 1265, 1104, 1031, 803, 596 cm$^{-1}$; HR-ESITOF-MS m/z 473.12243 $[M + Na]^+$, calcd. for $C_{25}H_{22}O_8Na$ 473.12069; EIMS (70 eV) m/z (%) 450 $[M]^+$ (46.6), 344 (63.2), 343 (54.6), 221 (76.6), 151 (53.7), 147 (73.2), 123 (22.8), 107 (36.5), 73 (100), 55(28.2). $^1$H and $^{13}$C NMR, see Table 1.

3,3′,5-Trihydroxy-4′,7-dimethoxyflavone (**2**, ombuine): m.p. 201–203 °C (ethyl acetate/n-hexane); IR (KBr), $\nu^*_{max}$ (cm$^{-1}$): 3500, 3482, 3296, 1655, 1611, 1500, 1311, 1159, 1042, 796, 638; EIMS (70 eV) m/z (%) 330 $[M]^+$ (100); $^1$H NMR (DMSO-$d_6$, 500 MHz), δ (ppm): 12.46 (s, O<u>H</u>), 9.75 (s, O<u>H</u>), 9.53 (s, OH), 7.79 (d, J = 2.0 Hz, H2′), 7.75 (dd J = 8.5 and

**Table 1: $^1$H and $^{13}$C NMR spectral data of 1 (acetone-$d_6$; 500 MHz for $^1$H and 125 MHz for $^{13}$C NMR)**

| C | $\delta_C$ (ppm) | $\delta_H$ (ppm) | J (Hz) | HMBC | NOESY |
|---|---|---|---|---|---|
| 2 | 156.7 | – | – | – | – |
| 3 | 139.2 | – | – | – | – |
| 4 | 180.0 | – | – | – | – |
| 5 | 162.0 | – | – | – | – |
| 6 | 95.6 | 6.59 s | – | H6/C8, C10, C5, C7 | H6/7-OC<u>H</u>$_3$ (st), H4″, 5-O<u>H</u> |
| 7 | 164.5 | – | – | – | – |
| 8 | 106.2 | – | – | – | – |
| 9 | 154.7 | – | – | – | – |
| 10 | 106.3 | – | – | – | – |
| 1′ | 123.0 | – | – | – | – |
| 2′ | 112.0 | 7.51 d | 2.0 | H2′/C1′, C6′, C3′ (w), C4′, C2 (w) | H2′/3′-OC<u>H</u>$_3$ (st), H8a, H4″, H6′ |
| 3′ | 148.3 | – | – | – | – |
| 4′ | 150.4 | – | – | – | – |
| 5′ | 116.0 | 6.93 d | 8.5 | H5′/C1′, C6″, C3′, C4′ (w) | H5′/4′-O<u>H</u>, H2″ |
| 6′ | 123.7 | 7.67 dd | 8.5; 2.0 | H6′/C2′, C4′ | H6′/3-OC<u>H</u>$_3$, H8a (w) ,H2′ |
| 8a | 22.9 | 4.21 s, 2 H | – | H8a/C8, C1″, C9, C6″, C7 | H8a/3′-OC<u>H</u>$_3$, 7-OC<u>H</u>$_3$ (st), H2″, H2′, H6′ (w), 6″-O<u>H</u> (w) |
| 1″ | 127.0 | – | – | – | – |
| 2″ | 128.8 | 6.74 dd | 7.4; 1.8 | H2″/ C4″, C6″, C8a | H2″/H8a, H5″, H4″ |
| 3″ | 120.3 | 6.67 td | 7.4; 1.2 | H3″/C5″, C1″, C4″ | H3″/H5″ (st) |
| 4″ | 127.7 | 7.01 td | 7.4; 1.8 | H4″/C2″, C6″ | H4″/H2″, H2′, H6 |
| 5″ | 115.5 | 6.90 dd | 8.0; 1.2 | H5″/C3″, C4″ | H5″/H3″ (st), H2″, 6″-O<u>H</u> |
| 6″ | 156.0 | – | – | – | – |
| 7-$OCH_3$ | 60.2 | 3.91 s | – | 7-OC<u>H</u>$_3$/C7 (st) | 7-OC<u>H</u>$_3$/H8a (st), 5-O<u>H</u>, H6 (st) |
| 3′-$OCH_3$ | 56.1 | 3.71 s | – | 3′-OC<u>H</u>$_3$/C3′ (st) | 3′-OC<u>H</u>$_3$/H2′ (st), H6, 3-OC<u>H</u>$_3$ (st) |
| 3-$OCH_3$ | 56.8 | 3.99 s | – | 3-OC<u>H</u>$_3$/C3 (st) | 3-OC<u>H</u>$_3$/H6′, 3′-OC<u>H</u>$_3$ (st) |
| 4′-OH | – | 8.39 s | – | – | 4′-O<u>H</u>/H5′ |
| 5-OH | – | 12.93 s | – | 5-O<u>H</u>/C6, C10, C5 | 5-O<u>H</u>/7-OC<u>H</u>$_3$, H6 |
| 6″-OH | – | 8.51 s | – | 6″-O<u>H</u>/C6″ (w), C1″ | 6″-O<u>H</u>/H5″, H8a (w) |

(st: strong, w: weak)

**Table 2: Extract of $^1$H and $^{13}$C NMR data (ppm) of 1 and 4 (acetone-$d_6$: 500 MHz for $^1$H and 125 MHz for $^{13}$C NMR)**

| Position C | 1 | | Pachypodol (4) | |
|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 6 | 6.59 s | 95.6 | 6.64 d | 98.5 |
| 7 | – | 164.5 | – | 166.6 |
| 8 | – | 106.2 | 6.30 d | 92.9 |
| 9 | – | 154.7 | – | 156.9 |

**Table 3: Cytotoxic activity of flavonoids 2–5**

| Sample | Cancer cell lines $IC_{50}$ values (μg/ml) | | |
|---|---|---|---|
| | KB | Hep-G2 | RD |
| Ombuine **(2)** | >5 | 1.5 | >5 |
| Chrysosplenol B **(3)** | 4.6 | 0.93 | >5 |
| Pachypodol **(4)** | 0.7 | 0.55 | 3.01 |
| Chrysosplenol C **(5)** | 4.3 | 0.57 | 2.09 |
| Control: elipithin (Sigma) | 0.002 | 0.001 | 0.001 |

2.0 Hz, H6′), 6.95 (d, J = 8.5 Hz, H5′), 6.79 (d, J = 2.2 Hz, H8), 6.36 (d, J = 2.2 Hz, H6, 3.88 (s, OC$\underline{H}_3$), 3.86 (s, OC$\underline{H}_3$) and $^{13}$C NMR (DMSO-$d_6$, 125 MHz), δ (ppm), 175.9 (C4), 164.8 (C7), 160.2 (C5), 156.0 (C9), 148.9 (C4′), 147.4 (C2), 147.0 (C3′), 136.0 (C3), 121.8 (C1′), 121.8 (C6′), 115.5 (C2′), 111.7 (C5′), 103.9 (C10), 97.4 (C6), 92.0 (C8), 56.0 (O$\underline{C}H_3$), 55.8 (O$\underline{C}H_3$).

4′,5-Dihydroxy-3,3′,6,7-tetramethoxyflavone (**3**, chrysosplenol B): m.p. 164–166 °C ($CHCl_3$), IR (KBr), $\nu^*_{max}$ ($cm^{-1}$): 3274, 2935, 1658, 1598, 1475, 1350, 1276, 1205, 809, 544; EIMS (70 eV) m/z (%) 374 $[M]^+$ (100); The compound was identified by analysis of the HMQC, HMBC experiments, $^1$H NMR ($CD_3OD$, 500 MHz), δ (ppm) 7.76 (1 H, d, J = 2.1 Hz, H2′), 7.70 (1 H, dd, J = 8.5 and 2.1 Hz, H6′), 6.97 (1 H, d, J = 8.5 Hz, H5′), 6.81 (1 H, s, H8), 3.99 (3 H, s, 7-OC$\underline{H}_3$), 3.97 (3 H, s, 3′-OC$\underline{H}_3$), 3.86 (3 H, s, 6-OC$\underline{H}_3$), 3.84 (3 H, s, 3-OC$\underline{H}_3$); $^{13}$C NMR ($CD_3OD$, 125 MHz), δ (ppm): 180.3 (C4), 160.6 (C7), 158.3 (C2), 154.0 (C9), 151.3 (C4′), 149.0 (2C: C3′ and (C5), 139.7 (C3), 133.4 (C6), 123.9 (C6′), 122.8 (C1′), 116.5 (C5′), 113.0 (C2′), 92.1 (C8), 61.1 (6-O$\underline{C}H_3$), 60.6 (3-O$\underline{C}H_3$), 57.0 (7-O$\underline{C}H_3$), 56.6 (3′-O$\underline{C}H_3$).

4′,5-Dihydroxy-3,3′,7-trimethoxyflavone (**4**, pachypodol): m.p. 177–178 °C ($CH_2Cl_2$), IR (KBr), $\nu^*_{max}$ ($cm^{-1}$): 3435, 3246, 1661, 1593, 1496, 1345, 1209, 1126, 1034, 798, 641; EIMS (70 eV) m/z (%) 344 $[M]^+$ (100); $^1$H NMR (acetone-$d_6$, 500 MHz), δ (ppm): 7.78 (1 H, d, J = 2.0 Hz, H2′), 7.70 (1 H, dd, J = 8.4 and 2.0 Hz, H6′), 7.00 (1 H, d, J = 8.4 Hz, H5′), 6.64 (1 H, d, J = 2.1 Hz, H6), 6.30 (1 H, d, J = 2.1 Hz), 3.95 (3 H, s, OC$\underline{H}_3$), 3.91 (3 H, s, OC$\underline{H}_3$), 3.90 (3 H, s, OC$\underline{H}_3$). $^{13}$C NMR (acetone-$d_6$, 125 MHz), δ (ppm): 179.6 (C4), 166.6 (C7), 162.9 (C5), 157.7 (C2), 156.9 (C9), 150.6 (C4′), 139.5 (C3), 123.4 (C6′), 122.8 (C2′), 116.1 (C5′), 106.6 (C10), 98.5 (C6), 92.9 (C8), 60.3 (O$\underline{C}H_3$), 56.5 (O$\underline{C}H_3$), 56.4 (O$\underline{C}H_3$).

4′,5,6-Trihydroxy-3,3′,7-trimethoxyflavone (chrysospenol C): m.p. 208–209 °C ($CH_2Cl_2$–MeOH), IR (KBr), $\nu^*_{max}$ ($cm^{-1}$): 3489, 3322, 1677, 1601, 1602, 1512, 1483, 1359, 1249, 1208, 1033, 820. EIMS (70 eV) m/z (%) 360 $[M]^+$ (100); $^1$H NMR (DMSO-$d_6$, 500 MHz), δ (ppm): 12.35 (1 H, s, 5-OH), 9.89 (1 H, s, 4′-OH), 8.70 (1 H, s, 6-OH), 7.67 (1 H, d, J = 2.0 Hz, H2′), 7.62 (1 H, dd, J = 8.4 and 2.1 Hz, H6′), 6.96 (1 H, d, J = 8.4 Hz, H5′), 6.89 (1 H, s, H8), 3.91 (3 H, s, 7-OC$\underline{H}_3$), 3.87 (3 H, s, 3′-OC$\underline{H}_3$), 3.81 (3 H, s, 3-OC$\underline{H}_3$) and $^{13}$C NMR (DMSO-$d_6$, 125 MHz), δ (ppm): 178.1 (C4), 155.5 (C2), 154.5 (C7), 149.7 (C4′), 148.8 (C9), 147.5 (C3′), 145.6 (C5), 137.6 (C3), 129.6 (C6), 122.6 (C6′), 121.0 (C1′), 115.6 (C5′), 112.0 (C2′), 105.5 (C10), 91.0 (C8), 59.7 (3-O$\underline{C}H_3$), 56.3 (7-O$\underline{C}H_3$), 55.8 (3′-O$\underline{C}H_3$).

### *3.3. Cytotoxic assays*

Cytotoxic assays were carried out in the Institute of Natural Product Chemistry-VAST, Hanoi, Viet Nam according to Likhiwitayawuid et al. (1993) and Skehan et al. (1990), at different concentrations in 96-well plates. Cell lines were cultured in Dulbecco's modified minimum essential medium (D-MEM) (GIBCO) supplemented with 10% feral bovine serum (FBS) and 100 units/ml penicilin G, 100 μg/ml steptomicin sulfate, 0.25 μg/ml amphotericin B (Fungizone) (PSF) (GIBCO). The plates, included cells + cultured medium + samples in DMSO were kept for 3 days at 37 °C in a $CO_2$ incubator. After that, cells were fixed in the plastic substratum by addition of 50 μl of cold 50% aqueous trichloroacetic acid fixed cells, washed with tap $H_2O$, stained by addition of 4% sulforhodamine B dissolved in 1% HOAc. Free sulforhodamine B solution was then removed by washing with 1% aqueous HOAc. The plates were placed on shaker for 5 min, and the absorption was determined at 510–540 nm using ELISA plate reader. $IC_{50}$ values were calculated by table curve program with logarit values based on different concentration scales of samples and OD values.

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