PHD DISSERTATION

BIOACTIVE CONSTITUENTS FROM *LINARIA JAPONICA* AND *SPILANTHES ACMELLA*

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INDEX

CHAPTER 1

LINARIA JAPONICA

1.1. Introduction

Linaria japonica Miq. (japanese toadflax) is a perennial herb, grayish and glabrous that is distributed to northern Shikoku, northward from Tottori prefecture along the Japan Sea side and Chiba prefecture along the Pacific Ocean side of Honshu to Hokkaido. It grows on the sandy shores and can reach 20 – 30 cm in height. Their stems are ascending and sometime branching. The leaves are elliptic, fleshy, sessile, ovate, obovate or oblong, 1.5 – 3 x 0.5 – 1.5 cm, base obtuse to subcuneate, apex obtuse to subacute, veins obscurely 3-campylodromous and arranged in opposite or 3 – 4 often irregularly whorled or alternate upward. The large-yellowish-white flowers bloom on the terminal racemes from August to October. They have inflorescences racemose, bracts similar to but much smaller than leaves, pedicel 3 – 5 mm, calyx lobes ovate to lanceolate with 2.5 – 4 x 1.5 – 2.5 mm. Their corolla are bright yellow, 1.2 – 1.7 cm (excluding spur), spur 3 – 6 mm, straight, lateral lobes of lower lip 3 – 5 mm wide, middle lobe narrower, upper lip longer than lower, capsule globose, ca. 6 mm in diam. The seeds are reniform, ca. 2.5 x 1.5 m and their margin are thickened.[1)] It belongs to the Scrophulariaceae (Figwort family) and has japanese name as un ran (ウンラン, 海蘭). Traditionally it used as diuretic and purgative.[2)]

Figure 1. *Linaria japonica*
(http://www.botanic.jp/plants-aa/unran.htm)

The isolation of flavonoid glycosides; linarin, pectolinarin, linariin and unranin,[3)] iridoid glucosides; linarioside, antirride and antirrhinoside,[4,5)] and diterpenoid; linaridial and linarienone[6,7,8,9,10)] from *Linaria japonica* Mig. were reported earlier. On our reinvestigation of the same plant, collected in sandy seashore areas of Tottori Prefecture, new flavonoids glycosides; isolinariin A and B,[11)] phenylethanoids; glucopyranosyl (1→6) martynoside, unranosides A, B, C,[12)] iridoid glucosides; 6-β-hydroxyantirrhide, linarioloside, 5-

deoxyteuhircoside, seco-linarioside, linarioside, linaride, 7*β*-hydroxy-8-epi-iridodial glucoside, phenolic glucosides; salidroside, syringin, *β*-D-glucopyranose-l-ferulate,[13] iridolinarins A, B, C,[14] iridolinaroside A, B, C, D,[15] monoterpene glucosides (6,7-dihydrofoliamenthoic acid diglucoside and 2,6-dimethyloctan-1,8-diol diglucoside) and ionol glucosides; linarionoside A, B, C[16] have been isolated.

Further investigation of the non-polar fraction, *i.e.* a mixture of hexane and ethyl acetate layers of the same plant, five new diterpenoids (**1 – 5)** and three new flavonoid glycosides (**6 − 8**) along with two known diterpenoids (**9 – 10),** two known flavonoid glycosides (**11 − 12**) and three known flavonoids (**13 − 15**) were isolated by various chromatographic techniques. The structures of these compounds were determined as follows by spectrometric analysis. Herein, described are the isolation and structural elucidation of them as well as the cytotoxic activities against lung cancer (A549) cytotoxic cell lines, *Leishmania major* parasites, collagenase and AGEs formation.

1.2. Extraction and Isolation of Chemical Constituents

The air-dried plants (2.30 kg) were extracted with methanol (15 liters x 2). On evaporation of the methanol extract to 5 liters, 125 g of precipitate was obtained on filtration. The methanol solution was adjusted to 95% aq. methanol by the addition of water and then extracted with *n*-hexane (1.5 liters x 2, 35.0 g). The concentrated methanol layer was suspended in 1.5 liters of water and then extracted with ethyl acetate (1.5 liters x 2, 49.7 g) and 1-butanol (1.5 liters x 3, 15.1 g), successively.

The mixture of *n*-hexane and ethyl acetate layer (60.5 g) was proceeded on silica gel column chromatography (300 g) with increasing amounts of methanol in chloroform [*n*-hexane-chloroform (1:1), 4l, chloroform-methanol (50:1, 40:1, 30:1, 20:1, 15:1, 10:1, 7:1, 5:1, 3:1, 2:1, 2l), 500 ml fractions being collected], yielding 12 fractions (Fr. Lj1 – Lj12).

The fraction Lj3 (9.61 g) was subjected by open reversed phase (ODS) column chromatography in 10% aq. methanol (400 ml) – 100% methanol (400 ml), linear gradient, lead 19 fractions (Fr. Lj3-1 – Lj3-19). The residue of fraction Lj3-9 (26.9 mg) was purified by prep. HPLC, 45% aq. methanol to give **3** (linarenone C, 1.23 mg). The residue of fraction Lj3-11 (62.9 mg) also was purified by recrystalization to give **13** (pectolinarigenin, 4.01 mg). Then fraction Lj3-12 (74.4 mg) also was purified by prep. HPLC, 67.5% aq. methanol to give **2** (linarenone B, 4.30 mg). The other residue of fraction Lj3-13 (48.7 mg) was purified by prep. HPLC, 80% aq. methanol to give **9** (linarienone, 3.50 mg). The fraction Lj3-14 (361 mg) was purified by prep. HPLC, 72.5% aq. acetone to give **5** (linarenone E, 7.17 mg).

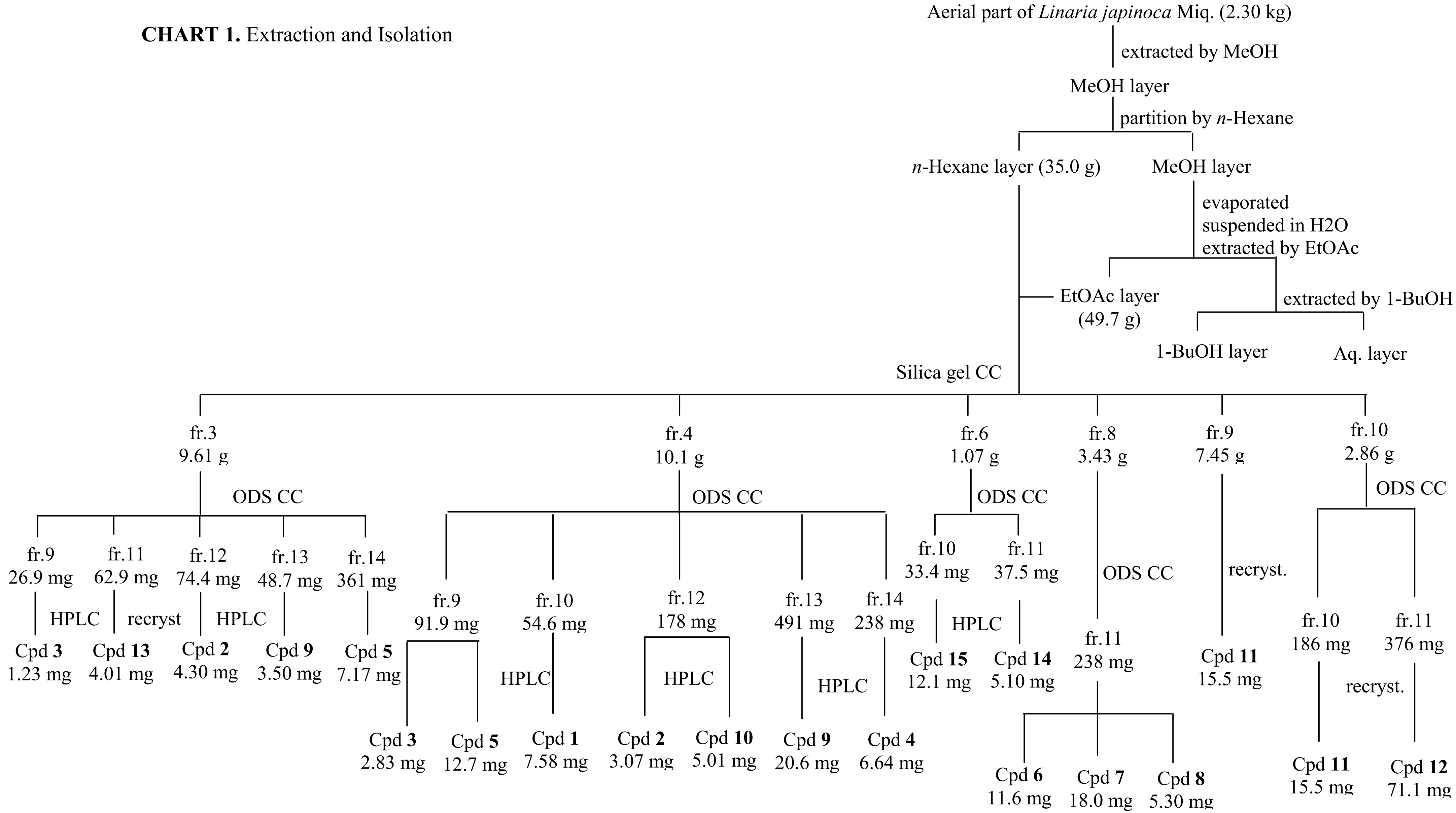
The fraction Lj4 (10.1 g) was subjected by open reversed phase (ODS) column chromatography in 10% aq. methanol (400 ml) – 100% methanol (400 ml), linear gradient, lead 19 fractions (Fr. Lj4-1 – Lj4-19). The other residue of fraction Lj4-9 (91.9 mg) was purified by prep. HPLC, 45% aq. acetone to give **3** (linarenone C, 2.83 mg) and **5** (linarenone E, 12.7 mg). The residue of fraction Lj4-10 (54.6 mg) was purified by prep. HPLC, 65% aq. methanol to give **1** (linarenone A, 7.58 mg). The residue of fraction Lj4-12 (178 mg) also was purified by prep. HPLC, 67.5% aq. methanol. Two peaks which appeared at 18 and 35 minute were collected to give **10** (desacetyl-linarienone, 5.01 mg) and **2** (linarenone B, 3.07 mg). The fraction Lj4-13 (491 mg) finally was purified by prep. HPLC, 80% aq. methanol to give **9** (linarienone, 20.6 mg). Then the fraction Lj4-14 (238 mg) was purified by prep. HPLC, 65% aq. acetone to give **4** (linarenone D, 6.64 mg).

The fraction Lj6 (1.07 g) was subjected by open reversed phase (ODS) column chromatography in 10% aq. methanol (400 ml) – 100% methanol (400 ml), linear gradient, lead 19 fractions (Fr. Lj6-1 – Lj6-19). The residue of fraction Lj6-10 (33.4 mg) was purified by prep. HPLC, 67.5% aq. methanol to give **15** (luteolin, 12.1 mg). The residue of fraction Lj6-11 (37.5 mg) also was purified by prep. HPLC, 45% aq. methanol to give **14** (apigenin, 5.10 mg).

The fraction Lj8 (3.43 g) was subjected by open reversed phase (ODS) column chromatography in 10% aq. methanol (400 ml) – 100% methanol (400 ml), linear gradient, lead 19 fractions (Fr. Lj8-1 – Lj8-19). The residue of fraction Lj8-11 (238 mg) was purified by prep. HPLC, 55% aq. methanol. Three peaks which appeared at 18, 25 and 35 minute were collected to give **6** (isolinariin C, 11.6 mg), **7** (isolinariin D, 18.0 mg) and **8** (isolinariin E, 5.30 mg).

The fraction Lj9 (7.45 g) was purified by recrystalization to give **11** (linariin, 15.5 mg). The other fraction Lj10 (2.86 g) was subjected by open reversed phase (ODS) column chromatography in 10% aq. methanol (400 ml) – 100% methanol (400 ml), linear gradient, lead 19 fractions (Fr. Lj10-1 – Lj10-19). The residue of fraction Lj10-10 (186 mg) was purified by recrystalization to get **11** (linariin, 15.5 mg). Then the residue of fraction Lj10-11 (376 mg) also was purified by recrystalization to give **12** (pectolinarin, 71.1 mg).

CHART 1. Extraction and Isolation



1.3. Structural Elucidations of Chemical Constituents

The mixture of *n*-hexane and ethyl acetate layers from methanol extract of aerial part of *Linaria japonica* was fractionated by silica gel and ODS column chromatography, then futher purified by HPLC to afford 15 compounds (Fig. 3). The known compounds were identified as linarienone (**9**), desacetyl-linarienone (**10**), linariin (**11**), pectolinarin (**12**), pectolinarigenin (**13**), apigenin (**14**) and luteolin (**15**) by comparing the physical and spectroscopic data in literature.[9,11,17,18] Desacetyl-linarienone (**10**) was isolated for the first time as naturally. The NMR spectroscopic analysis of isolated compounds established structural similarities and indicated the presence of a *cis*-clerodane skeleton (**1** – **5**, **9** and **10**) and flavonoids or flavonoid glycosides (**6** – **8**, **11** – **15**). We report that three new flavonoid glycosides (**6** – **8**) were found to be glycosylated with the same disaccharide, consisting D-glucose and L-rhamnose that have different position of acetoxy groups.

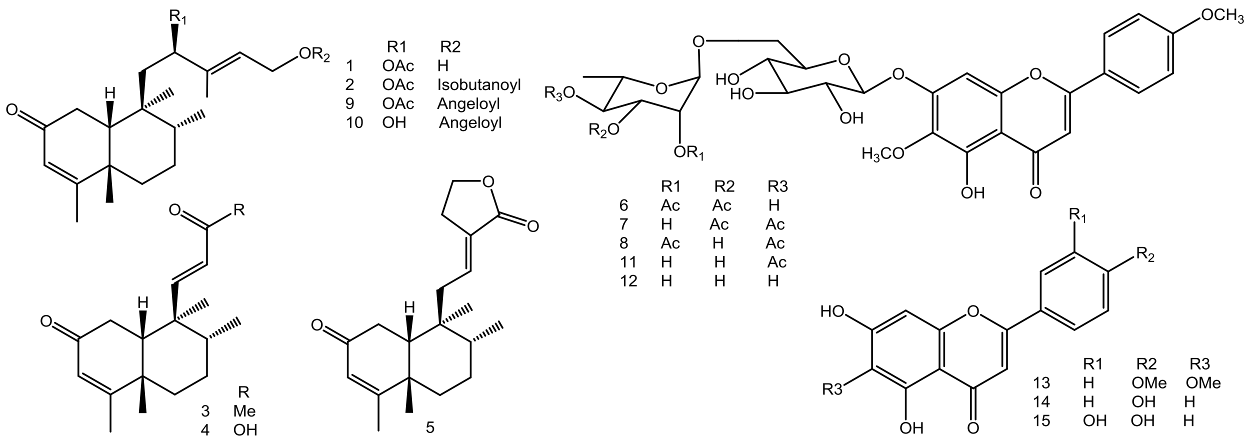


Figure 3. Isolated compounds of *Linaria japonica*

Linarenone A (**1**) was obtained as colorless powder with molecular formula of $C_{22}H_{34}O_4$ as determined by HR-ESI-MS at *m/z* 385.2348 $[M+Na]^+$ (calcd. 385.2349). The IR spectrum has absorption bands at 3340, 1732 and 1237 cm^{-1} indicating the presence of hydroxy and ester carbonyl group. The ^1H NMR spectrum (Table 1) displayed signals due to two tertiary methyls at δ_H 0.56 (s) and 1.23 (s), one secondary methyl at δ_H 0.84 (d, J = 6 Hz), two olefinic methyls at δ_H 1.64 (s) and 1.93 (s), eight methylene protons at 1.11 (br qd, J = 13, 3 Hz), 1.19 (ddd, J = 14, 11, 3 Hz), 1.30 (dq, J = 13, 3 Hz), 1.61 (dd, J = 16, 2 Hz), 1.73 (dd, J = 16, 8 Hz), 2.06 (ddd, J = 14, 3, 3 Hz), 2.47 (br d, J = 18 Hz), and δ_H 2.71 (dd, J = 18, 6 Hz), two protons of oxygenated methylene at δ_H 4.15 (d, J = 6 Hz), two methine protons at δ_H 1.42 (m) and 1.98 (br d, J = 6 Hz), and an oxygenated methine proton at δ_H 5.19 (dd, J = 8, 2 Hz), one acetyl methyl proton at δ_H 2.01 (s), two olefinic protons at δ_H 5.59 (t, J = 6 Hz) and 5.83 (s).

Table 1
^{1}H NMR spectroscopic data of **1 – 5**

Position	**1**	**2**	**3**	**4**	**5**
1-α	2.47 br d (18)	2.46 br d (18)	2.25 br d (18)	2.28 br d (18)	2.48 br d (18)
β	2.71 dd (18, 6)	2.71 dd (18, 6)	2.66 dd (18, 6)	2.68 dd (18, 6)	2.72 dd (18, 6)
2	-	-	-	-	-
3	5.83 s	5.83 s	5.85 s	5.86 s	5.84 s
4	-	-	-	-	-
5	-	-	-	-	-
6-α	2.06 ddd (14, 3, 3)	2.07 ddd (14, 3, 3)	2.16 ddd (14, 3, 3)	2.16 ddd (14, 3, 3)	2.07 ddd (14, 3, 3)
β	1.19 ddd (14, 11, 3)	1.18 ddd (14, 11, 3)	1.29 ddd (14, 11, 3)	1.30 ddd (14, 11, 3)	1.22 ddd (14, 11, 3)
7-α	1.11 br qd (13, 3)	1.09 br qd (13, 3)	1.14 br qd (13, 3)	1.14 br qd (13, 3)	1.12 br qd (13, 3)
β	1.30 dq (13, 3)	1.33 dq (13, 3)	1.46 dq (13, 3)	1. 46 dq (13, 3)	1.38 dq (13, 3)
8	1.42 m	1.43 m	1.49 m	1.48 m	1.55 m
9	-	-	-	-	-
10	1.98 br d (6)	1.97 br d (6)	1.86 br d (6)	1.86 br d (6)	1.81 br d (6)
11	1.61 dd (16, 2)	1.62 dd (16, 2)	6.44 d (16)	6.69 d (16)	2.16 ddt (16, 6, 2)
	1.73 dd (16, 8)	1.73 dd (16, 8)	-	-	2.32 dd (16, 8)
12	5.19 dd (8, 2)	5.21 dd (8, 2)	6.03 d (16)	5.75 d (16)	6.75 ddt (8, 6, 3)
13	-	-	-	-	-
14	5.59 t (6)	5.53 t (6)	2.24 s	-	2.86 br t (7)
15	4.15 d (6)	4.56 d (6)	-	-	4.37 t (7)
16	1.64 s	1.68 s	-	-	-
17	0.84 d (6)	0.84 d (6)	0.69 d (6)	0.70 d (6)	0.81 d (6)
18	0.56 s	0.56 s	0.75 s	0.76 s	0.66 s
19	1.23 s	1.23 s	1.24 s	1.24 s	1.20 s
20	1.93 s	1.93 s	1.95 s	1.95 s	1.93 s
12 –OAc	2.01 s	2.01 s	2.01 s		
1′		-			
2′		2.52 septet (7)			
3′, 4′		1.14 d (7)			

Recorded at 600 MHz in $CDCl_3$. Chemical shifts (δ) are expressed in ppm and *J* values are presented in Hz in parenthesis

The ^{13}C NMR spectrum (Table 2) of **1** showed 22 carbon resonances that classified by analysis of its chemical shift values and its HSQC spectrum as five methyls (δ_C 12.3, 16.1, 18.2, 20.3, 31.8), four methylenes (δ_C 28.1, 35.5, 36.5, 39.7), an oxygenated methylene (δ_C 58.7), two methines (δ_C 37.6, 47.5), an oxygenated methine (δ_C 74.5), two olefinic methines (δ_C 125.3, 128.3), four quaternary carbons (δ_C 39.2, 40.5, 137.7, 168.8), an acetoxy carbon signals at δ_C 21.0 and δ_C 169.9, and a ketone (δ_C 198.6).

The NMR spectroscopic data of **1** closely resembled that of linarienone (**9**),[9] except for some differences in the chemical shift values at C-13, 14 and 15 (δ_C 140.0, 121.0 and 60.2, respectively). The proton (δ_H 4.15) and carbon resonance (δ_C 58.7) of **1** indicated that C-15 have hydroxy group. The acetate substituent was placed at C-12 on the basis of the HMBC correlation of H-12 (δ_H 5.19) to carbon signal at δ_C 169.9 (12-OAc) (Fig. 4). The A/B ring junction in **1** were deduced to be *cis* on the basis of the ^{13}C NMR chemical shifts of the angular methyl (δ_C 31.8 for C-19), which were found to be in the range δ_C 11-19 for *trans* and higher than δ_C 20 for *cis*-clerodanes.[19)]

Table 2

^{13}C NMR spectroscopic data of **1 – 5**

Position	**1**	**2**	**3**	**4**	**5**
1	35.5	35.6	36.6	36.3	36.2
2	198.6	198.5	198.3	198.4	198.7
3	128.3	128.4	128.7	128.5	128.8
4	168.8	168.1	167.1	167.2	168.6
5	39.2	39.2	38.3	38.1	39.7
6	36.5	36.5	36.5	36.2	36.7
7	28.1	28.1	27.5	27.2	28.4
8	37.6	37.6	40.5	40.1	36.7
9	40.5	40.5	45.7	45.7	42.2
10	47.5	47.5	49.8	49.5	48.5
11	39.7	39.5	156.3	159.9	37.8
12	74.5	74.3	130.1	120.5	136.7
13	137.7	139.9	197.9	169.8	127.4
14	125.3	120.7	28.2	-	25.6
15	58.7	60.2	17.0	16.7	65.6
16	12.3	12.5	13.4	13.1	171.3
17	16.1	16.2	31.9	31.7	16.5
18	18.2	18.2	20.5	20.3	18.6
19	31.8	31.7			31.9
20	20.3	20.3			20.8
12 – OAc	169.9	169.8			
	21.0	21.2			
1′		177.0			
2′		33.7			
3′, 4′		18.8			

Recorded at 125 MHz in $CDCl_3$. Chemical shifts (δ) are expressed in ppm.

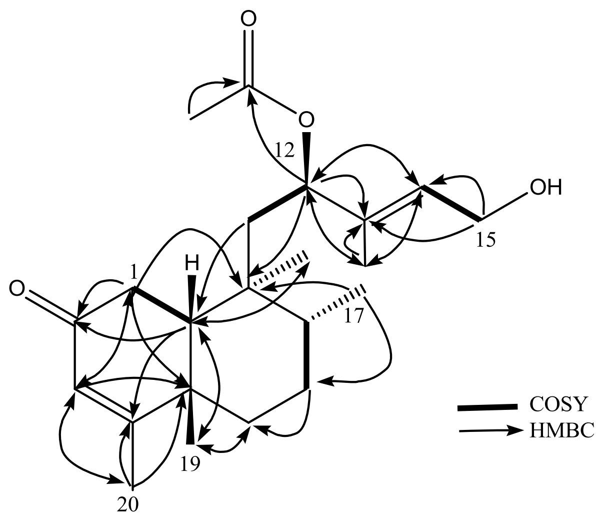


Figure 4. HMBC and COSY correlations of **1**

Its relative configuration of **1** was established by NOESY analysis. The correlation of H-15/Me-16 suggested as *E* configuration. Another correlations were observed between H-10/Me-19, H-10/H-12 and Me-17/Me-18, which suggested the orientation of H-10, H-12, Me-19 to be *β*-oriented and those of Me-17, Me-18 to be *α*–oriented (Fig. 5). Next, the absolute stereochemistry of the ring moiety of **1** was determined by an analysis of the CD spectrum. The significant positive cotton effect at 331 nm ($\Delta\varepsilon$ = +1.02) showed the same absolute stereochemistry with linarienone (**9**).

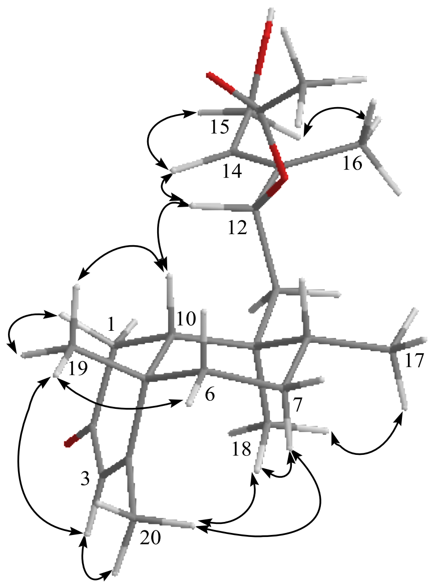


Figure 5. Key NOESY correlations of **1**

In addition, from the biogenetic point of view, the remaining stereochemistry at C-12 of **1** should be identical with co-occurring *cis*-clerodane derivatives. This hypothesis was confirmed by the alkaline hydrolysis of **1** and **9**. The deacyl compounds (**1a** and **9a**) were revealed to be identical by spectroscopic and HPLC analysis. Hence, on the basis of above spectrum analysis, the structure of **1** was determined as (5*S*,8*R*,9*S*,10*R*,12*R*)-2-oxo-12,15-dihydroxy-*cis*-clerod-3*Z*,13(14)*E*-diene 12-acetate.

Linarenone B (**2**) was also a clerodane diterpenoid. It was obtained as colorless solid with molecular formula of $C_{26}H_{40}O_5$ as determined by HR-ESI-MS at *m/z* 455.2766 $[M+Na]^+$ (calcd. 455.2768). The ^1H and ^{13}C NMR spectrum (Table 1 and 2) of **2** were similar to **1**, except for the appearance of isobutanoyl moiety, *i.e*. an ester carbonyl at δ_C 177.0 (C-1′), a methylene at δ_C 33.7 (C-2′), two methyls at δ_C 18.8 (C-3′ and C-4′), and an esterified methylene at δ_H 4.56 (d, *J* = 6 Hz, H-15), δ_C 60.2 (C-15). It suggested that there was a isobutanoyl moeity at C-15, which was also supported by HMBC correlations of H-15 to C-1′ (Fig. 6).

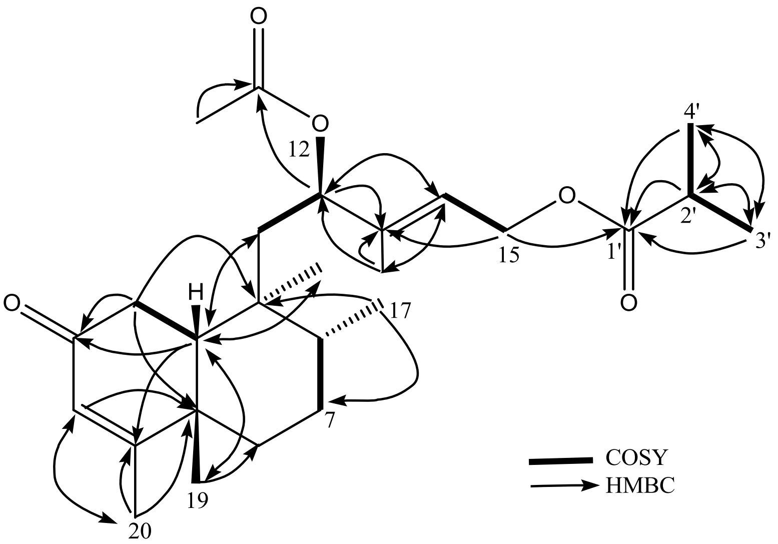


Figure 6. HMBC and COSY correlations of **2**

The relative and absolute stereochemistry of **2** was revealed to be the same as **1** by NOESY (Fig. 7), CD spectrum and a mild alkaline hydrolysis of **2** by which the deacyl derivative of **2** (**2a**) was identical to **1a** and **9a**, thus the structure of **2** was deduced as (5*S*,8*R*,9*S*,10*R*,12*R*)-2-oxo-15-isobutanoyl-*cis*-clerod-3*Z*,13(14)*E*-diene 12-acetate.

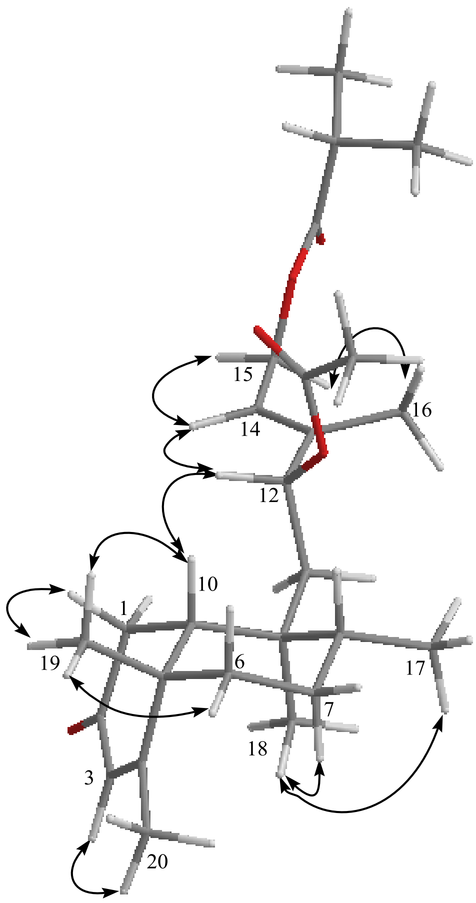


Figure 7. Key NOESY correlations of **2**

Linarenone C (**3**) was also colorless amorphous solid and displayed an $[M+Na]^+$ ion peak at *m/z* 297.1828 (calcd. 297.1825) corresponding to a molecular formula of $C_{18}H_{26}O_2$. The ^1H and ^{13}C NMR spectrum (Table 1 and 2) displayed two *trans* coupled doublets with J = 16 Hz at δ_H 6.03 (H-12) and δ_H 6.44 (H-11), a singlet methyl at δ_H 2.24 (Me-14), a carbonyl group at δ_C 197.9 (C-13), which is characteristic as a methyl ketone.

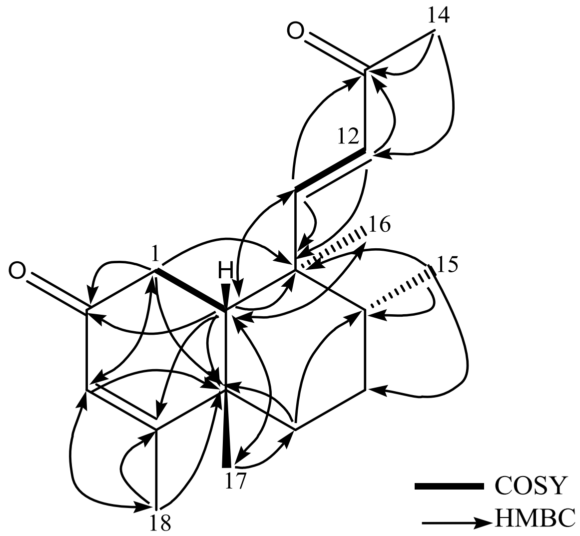


Figure 8. HMBC and COSY correlations of **3**

The planer structure of **3** was determined by ^1H – ^1H COSY and HMBC spectra (Fig. 8) as 2-oxo-14,15-bisnor-3,11*E*-kolavadien-13-one,[20] however, the chemical shift of C-19 has quite different (δ_C 31.9 for **3**, δ_C 18.9 for 2-oxo-14,15-bisnor-3,11*E*-kolavadien-13-one). It indicated that 3 is an epimer of C-19, *i.e.* a *cis*-clerodane diterpenoid.

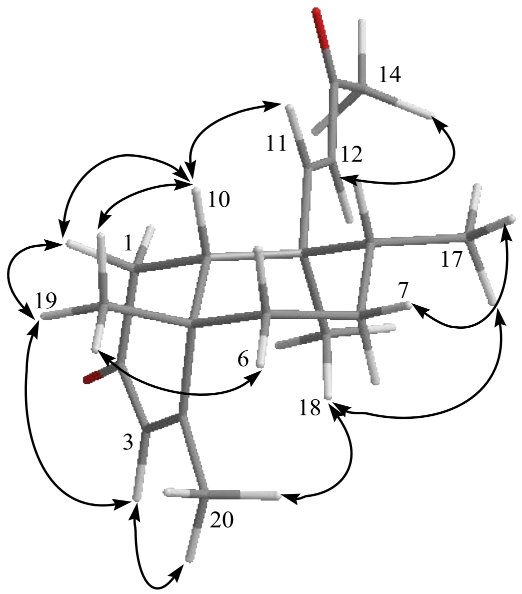


Figure 9. Key NOESY correlations of **3**

The relative configuration of **3** was deduced on the basis of NOESY correlations of H-10/H-11 and H-10/Me-19, which indicated *β*-orientation, and the correlation between Me-17/Me-18 for *α*-orientation (Fig. 9). The significant positive cotton effect of CD spectrum at 334 nm (Δ*ε*= +1.16) is similar to **1**. Thus the absolute stereochemistry of **3** should be (5*S*,8*R*,9*S*,10*R*,3*Z*,11*E*)-2-oxo-14,15-bisnor-3*Z*,11*E*-kolavadien-13-one.

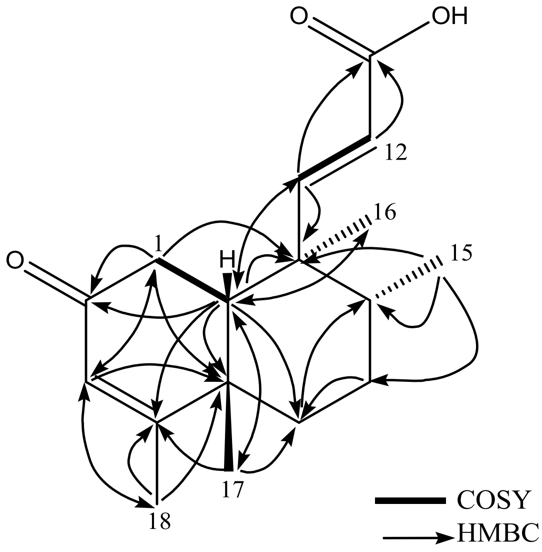


Figure 10. HMBC and COSY correlations of **4**

Linarenone D (**4**), a colorless amorphous solid, was exhibited a HR-ESI-MS $[M+Na]^+$ ion peak at *m/z* 299.1618 (calcd. 299.1618) suggesting to a molecular formula of $C_{17}H_{24}O_3$. The NMR spectroscopic data (Table 1 and 2) of **3** and **4** indicated differences regarding the C-13 substituent. The carbon signal at δ_C 169.8 (C-13), the upfield shift to δ_H 5.75 (d, *J* = 16 Hz, H-12) and δ_C 120.5 (C-12) and the downfield shift to δ_H 6.69 (d, *J* = 16 Hz, H-11) and δ_C 159.9 (C-11) indicated a carboxylic acid at the C-13 position. It also supported by IR absorption bands at 1715 cm^{-1} (COOH group). The relative and absolute configuration of **4** was assigned to be the same as that of **3**, based on 1D and 2D NMR analysis including NOESY (Fig. 11) and CD spectrum (Δ*ε*= +1.22 at 325 nm). Thus, the structure of **4** was elucidated as (5*S*,8*R*,9*S*,10*R*)-2-oxo-14,15-bisnor-3*Z*,11*E*-kolavadien-13-carboxylic acid.

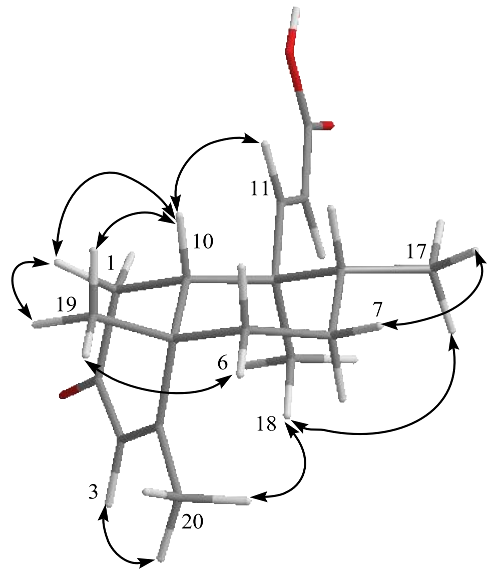


Figure 11. Key NOESY correlations of **4**

Linarenone E (**5**), isolated as a colorless solid, was assigned the molecular formula of $C_{20}H_{28}O_3$ as determined by HR-ESI-MS at *m/z* 339.1929 $[M+Na]^+$ (calcd. 339.1931). The IR absorption band at 1754 cm^{-1} indicative of the present of *γ*-lactone moiety. The ^1H and ^{13}C NMR spectra (Table 1 and 2) also revealed a *cis*-clerodane framework, with two tertiary methyls [δ_H/δ_C 0.66 (s)/18.6 (Me-18) and 1.20 (s)/31.9 (Me-19)], a secondary methyl [δ_H 0.81 (d, $J = 6$ Hz); δ_C 16.5 (Me-17)], an olefinic methyl [δ_H 1.93 (s); δ_C 20.8 (Me-20)], an olefinic methine [δ_H 6.75 (ddt, $J = 8, 6, 3$ Hz); δ_C 136.7 (C-12)], a typical *γ*-lactone ring [δ_C 127.4 (C-13), δ_H 2.86 (br t, $J = 7$ Hz, H-14), δ_C 25.6 (C-14), δ_H 4.37 (t, $J = 7$ Hz, H-15), δ_C 65.6 (C-15) and δ_C 171.3 (C-16)] and a six membered ring ketone [δ_C 198.7 (C-2)].

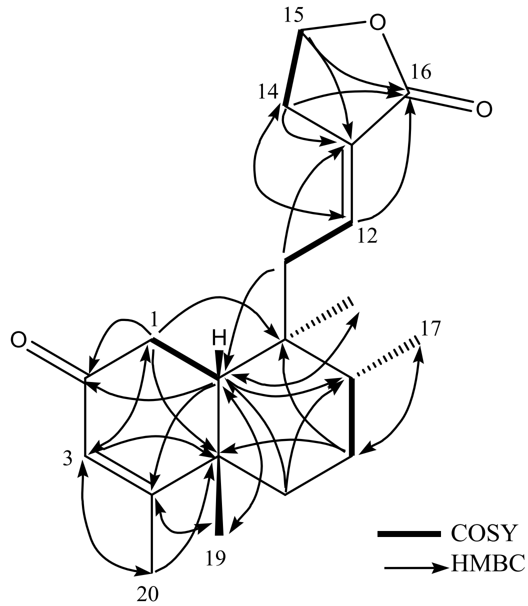


Figure 12. HMBC and Cosy correlations of **5**

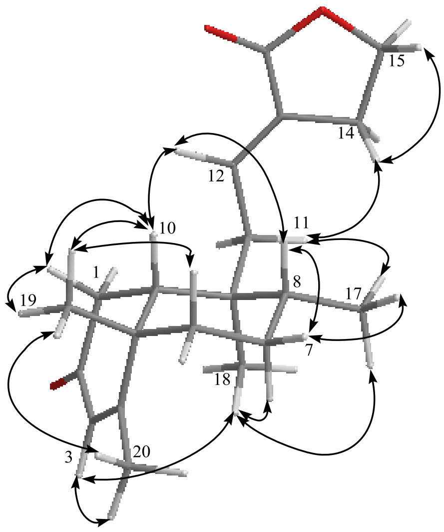


Figure 13. Key NOESY correlations of **5**

The γ-lactone was connected to C-12 according to the HMBC correlations of H-12 to carbon at δ_C 171.3 (C-16) and 25.6 (C-14), together with the correlations of H-11, 14 and 15 to the carbon at δ_C 127.4 (C-13) (Fig. 12). The NOESY experiment revealed its relative structure as a *cis*-clerodane framework. Especially, the NOESY correlation of H-10 to Me-19 and Me-17 to Me-18 indicated that H-10/Me-19 were β-oriented and Me-17/Me-18 were α-oriented (Fig. 13). Finally the absolute stereochemistry of **5** was established as 5*S*, 8*R*, 9*S* and 10*R* by means of the CD spectrum ($\Delta\varepsilon$= +1.13 at 327 nm) that was similar to **1**. Accordingly, the structure of **5** was determined as (5*S*,8*R*,9*S*,10*R*)-2-oxo-*cis*-cleroda-3*Z*,12*E*-dien-15,16-olide.

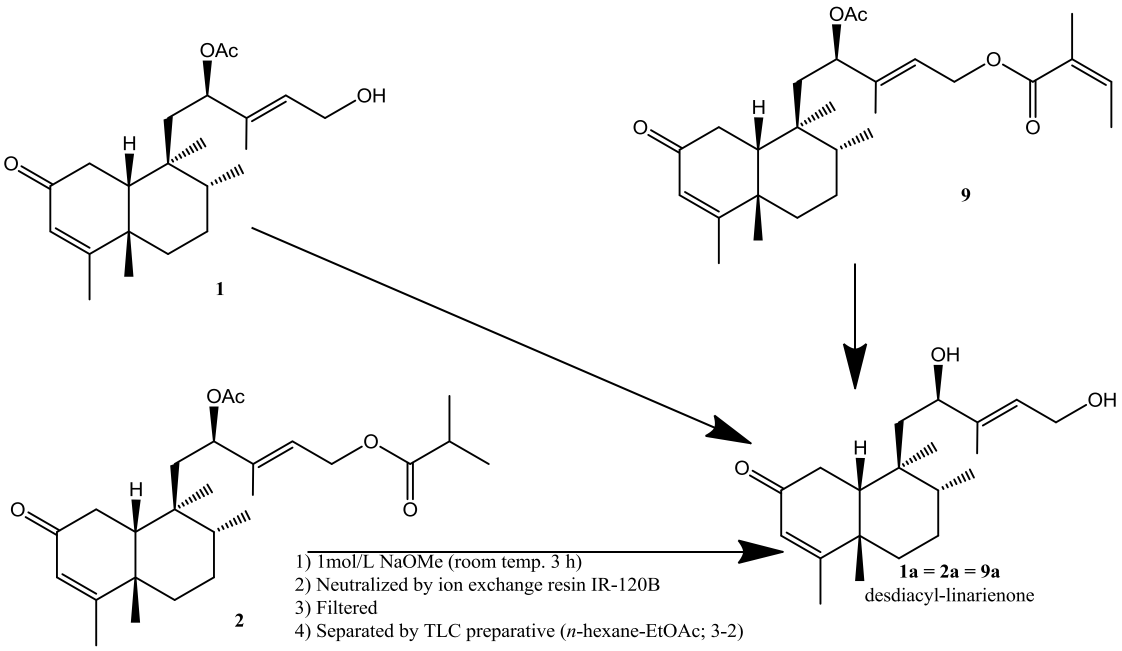


Figure 14. Alkaline hydrolysis reaction of **1**, **2** and **9**

On mild alkaline treatment, the new diterpenoids **1**, **2** and linarienone (**9**) was converted to desacetyl-linarienone (**10**), $C_{25}H_{38}O_4$, and desdiacyl-linarienone (**1a, 2a** and **9a**), $C_{20}H_{32}O_3$. A three of acetyl group at C-12 (**1, 2, 9**), butanoyl function (**2**) and angeloyl function (**9**) at C-15 are lost in desdiacyl-linarienone (**1a, 2a** and **9a**) (Fig. 14).

Isolinariin C (**6**), $[\alpha]_D$ -4.81, was obtained as pale yellow powder with molecular formula of $C_{33}H_{38}O_{17}$ as determined by HR-ESI-MS at *m/z* 729.1998 $[M+Na]^+$ (calcd. 729.2001). The IR spectrum indicated the presence of hydroxyl (3437 cm^{-1}), methylene (2933 cm^{-1}), ester (1746 cm^{-1}), α,β-unsaturated (1653 and 1606 cm^{-1}), aromatic ring (1509 and 1566 cm^{-1}) and enol ether (1251 cm^{-1}) groups. A broad carbonyl stretching band in the region 1100–1600 cm^{-1} suggested a glycosidic nature. Their IR and UV spectrum show similar absorbantion maxima to those of linariin.[11,17,18)]

The ^1H NMR spectrum (Table 3) displayed signals due to a doublet methyl of rhamnose moiety at δ_H 1.17 (d, J = 6 Hz), two singlet methyl signals of acetyl group at δ_H 1.75 (s) and 1.93 (s), two oxygenated methylene protons resonanted of glucose at δ_H 3.76 (m) and 4.05 (d, J = 10 Hz), six singlet protons of two methoxy groups at δ_H 3.89 (s), two anomeric proton of sugars at δ_H 4.72 (br s) and 5.19 (d, J = 7 Hz), two aromatic protons at δ_H 6.68 (s) and 6.89 (s), and AA'BB' type coupling system at δ_H 7.08 (d, J = 8 Hz) and 7.95 (d, J = 8 Hz).

The ^{13}C NMR spectrum (Table 3) of **6** showed 33 carbon resonances that classified by analysis of its chemical shift values and its HSQC spectrum as a methyl of rhamnose moeity carbon (δ_C 18.0), two acetoxy carbons (δ_C 20.6, 171.5 and δ_C 20.8, 172.1), two methoxy carbons (δ_C 56.2, 61.7), an oxygenated methylene (δ_C 67.5), eight oxygenated methine carbons of sugar (δ_C 70.0, 71.1, 71.3, 71.6, 73.3, 74.8, 77.3, 77.9), four olefinic methine carbons (δ_C 95.5, 104.5, 115.8, 129.8), two anomeric carbons (δ_C 99.3, 101.6), two quaternary carbons (δ_C 108.0, 124.7), six olefinic quaternary carbons (δ_C 134.0, 154.3, 154.3, 158.0, 164.5, 166.9) and a carbonyl carbon at δ_C 184.6 (C-4) supporting the flavone type carbon framework of the molecule. While five signals were corresponded to C1-C5 of β-D-glucopyranose, the anomeric carbon signal, resonating at δ_C 101.6, was indicative of the participation of this carbon in an ester linkage. The highly deshielded chemical shift of an anomeric proton signal (δ_H 5.19) was in accordance with this assumption and its coupling constant (J = 7 Hz) recommended the β mode of linkage. The other anomeric carbon signal (δ_C 99.3) was indicative of α-L-rhamnopyranose. The shielded chemical shift of an anomeric proton signal (δ_H 4.72) was in accordance with this assumption and recommended the α mode of linkage.

Table 3.
The ^{13}C and ^{1}H NMR spectroscopic data of **6** – **8**

Position	**6**		**7**		**8**	
	δ_C	δ_H Multi (*J* in Hz)	δ_C	δ_H Multi (*J* in Hz)	δ_C	δ_H Multi (*J* in Hz)
2	166.9	-	166.5	-	166.9	-
3	104.5	6.68 s	104.5	6.68 s	104.6	6.70 s
4	184.6	-	184.5	-	184.6	-
5	154.3	-	154.4	-	154.5	-
6	134.0	-	134.6	-	134.7	-
7	158.0	-	157.8	-	157.8	-
8	95.5	6.89 s	95.8	6.90 s	96.1	6.95 s
9	154.3	-	154.4	-	154.5	-
10	108.0	-	107.8	-	107.9	-
1′	124.7	-	124.6	-	124.6	-
2′, 6′	129.8	7.95 d (8)	129.6	7.94 d (8)	129.7	7.97 d (8)
3′, 5′	115.8	7.08 d (8)	115.9	7.07 d (8)	115.9	7.09 d (8)
4′	164.5	-	164.6	-	164.7	-
6-OCH_3	61.7	3.89 s	61.7	3.90 s	61.7	3.90 s
4′-OCH_3	56.2	3.89 s	56.3	3.89 s	56.3	3.88 s
1″	101.6	5.19 d (7)	101.6	5.20 d (7)	101.8	5.19 d (7)
2″	74.8	3.58 t (8)	74.9	3.59 t (8)	75.0	3.58 t (8)
3″	77.9	3.52 t (9)	78.0	3.53 m	77.9	3.52 t (9)
4″	71.6	3.43 m	71.3	3.45 m	70.9	3.48 m
5″	77.3	3.73 m	77.2	3.73 m	76.9	3.69 m
6″	67.5	4.05 d (10)	67.1	4.03 d (10)	66.8	3.99 m
		3.76 m		3.76 m		3.75 m
1‴	99.3	4.72 br s	101.6	4.74 br s	98.8	4.73 br s
2‴	71.1	5.15 dd (3, 2)	70.0	3.98 m	73.9	5.06 dd (3, 2)
3‴	73.3	5.01 dd (10, 3)	73.4	5.02 dd (10, 3)	68.6	3.97 dd (10, 3)
4‴	71.3	3.42 d (10)	72.6	4.98 d (10)	75.5	4.78 d (10)
5‴	70.0	3.77 m	67.7	3.84 m	67.7	3.77 m
6‴	18.0	1.17 d (6)	17.7	0.96 d (6)	17.7	0.92 d (6)
2‴-OAc	20.6	1.93 s	-	-	20.8	2.03 s
	171.5				172.1	
3‴-OAc	20.8	1.75 s	20.8	1.82 s	-	-
	172.1		171.9			
4‴-OAc	-	-	20.9	1.96 s	21.1	2.03 s
			171.9		172.4	

Recorded at 600 and 125 MHz in CD_3OD. Chemical shifts (δ) are expressed in ppm MHz

The NMR spectroscopic data of **6** closely resembled that of linariin,[18)] except for some differences in the chemical shift values at C-2‴ and 3‴. The deshielded proton at δ_H 5.15 and carbon at δ_C 71.1 of **6** suggested location of the acetoxy group at C-2‴. It also happened at the proton (δ_H 5.01) and carbon resonance (δ_C 73.3) of **6** indicated that C-3‴ attach acetoxy group. We also determined that the acetate substituents were placed at C-2‴ and 3‴ on the basis of the HMBC correlation of H-2‴ (δ_H 5.15) and 3‴ (δ_H 5.01) to carbon signal at δ_C 171.5 (2‴OAc) and 172.1 (3‴OAc), respectively (Fig. 15).

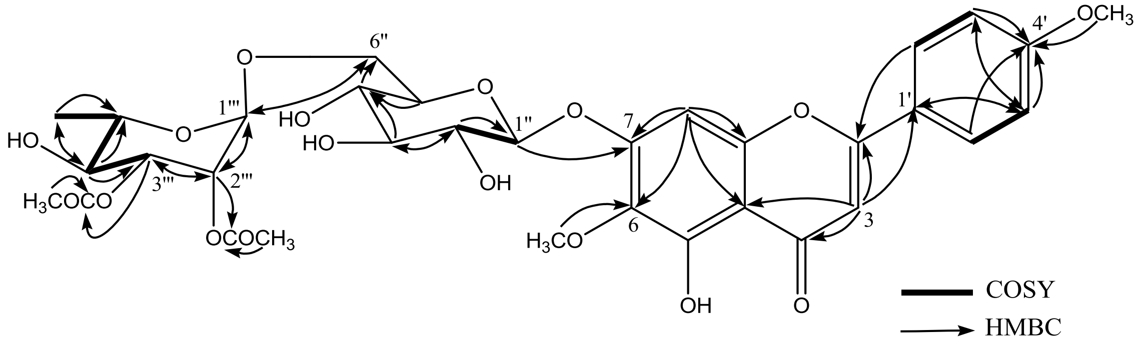


Figure 15. HMBC and COSY correlations of **6**

The D-glucose moeity was connected to C-7 according to the HMBC correlations of H-1″ to carbon at δ_C 158.0 (C-7), and the L-rhamnose moeity was connected to C-6″ of D-glucose moeity according to their correlations of H-1‴ to the carbon at δ_C 67.5 (C-6″). The HMBC spectrum exhibited correlation between the proton signal at δ_H 6.68 and the three quartenary carbon resonances at δ_C 124.7, 166.9 and 184.6 which follow us to assign this proton at position 3 of the flavone core.[21)] Mild acid hydrolysis of **6** with 1N HCl showed initial removal of a pectolinarigenin, D-glucose unit followed by one of L-rhamnose (Fig. 18).[22)] Based on the above NMR data and chemical reactions, the structure of **6** was determined as pectolinarigenin-7-*O*-(2,3-diacetyl-α-L-rhamnopyranosyl)-(1→6)-β-D-glucopyranoside.

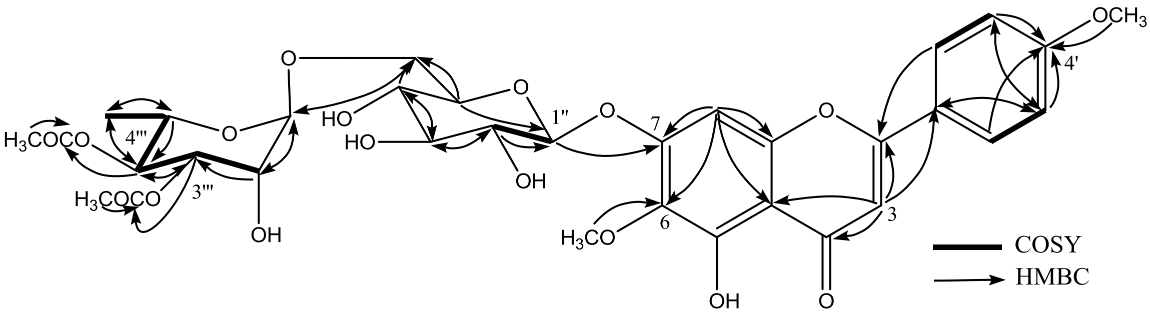


Figure 16. HMBC and COSY correlations of **7**

Isolinariin D (**7**), $[\alpha]_D$ -8.56, was also a flavonoid glycoside. It was obtained as pale yellow powder, whose molecular formula was determined to be $C_{33}H_{38}O_{17}$ from its positive-ion mode HR-ESI-MS data at *m/z* 729.1997 $[M+Na]^+$ (calcd. 729.2001). The ^1H and ^{13}C NMR spectrum (Table 3) of **7** were very similar to those of **6**, except for the position of two acetyl group resonances [δ_H 1.82 (3H, s); δ_C 20.8, 171.9 and δ_H 1.96 (3H, s); δ_C 20.9, 171.9], shielded of H-2‴ [δ_H 3.98 (1H, m)] and C-2‴ (δ_C 70.0) and deshielded of H-4‴ [δ_H 4.98 (1H, d, 10 Hz)] and C-4‴ (δ_C 72.6). The position of two acetyl groups were deduced to be at C-3‴ and C-4‴ by analysis of the HMBC data showing correlations of H-3‴ and H-4‴ to two carbons at δ_C 171.9 (Fig. 16). The acid hydrolysis of **7** (Fig. 18) yielded the aglycone (pectolinarigenin), D-glucose and L-rhamnose, thus the structure of **7** was determined as pectolinarigenin-7-*O*-(3,4-diacetyl-*α*-L-rhamnopyranosyl)-(1→6)-*β*-D-glucopyranoside.

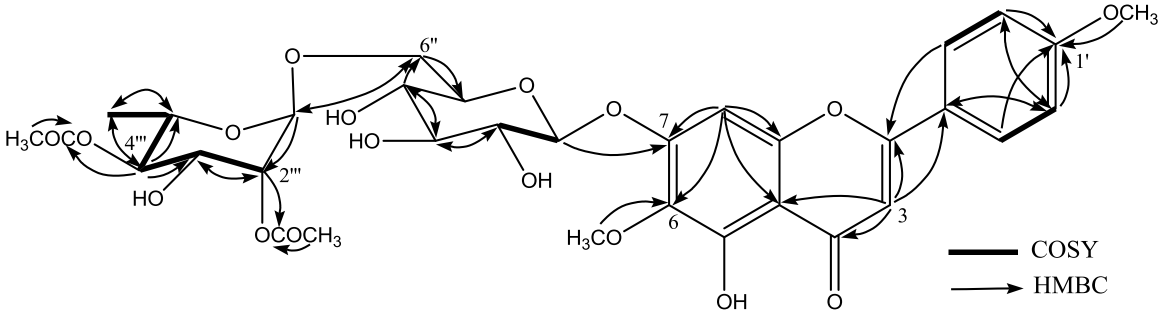


Figure 17. HMBC and COSY correlations of **8**

Isolinariin E (**8**), $[\alpha]_D$ -6.56 was also pale yellow powder. The NMR spectral data together with molecular ion at *m/z* 729.1997 $[M+Na]^+$ in HR-ESI-MS indicated that **8** was closely related to **6** except that one of the acetyl groups in **8** has different position. The deshielded of H-4‴ [δ_H 4.78 (1H, d, 10 Hz)] and C-4‴ (δ_C 75.5) and shielded of H-3‴ [δ_H 3.97 (1H, dd, 10, 3 Hz)] and C-3‴ (δ_C 68.6) suggested that the acetylated position was changed from C-3‴ to C-4‴ in **8**. That was further supported by a correlation between the proton at δ_H 4.78 (H-4‴) and carbon signal at δ_C 172.4 (Fig. 17) in the HMBC spectrum. The acid hydrolysis of **8** also showed the aglycone (pectolinarigenin), D-glucose and L-rhamnose (Fig. 18), therefore the structure of **8** was deduced as pectolinarigenin-7-*O*-(2,4-diacetyl-*α*-L-rhamnopyranosyl)-(1→6)-*β*-D-glucopyranoside.

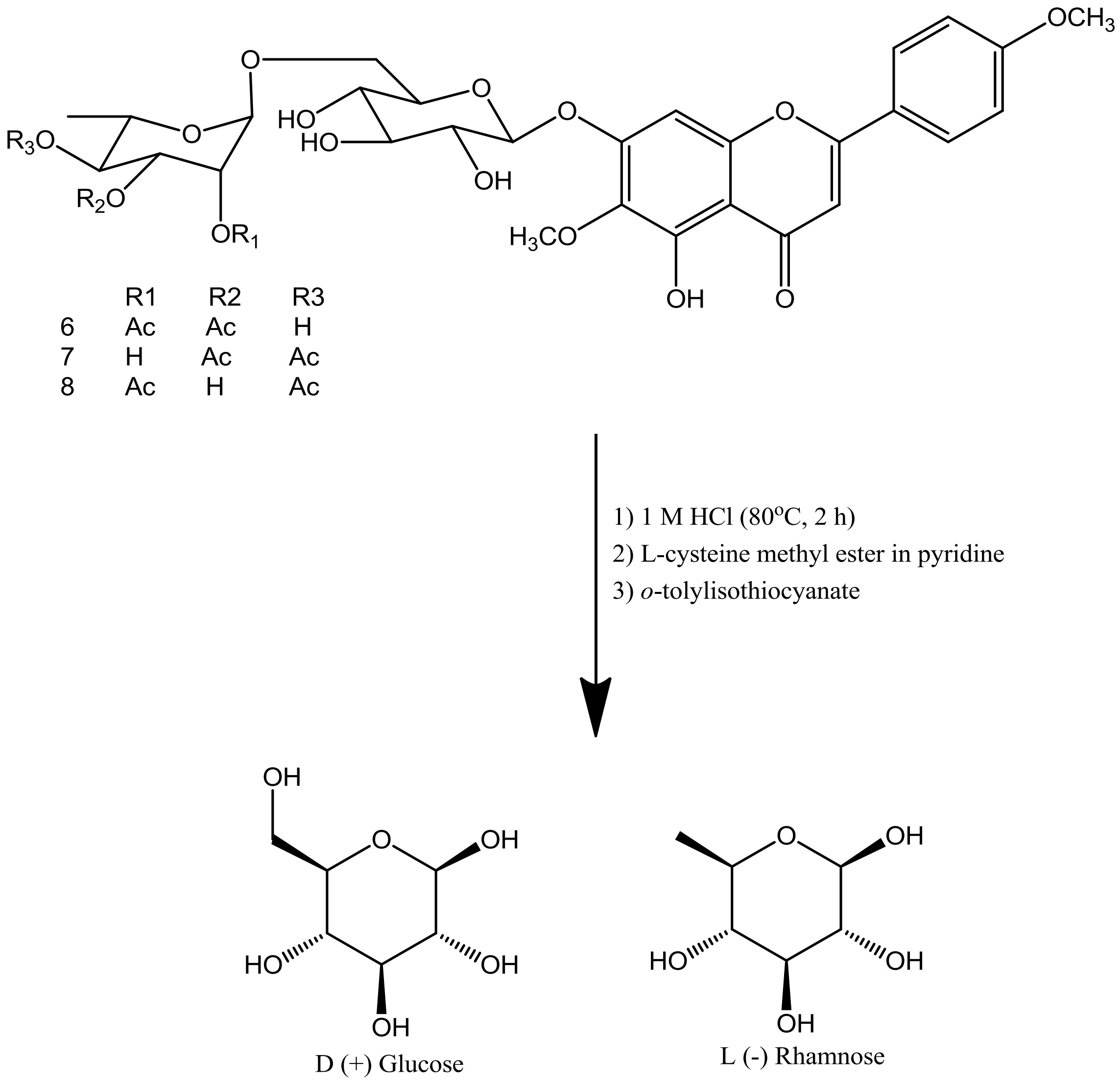


Figure 18. Acid hydrolysis of **6**, **7** and **8**

1.4. Bioassay of Chemical Constituents

1.4.1. A549 cytotoxic activity

According to some literatures, flavonoid has been proven to be potential drug leads most notably with anti cancer effects. It exhibited significant cytotoxic activity against several human cancer lines *in vitro.*[23,24,25] Triterpenoids also have been reported to act as selective catalytic inhibitors of human DNA topoisomerases which it play important roles in replication, transcription, recombination and chromosome segregation at mitosis.[26] Betulin, triterpenoid, elicits anticancer effects in tumor primary cultures and cell lines in vitro whereas, it exhibited anti proliferative effect, altered tumor cells morphology, decreased their motility and induced apoptotic cell death.[27] On the other hand, they have been reported that *β*-amyrin exhibited weak cytotoxicity against A549 and HL-60 cancer cell lines.[28]

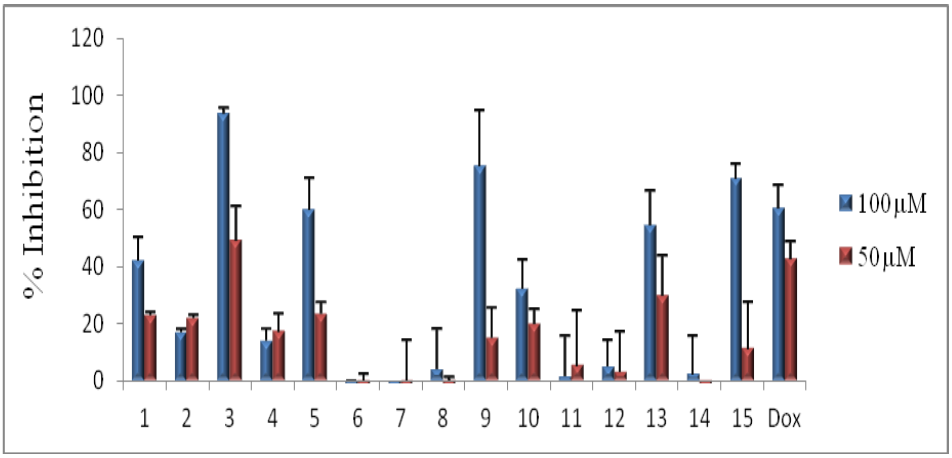


Figure 19. A549 cell lines inhibitory activity of **1** – **15**

The isolated compounds (**1** – **15**) of the mixture of hexane-ethyl acetate layer of *Linaria japonica* were evaluated for cytotoxicity evaluation toward A549 (human lung cancer) cell lines (Table 4, Fig. 19) using doxorubicin as a positive control (IC_{50} value of 0.70 μM). The result showed that linarenone C (**3**) exhibited moderated cytotoxicity with the IC_{50} value of 51.2 μM. Then linarenone E (**5**), linarienone (**9**), pectolinarigenin (**13**) and luteolin (**15**) found to be active (IC_{50} of 86.5, 79.0, 91.1 and 82.6 μM, respectively). The present of glucose and rhamnose of **6**, **7**, **8**, **11** and **12** were found to be inactive. The replacement by an acetyl group at C-12 of **3** resulted in the enhancement of activity compare to that having carboxylic acid group at this position of **4** (inactive).

Table 4.
Anti-Leishmania activity of **1 – 15** from *Linaria japonica*

Isolated Compounds	**A549 (IC_{50}, μM)**	***L. major* (IC_{50}, μM)**
Linarenone A **1**	-	56.7 ± 1.8
Linarenone B **2**	-	89.4 ± 7.2
Linarenone C **3**	51.2 ± 2.6	-
Linarenone D **4**	-	-
Linarenone E **5**	86.5 ± 5.7	97.3 ± 5.9
Isolinariin C **6**	-	-
Isolinariin D **7**	-	-
Isolinariin E **8**	-	-
Linarienone **9**	79.0 ± 9.9	50.3 ± 1.6
Desacetyllinarienone **10**	-	52.7 ± 2.6
Linariin **11**	-	-
Pectolinarin **12**	-	-
Pectolinarigenin **13**	91.1 ± 6.7	-
Apigenin **14**	-	-
Luteolin **15**	82.6 ± 5.4	77.7 ± 5.4
Doxorubin	0.70 ± 0.1	n.d
Miltefosine	n.d	17.8 ± 1.1

–: > 100 μM, n.d: not determined

1.4.2. *Leishmania major* activity

The leishmaniases are a complex of diseases caused by different species of the protozoan parasite Leishmania and are a major public health problem in many developing countries, where 350 million people live at risk of infection.[29)] There is no approved vaccine for clinical use. Despite a few research achievements, first-line chemotherapy is still based on pentavalent antimonials, developed more than 50 years ago, which are toxic and prone to drug resistance.[30)] Recently, several natural products with antileishmanial activity, including naphthoquinones, lignans, neolignans, alkaloids and triterpenoids have been reported.[31)] However, there have been few studies on the antileishmanial activity of the flavonoid class of natural polyphenols. These few studies include that of luteolin, a common flavonoid in the human diet, which was recently described as a promising antileishmanial drug.[32)] Proanthocyanidins also show antileishmanial activity, as well as modulatory effects on nitric oxide and tumor necrosis factoralpha release in RAW 264.7 cells,[33)] and a methoxychalcone isolated from inflorescences of *Piper aduncum* reportedly has significant antileishmanial activity as well.[34)] Quercitrin, previously isolated from an active flavonoid fraction of *K. pinnata*, was an additional potent antileishmanial compound, with a low toxicity profile.[35)]

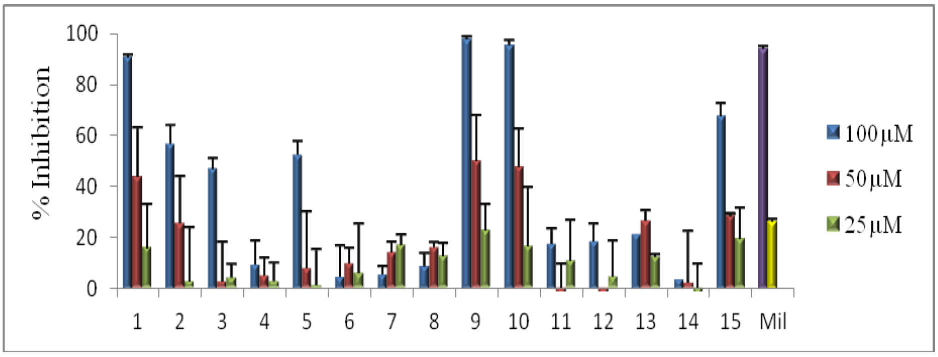


Figure 20. *Leishmania major* inhibitory activity of **1** – **15**

Although a broad spectrum of biological activities has already been demonstrated for flavonoids, few studies have been devoted to the antileishmanial activity of this class of natural chemical constituents. In order to evaluate their antileishmanial activity, the isolated compounds (**1** – **15**) were thus tested at three different concentrations on intracellular *L. major* (Table 4, Fig. 20) and miltefosine was used as positive control (IC_{50} values of 17.8 µM). linarenone A (**1**), linarienone (**9**) and desacetyl-linarienone (**10**) showed moderate inhibition (IC_{50} 56.7, 50.3 and 52.7 µM, respectively) and linarenone B (**2**) and linarenone E (**5**) found to be active (IC_{50} 89.4 and 97.3 µM, respectively). It is noteworthy that **1** and **9** were relatively selective against *L. major* than cytotoxicity. The presence of hydroxy group at position 5, 7, 3′ and 4′ of luteolin (**15**) showed moderated inhibition with IC_{50} value of 77.7 µM. The other flavonoids did not show their inhibitory activity then they were explored for another activities that related with anti aging such as collagenase and AGEs formation inhibitory activity.

1.4.3. Collagenase

The process of skin aging has been divided into two categories: instrinsic and extrinsic aging. Intrinsic aging or natural aging is caused by changes in elasticity of the skin over time. Extrinsic skin aging is predominately a result of exposure to solar radiation (photoaging). UV exposure cause physical changes to the skin due to alteration that occur in the connective tissue via the formation of lipid peroxides, cell contents, enzymes and reactive oxygen species (ROS). Lipid peroxides can be metabolised to form secondary products which damage the extracellular matrix (ECM) while ROS are credited with involvement in the loss of skin elasticity.[36, 37, 38)]

Eighty percent of skin dry weight is collagen which is responsible for the tensile strength of the skin. Collagen fibres, elastin fibres and glycoaminoglycans are produced by fibroblasts and are primarily affected by photoaging resulting in visible changes in the skin such as wrinkles, pigmentation and changes in thickness. ROS are also capable of inducing expression of proteinase which are responsible for remodelling the extracelullar matrix such as matrix metalloproteinases (MMPs) and serine protease.[39)]

MMPs are part of a group of transmembrane zinc containing endopeptidases which include collagenase and gelatinases. Collagenase is enzyme that known to be a member of the matrix metalloproteinase (MMPs). They are highly induced in inflames skin as well as in photoaged skin, and they breakdown the dermal matrix proteins such as collagen and elastin; this possibly leads to the prolonged skin damage and wrinkle formation.[40)] Therefore, the agents that inhibit collagenase and/or elastase activity may have beneficial effects for maintaining healthy skin by preventing dermal matrix degradation.

The effects of flavonoids on MMP have been previously examined. Flavan and several flavonoids were reported to inhibit gelatinases (MMP-2 and MMP-9).[41)] The direct modulatory effect of flavonoids on collagenase or MMP-1 (mammalian collagenase-1) has rarely been demonstrated, despite the importance of MMP-1 and collagen breakdown in inflammatory skin disease and photoaging.

Therefore, we investigated the inhibitory activity of some different types of flavonoids againts collagenase from *Clostridium histolyticum* to elucidate their therapeutic potential against skin inflammation and degrades ECM. This bacterial collagenase hydrolyses triple-helical collagen in both physiological and *in vitro* conditions using synthetic peptides as substrates. We found that several derivatives, particularly the flavonoids, demonstrated significant inhibition of collagenase.

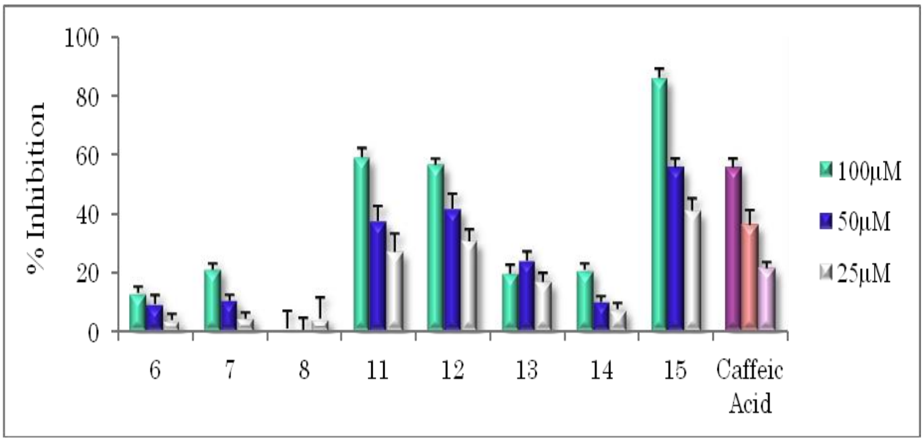


Figure 21. Collagenase inhibitory activity of **6 – 8**, **11 – 15**

The isolated compounds (**6 – 8, 11 – 15**) were tested and revealed to have, more or less, an inhibitory action on the collagenase obtained from *Clostridium histolyticum* (Table 5, Fig. 21). The result showed that linariin (**11**) and pectolinarin (**12**) exhibited significant inhibition with IC_{50} value of 79.4 and 78.6 μM, respectively. luteolin (**15**, 40.5 μM) showed stronger inhibitory activity than that of a positive control, caffeic acid (IC_{50} value of 0.12 mM). The other compounds did not show inhibitory activity.

Table 5.
Anti-AGEs and collagenase activities of **6 – 8, 11 – 15** from *Linaria japonica*

Isolated Compounds	AGEs (IC_{50}, μM)	Collagenase (IC_{50}, μM)
Isolinariin C **6**	34.8 ± 5.6	-
Isolinariin D **7**	35.0 ± 8.8	-
Isolinariin E **8**	19.5 ± 2.0	-
Linariin **11**	-	79.4 ± 3.8
Pectolinarin **12**	-	78.6 ± 2.4
Pectolinarigenin **13**	-	-
Apigenin **14**	-	-
Luteolin **15**	28.3. ± 6.8	40.5 ± 3.2
Aminoguanidine	1290 ± 31.5	n.d
Caffeic acid	n.d	120 ± 1.8

–: > 100 μM, n.d: not determined

1.4.4. AGEs formation

Reactive Oxygen Species (ROS) include oxygen ions, free radicals and peroxides. ROS are generally very small molecules and are highly reactive due to the presence of unpaired electrons. They form as a natural by-product of the normal metabolism of oxygen. During times of environmental stress, ROS levels can increase dramatically, causing significant damage to cell structures. This is known as oxidative stress, which is the major cause of degenerative disorders including aging and disease. Lipid peroxidation results from ROS damage to cell membranes, leading to premature aging, skin cancer and cell death.[42, 43)]

Collagen and elastin proteins are highly susceptible to an internal chemical reaction within the body called glycation. This is a non-enzyme mediated reaction that takes place between free amino groups in proteins and a sugar such as glucose. The same glucose that provides energy for our cells can react with proteins (such as collagen), resulting in the formation of Advanced Glycation End-products (AGEs) and ROS; these contribute to crosslinking of protein fibers, the loss of elasticity and changes in the dermis associated with the aging process.[42)]

AGEs, advanced glycation end products, are well known to be a cause of aging as well as readily form and accumulate with sustained hyperglycemia, contribute to the development of diabetic complications and considered a potential therapeutic target. They are formed during non-enzymatic reaction involving proteins and sugars, *i.e.* the Maillard or browning reaction. The non-enzymatic reaction of reducing sugars with protein leads to a variety of fluorescent and non-fluorescent advanced glycation end products (AGEs) involving free radical and carbonyl intermediates. Glycation occurs physiologically in the course of aging and in various pathological processes. When AGEs form in the skin, they activate a receptor site and form a complex known as Receptor-AGE (R-AGE) that signals cellular processes related to inflammation and subsequent disease.[42,44)]

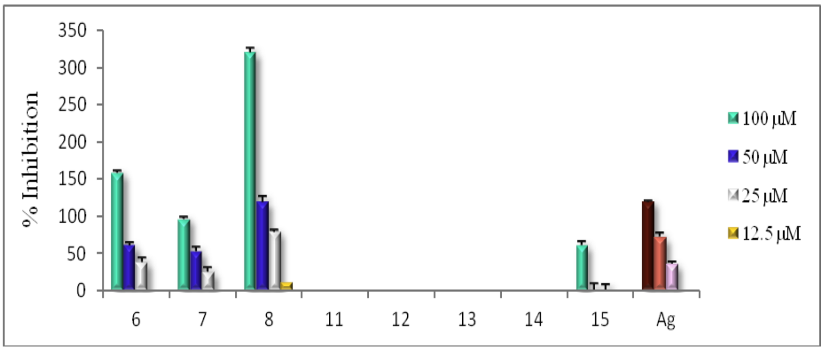


Figure 22. AGEs formation inhibitory activity of **6 – 8, 11 – 15**

Inhibitory effects of isolated compounds (**6** – **8, 11** – **15**) on AGEs formation were evaluated using a fluorescent method[44)] (Table 5, Fig. 22). The result showed that new compounds **6** (34.8 μM), **7** (IC_{50} of 35.0 μM), **8** (19.5 μM) and luteolin (**15**, 28.3 μM) showed stronger inhibitory activity than that of a reference compound, aminoguanidine (IC_{50} value of 1.29 mM). The other compounds did not show inhibitory activity. So, the present of acetoxy goups at rhamnose make stronger inhibition activity of AGEs formation in flavonoids.

1.5. Experimental Section

1.5.1. General experimental procedures

^{1}H and ^{13}C-NMR spectra were taken on on a Bruker Ultrashield 600 at 600 MHz and 150 MHz with TMS as an internal standard. IR and UV spectra were measured on a HORIBA FT-720 and JASCO V-520 UV/Vis spectrophotometer, respectively. Optical rotations and CD spectra were measured on a JASCO P-1030 digital polarimeter and a JASCO J-720 spectropolarimeter, respectively. Positive ion HR-ESI-MS was performed with an Applied Biosystems QSTAR XL NanoSprayTM System. Silica gel open and reversed phase [octadecyl silica gel (ODS)] column chromatography were performed on silica gel 60 (E. Merck, Darmstadt, Germany), and Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan; Φ = 35 mm, L = 350 mm), respectively. HPLC was performed on an ODS column (Inertsil, GL Science, Tokyo, Japan; Φ = 6 mm, L = 250 mm) and the eluate was monitored with a JASCO RI-930 intellegent detector and a JASCO PU-1580 intelligent pump.

1.5.2. Plant material

Whole plants of *Linaria japonica* were collected in late July 1990 in seashore areas of Tottori Prefecture, and a voucher specimen (90-LJ-Tottori) was deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Hiroshima University.

1.5.3. Isolated Compounds

*Linarinone A (**1**)*

Colorless amorphous solid; $[\alpha]_D^{26}$ +10.0 (*c* 0.50, $CHCl_3$); UV (EtOH) λ_{max} (log *e*) nm: 230 (3.72); CD (*c* 2.76 x 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm): +1.02 (331); IR (film) ν_{max} cm^{-1}: 3340, 2929, 1732, 1716, 1653, 1456, 1375, 1237, 1029; ^{1}H NMR and ^{13}C NMR, see Table 1 and 2; positive HRESIMS *m/z* 385.2348 $[M+Na]^+$ (calcd. for $C_{22}H_{34}O_4Na$: 385.2349).

*Linarenone B (**2**)*

Colorless amorphous solid; $[\alpha]_D^{31}$ +5.7 (*c* 0.20, $CHCl_3$); UV (EtOH) λ_{max} (log *e*) nm: 224 (3.45); CD (*c* 1.94 x 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm): +0.95 (333); IR (film) ν_{max} cm^{-1}: 2924, 1733, 1716, 1653, 1456, 1375, 1239, 1027; ^{1}H NMR and ^{13}C NMR, see Table 1 and 2; positive HRESIMS *m/z* 455.2766 $[M+Na]^+$ (calcd. for $C_{26}H_{40}O_5Na$: 455.2768).

*Linarenone C (**3**)*

Colorless amorphous solid; $[\alpha]_D^{31}$ +15.5 (*c* 0.21, $CHCl_3$); UV (EtOH) λ_{max} (log *e*) nm: 232 (3.45); CD (*c* 2.33 x 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm): +1.16 (334); IR (film) ν_{max} cm^{-1}: 2924, 1731, 1698, 1655, 1619, 1456, 1376, 1258, 1033; ^{1}H NMR and ^{13}C NMR, see Table 1 and 2; positive HRESIMS *m/z* 297.1825 $[M+Na]^+$ (calcd. for $C_{18}H_{26}O_2Na$: 297.1825).

*Linarenone D (**4**)*

Colorless amorphous solid; $[\alpha]_D^{27}$ +12.6 (*c* 0.44, $CHCl_3$); UV (EtOH) λ_{max} (log *e*) nm: 218 (3.62); CD (*c* 3.21 x 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm): +1.22 (325); IR (film) ν_{max} cm^{-1}: 2925, 1728, 1698, 1649, 1456, 1376, 1260, 1034; ^{1}H NMR and ^{13}C NMR, see Table 1 and 2; positive HRESIMS *m/z* 299.1618 $[M+Na]^+$ (calcd. for $C_{17}H_{24}O_3Na$: 299.1618).

*Linarenone E (**5**)*

Colorless amorphous solid; $[\alpha]_D^{27}$ +48.8 (*c* 0.82, MeOH); UV (EtOH) λ_{max} (log *e*) nm: 239 (2.97); CD (*c* 5.21 x 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm): +1.31 (327); IR (film) ν_{max} cm^{-1}: 2923, 1754, 1660, 1435, 1377, 1256, 1206, 1031; ^{1}H NMR and ^{13}C NMR, see Table 1 and 2; positive HRESIMS *m/z* 339.1929 $[M+Na]^+$ (calcd. for $C_{20}H_{28}O_3Na$: 339.1931).

*Isolinariin C (**6**)*

Pale yellow powder; $[\alpha]_D^{25}$ -4.81 (*c* = 0.77, methanol); UV (EtOH) λmax (log ε) nm: 324 (3.83), 274 (3.86), 232 (3.83); IR (film) ν_{max} cm^{-1}: 3437, 2933, 1746, 1653, 1606, 1460, 1361, 1251, 1182, 1054; ^{1}H NMR and ^{13}C NMR, see Table 3; positive HR-ESI-MS *m/z* 729.1998 $[M+Na]^+$(calcd. for $C_{33}H_{38}O_{17}Na$: 729.2001).

Isolinariin D (7)

Pale yellow powder; $[\alpha]_D^{26}$ -8.56 (*c* = 1.20, methanol); UV (EtOH) λmax (log ε) nm: 322 (3.85), 272 (3.85), 233 (3.86); IR (film) ν_{max} cm^{-1}: 3443, 2932, 1735, 1653, 1607, 1458, 1360, 1250, 1182, 1044; ^{1}H NMR and ^{13}C NMR, see Table 3; positive HR-ESI-MS *m/z* 729.1997 $[M+Na]^+$(calcd. for $C_{33}H_{38}O_{17}Na$: 729.2001).

*Isolinariin E (**8**)*

Pale yellow powder; $[\alpha]_D^{26}$ -6.56 (*c* = 0.35, methanol); UV (EtOH) λmax (log ε) nm: 319 (3.78), 276 (3.76), 229 (3.78); IR (film) ν_{max} cm^{-1}: 3361, 2931, 1735, 1652, 1603, 1457, 1360, 1250, 1182, 1051; ^{1}H NMR and ^{13}C NMR, see Table 3; positive HR-ESI-MS *m/z* 729.1997 $[M+Na]^+$(calcd. for $C_{33}H_{38}O_{17}Na$: 729.2001).

*Linarienone (**9**)*[9)]

Colorless amorphous solid; $[\alpha]_D^{26}$ +23.5 (*c* 0.17, MeOH); CD (*c* 2.77 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm): +0.76 (334); ^{1}H NMR ($CDCl_3$, δ): 0.55 (3H, s, H_3-18), 0.82 (3H, d, *J* = 6 Hz, H_3-17), 1.08 (1H, br qd, *J* =13, 3 Hz, H-7α), 1.18 (1H, ddd, *J* =14, 11, 3 Hz, H-6β), 1.21 (3H, s, H_3-19), 1.32 (1H, dq, *J* =13, 3 Hz, H-7β), 1.43 (1H, m, H-8), 1.62 (1H, dd, 16, 2 Hz, H-11), 1.69 (3H, s, H_3-16), 1.71 (1H, dd, 16, 8 Hz, H-11), 1.85 (3H, s, H_3-5′), 1.92 (6H, br s, H_3-20 and

H_3-4′), 1.95 (1H, br d J = 6 Hz, H-10), 2.00 (3H, s, 12-OAc), 2.05 (1H, ddd, J =14, 3, 3 Hz, H-6α), 2.45 (1H, br d, J = 18 Hz, H-1α) and 2.70 (1H, dd, J = 18, 6 Hz, H-1β), 4.63 (2H, d, J = 6 Hz, H_2-15), 5.21 (1H, dd, J = 7, 2 Hz, H-12), 5.58 (1H, t, J = 6 Hz, H-14), 5.82 (1H, s, H-3), 6.02 (1H, dd, J = 7, 1 Hz, H-3′) and ^{13}C NMR ($CDCl_3$, δ): 12.7 (CH_3-16), 16.4 (CH_3-17), 18.4 (CH_3-18), 20.6 (CH_3-20), 20.6 (CH_3-5′), 21.2 (CH_3-4′ and OAc-12), 28.3 (CH_2-7), 31.9 (CH_3-19), 35.7 (CH_2-1), 36.7 (CH_2-6), 37.7 (CH-8), 39.4 (CH_2-11), 40.7 (C-5), 42.6 (C-9), 47.7 (CH-10), 60.2 (CH_2-15), 74.5 (CH-12), 120.8 (CH-14), 121.0 (CH-3), 127.8 (CH-3′), 128.5 (C-2′), 137.8 (C-4), 140.0 (C-13), 167.9 (OAc-12), 170.0 (C-1′), 198.9 (C-2); positive HR-ESI-MS *m/z* 467.2773 [M+Na]+ (calcd. for $C_{27}H_{40}O_5Na$: 467.2768).

*Desacetyl-linarienone (**10**)*[9)]

Colorless amorphous solid; $[\alpha]_D^{30}$ +67.5 (c = 0.04, MeOH); UV (EtOH) λmax (log ε) nm: 247 (3.83); CD ($c = 1.24 \times 10^{-5}$ M, MeOH) $\Delta\varepsilon$ (nm): +8.61 (327); IR (film) ν_{max} cm^{-1}: 3430, 2926, 1714, 1650, 1456, 1225, 1080; ^{1}H NMR ($CDCl_3$, δ): 0.56 (3H, s, H_3-18), 0.86 (3H, d, J = 6 Hz, H_3-17), 1.12 (1H, br qd, J = 13, 3 Hz, H-7α) , 1.22 (3H, s, H_3-19), 1.24 (1H, ddd, J = 14, 11, 3 Hz, H-6β), 1.35 (1H, dq, J = 13, 3 Hz, H-7β), 1.60 (2H, m, H-11), 1.72 (3H, s, H_3-16), 1.87 (3H, s, H_3-5′), 1.93 (3H, s, H_3-20), 1.96 (3H, dd, J = 7, 1 Hz, H_3-4′), 2.02 (1H, br d, J = 6 Hz, H-10), 2.06 (1H, ddd, J = 14, 3, 3 Hz, H-6α), 2.45 (1H, br d, J = 18 Hz, H-1α), 2.69 (1H, dd, J = 18, 6 Hz , H-1β), 4.16 (1H, t, J = 6 Hz, H-12), 4.67 (2H, t, J = 7 Hz, H_2-15), 5.59 (1H, t, J = 7 Hz, H-14), 5.83 (1H, s, H-3), 6.04 (1H, dd, J = 7, 1 Hz, H-3′); positive HR-ESI-MS *m/z* 425.2661 $[M+Na]^+$ (calcd. for $C_{25}H_{38}O_4Na$: 425.2662).

*Linariin (**11**)*[18)]

Pale yellow powder; ^{1}H NMR (pyridine-d$_5$, δ): 1.27 (3H, d, J = 6 Hz, H-6‴), 2.00 (3H, s, H-4‴OAc), 3.70 (3H, s, H-4′OCH_3), 4.07 (3H, s, H_3-6OCH_3), 4.40 (1H, m, H-2″), 4.41 (1H, m, H-3″), 4.17-4.22 (4H, m, H-4″, H_2-6″, H-5‴), 4.34 (1H, m, H-5″), 4.55 (1H, m, H-3‴), 4.63 (1H, m, H-2‴), 5.47 (1H, br s, H-1‴), 5.68 (1H, t, J = 10 Hz, H-4‴), 5.82 (1H, d, J = 7 Hz, H-1″), 6.92 (1H, s, H-3), 7.23 (2H, d, J = 8 Hz, H-3′, 5′), 7.36 (1H, s, H-8), 8.03 (2H, d, J = 8 Hz, H-2′, 6′) and ^{13}C NMR (pyridine-d$_5$, δ): 17.9 (CH_3-6‴), 21.1 & 170.7 (OAc-4‴), 55.5 (CH_3O-4′), 60.9 (CH_3O-6), 67.2 (CH-5‴), 67.5 (CH_2-6″), 70.3 (CH-3‴), 71.1 (CH-4″), 72.1 (CH-2‴), 74.7 (CH-2″), 75.7 (CH-4‴), 77.6 (CH-5″), 78.5 (CH-3″), 95.2 (CH-8), 102.1 (CH-1‴), 102.6 (CH-1″), 104.4 (CH-3), 107.2 (C-10), 115.2 (CH_2-3′, 5′), 123.9 (C-1′), 128.8 (CH_2-2′, 6′), 134.1 (C-6), 153.2 (C-9), 154.1 (C-5), 157.8 (C-7), 163.1 (C-4′), 164.7 (C-2), 183.7 (C-4); positive HR-ESI-MS *m/z* 687.1897 $[M+Na]^+$(calcd. for $C_{31}H_{36}O_{16}Na$: 687.1896).

*Pectolinarin (**12**)*[11)]

Pale yellow powder; ^{1}H NMR (pyridine-d_5, δ): 1.55 (3H, d, J = 6 Hz, H-6‴), 2.00 (3H, s, H-4‴OAc), 3.70 (3H, s, H-4′OCH_3), 4.05 (3H, s, H_3-6OCH_3), 4.08 (2H, m, H-4″, 6″α), 4.18 (1H, m, H-4‴), 4.27 (2H, m, H-5″, 5‴), 4.37 (2H, m, H-2″, 3″), 4.54 (1H, dd, J = 10, 3 Hz, H-3‴), 4.65 (1H, dd, J = 3, 1 Hz, H-2‴), 4.74 (1H, m, H-6″β), 5.49 (1H, br s, H-1‴), 5.68 (1H, t, J = 10 Hz, H-4‴), 5.76 (1H, d, J = 7 Hz, H-1″), 6.88 (1H, s, H-3), 7.25 (2H, d, J = 8 Hz, H-3′, 5′), 7.39 (1H, s, H-8), 8.04 (2H, d, J = 8 Hz, H-2′, 6′), 13.61 (1H, s, OH-5) and ^{13}C NMR (pyridine-d_5, δ): 18.3 (CH_3-6‴), 55.2 (CH_3O-4′), 60.6 (CH_3O-6), 67.4 (CH_2-6″), 69.6 (CH-5‴), 71.9 (CH-2‴), 72.6 (CH-3‴), 73.8 (CH-4″), 74.4 (CH-2″), 75.7 (CH-4‴), 77.5 (CH-5″), 78.2 (CH-3″), 94.9 (CH-8), 102.2 (CH-1‴), 102.3 (CH-1″), 104.1 (CH-3), 106.9 (C-10), 114.9 (CH_2-3′, 5′), 122.9 (C-1′), 128.6 (CH_2-2′, 6′), 133.8 (C-6), 152.9 (C-9), 153.8 (C-5), 157.5 (C-7), 162.8 (C-4′), 164.5 (C-2), 182.9 (C-4); positive HR-ESI-MS *m/z* 645.1790 $[M+Na]^+$(calcd. for $C_{29}H_{34}O_{15}Na$: 645.1790).

*Pectolinarigenin (**13**)*[45)]

Pale yellow powder; ^{1}H NMR (pyridine-d_5, δ): 3.73 (3H, s, H-4′OCH_3), 3.96 (3H, s, H_3-6OCH_3), 6.93 (1H, s, H-3), 7.07 (2H, d, J = 8 Hz, H-3′, 5′), 6.89 (1H, s, H-8), 7.94 (2H, d, J = 8 Hz, H-2′, 6′), 13.83 (1H, s, OH-5) and ^{13}C NMR (pyridine-d_5, δ): 55.1 (CH_3O-4′), 59.9 (CH_3O-6), 94.9 (CH-8), 103.7 (CH-3), 105.0 (C-10), 114.5 (CH_2-3′, 5′), 123.7 (C-1′), 128.2 (CH_2-2′, 6′), 132.3 (C-6), 153.7 (C-9), 153.4 (C-5), 158.6 (C-7), 162.6 (C-4′), 163.7 (C-2), 182.8 (C-4); positive HR-ESI-MS *m/z* 337.0684 $[M+Na]^+$(calcd. for $C_{17}H_{14}O_6Na$: 337.0683).

*Apigenin (**14**)*[46)]

Pale yellow powder; ^{1}H NMR (pyridine-d_5, δ): 6.77 (1H, s, H-8), 6.84 (1H, br s, H-6), 6.93 (1H, br s, H-3), 7.23 (2H, d, J = 8 Hz, H-3′, 5′), 7.94 (2H, d, J = 8 Hz, H-2′, 6′), 13.80 (1H, s, OH-5) and ^{13}C NMR (pyridine-d_5, δ): 95.3 (CH-8), 100.5 (CH-6), 104.4 (CH-3), 105.4 (C-10), 117.3 (CH_2-3′, 5′), 122.8.7 (C-1′), 129.4 (CH_2-2′, 6′), 158.9 (C-9), 163.2 (C-4′), 163.7 (C-5), 165.1 (C-2), 166.4 (C-7), 183.2 (C-4).

*Luteolin (**15**)*[46)]

Pale yellow powder; ^{1}H NMR (pyridine-d_5, δ): 6.75 (2H, s, H-6, 8), 6.93 (1H, s, H-3), 7.30 (1H, d, J = 8 Hz, H-5′), 7.55 (1H, dd, J = 8, 2 Hz, H-6′), 7.93 (1H, d, J = 2 Hz, H-2′), 13.80 (1H, s, OH-5) and ^{13}C NMR (pyridine-d_5, δ): 95.3 (CH-8), 100.4 (CH-6), 104.3 (CH-3), 105.5 (C-10), 115.1 (CH-2′), 117.4 (CH-5′), 120.0 (CH-6′), 123.5 (C-1′), 148.3 (C-3′), 152.2 (C-4′), 159.0 (C-9), 163.7 (C-5), 165.4 (C-2), 166.3 (C-7), 183.3 (C-4).

*Desdiacyl-linarienone (**1a** = **2a** = **9a**)*[9)]

Colorless amorphous solid; $[\alpha]_D^{30}$ +62.4 (*c* 0.11, MeOH); UV (EtOH) λmax (log ε) nm: 248 (4.50); CD (*c* 1.24 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm): +1.62 (330); IR (film) ν_{max} cm^{-1}: 3382, 2927, 1730, 1717, 1649, 1456, 1379, 1258, 1004; ^{1}H NMR (CDCl$_3$, δ): 0.57 (3H, s, H_3-18), 0.86 (3H, d, *J* = 7 Hz, H_3-17), 1.10 (1H, br qd, *J* = 13, 3, H-7α), 1.23 (3H, s, H_3-19), 1.26 (1H, ddd, *J* = 13, 11, 3, H-6β) , 1.35 (1H, dq, *J* = 13, 3, H-7β), 1.54 (2H, m, H_2-11), 1.67 (3H, s, H_3-16), 1.94 (3H, s, H_3-20), 2.07 (1H, ddd, *J* = 14, 3, 3, H-6α), 2.09 (1H, m, H-10), 2.44 (1H, br d, *J* = 18 Hz, H-1α), 2.68 (1H, dd, *J* = 18, 6 Hz, H-1β), 4.13 (1H, m, H-12), 4.17 (2H, m, H_2-15), 5.59 (1H, t, *J* = 7 Hz, H-14), 5.84 (1H, s, H-3) and ^{13}C NMR (CDCl$_3$, δ): 12.6 (CH_3-16), 17.1 (CH_3-17), 19.4 (CH_3-18), 21.0 (CH_3-20), 28.9 (CH_2-7), 32.6 (CH_3-19), 36.4 (CH_2-1), 37.3 (CH_2-6), 38.2 (CH-8), 39.9 (C-5), 41.2 (C-9), 42.0 (CH_2-11), 47.9 (CH-10), 59.5 (CH_2-15), 74.3 (CH-12), 124.4 (CH-14), 129.1 (CH-3), 142.9 (C-13), 168.9 (C-4), 199.3 (C-2) ; positive HR-ESI-MS *m/z* 343.2246 $[M+Na]^+$ (calcd. for $C_{20}H_{32}O_3Na$: 343.2244).

1.5.4. Mild alkaline hydrolysis

A solution of linarienone (**9**) (11.5 mg) in MeOH - $CHCl_3$ (1:1, 1.8 ml) was added 1 mol/l NaOMe solution (0.2 ml), stirred at room temparature for 3 h. The mixture was neutralized with ion exchange resin IR-120B (ORGANO, H^+-form) and filtered off. The filtrate was evaporated to dryness under reduced pressure. Purification of the reaction product by preparative TLC (*n*-hexane – EtOAc = 3 : 2) furnished desacetyl-linarienone (**10**) (0.3 mg, R_f = 0.45) and desdiacyl-linarienone (**9a**) (1.2 mg, R_f = 0.18). This procedure also performed for **1** and **2** to produce **1a** and **2a**, respectively, which were identical to **9a** by HPLC (65% aq. MeOH, ODS Φ = 6 mm × 250, UV-Vis detector 248 nm, 1.5ml/min, t_R=16 min) and HR-ESI-MS analysis.[9)]

1.5.5. Acid hydrolysis to identification of sugar moeity of **6**, **7** and **8**

A solution of each isolinariin C (**6**), D (**7**) and E (**8**) (@ 5 mg) in 1 N HCl (0.2 ml) was heated at 90-100 °C in screw-capped vial for 2 h. The mixture was neutralized by addition of amberlite IRA400 (OH^- form) and filtered. The filtrate was dried in vacou, dissolved in 0.2 ml of pyridine containing L-cysteine methyl ester (15 mg/ml) and reacted at 60°C for 1 h. To the mixture, a solution (0.1 ml) of *o*-toryl isothiocyanate in pyridine (5 mg/ml) was added, and it was heated at 60°C for 1 h. The final mixture was directly analyzed by HPLC [Cosmosil 5C_{18} AR II (250 x 4.6 mm i.d., Nacalai Tes-que); 25% CH_3CN in 50 mM H_3PO_4;

flow rate 0.8 ml/min; column temperature 35°C; detection 250 nm]. The t_R of the peak at 18 min coincided with that of D-glucose. The t_R of the L-rhamnose was 30 min.[22)]

1.5.6. A549 growth inhibition assay

Human lung cancer cells (A549) were cultured in Dulbecco`s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, kanamycin (100 μg/ml) and amphotericin (0.5 μg/ml). Into a 96-well plate, aliquots of the DMSO solution of the test compounds (1% final concentration) were incubated with A549 cells (5×10^3 cells/well) in a CO_2 incubator at 37°C for 72 h. MTT was added into each well and the plate was further incubated for one and half hour. Absorbance was measured at 540 nm using a 2300 EnSpire Multimode plate reader (Perkin Elmer). DMSO was used as a negative control and doxorubicin as a positive control. The viability was compared to that of control cells incubated in the same medium without the test compounds. Measurements were performed in triplicate and the concentrations required for 50% inhibition (IC_{50}) of the intensity of absorbance were determined graphically [47)]

1.5.7. Anti-Leishmania major assay

Leishmania major promastigotes were cultured in M199 medium supplemented with 10% heat-inactivated fetal bovine serum and kanamycin (100 μg/mL). Into a 96-well plate, aliquots of the DMSO solution of the test compounds (1% final concentration) were incubated with *Leishmania major* cells (1×10^5 cells/well) in a CO_2 incubator at 27°C for 72 h. MTT was added into each well and further incubated overnight. Absorbance was measured at 540 nm using a 2300 EnSpire Multimode plate reader (Perkin Elmer). DMSO was used as a negative and amphotericin B as positive control. The viability was compared to that of control cells incubated in the same medium without the test compounds. Measurements were performed in triplicate and the concentrations required for 50% inhibition (IC_{50}) of the intensity of absorbance were determined graphically [47)]

1.5.8. Collagenase inhibition assay

Collagenase inhibitory activity was examined using the modified method described by Teramachi *et al*. (2005). Briefly, the test compounds, enzyme solution (final concentrations of collagenase: 10 μg/ml) and 50 mM Tricine buffer (pH 7.5) were added to 96-well microtitre plate, and preincubated for 10 min at 37 ºC. Afterwards, the substrate solution ((7-

methoxycoumarin-4-yl) acetyl–L-prolyl-L-leucylglycyl-L-leucyl-[N^{β}-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide) at final concentration of 10 μM was added to initiate the reaction. The fluorescence values were measured at an excitation of 320 nm and an emission of 405 nm after 0 min and 30 min incubation at 37 ºC using a fluorescence plate reader (EnSpire, PerkinElmer Japan). These assays were performed in triplicate using caffeic acid as a positive control and the concentrations required for 50% inhibition (IC_{50}) of the intensity of fluorescent were determined graphically.[48)]

1.5.9. Determination of AGEs formation in vitro

The reaction mixture, 10 mg/ml of bovine serum albumin in 50 mM phosphate buffer (pH 7.4) containing 0.02 % sodium azide, was added to a 0.5 M ribose solution. The reaction mixture was then mixed with test compounds. After incubation at 37 for 24 h, the fluorescent reaction products were assayed with a spectrofluorometric detector (EnSpire, PerkinElmer Japan; Ex: 370 nm, Em; 440 nm). Measurements were performed in triplicate and the concentrations required for 50% inhibition (IC_{50}) of the intensity of fluorescent were determined graphically.[44)]

CHAPTER 2

SPILANTHES ACMELLA

2.1. Introduction

Spilanthes acmella Murr. (Compositae) is a genus comprising of over 60 species that are widely distributed in tropical and subtropical regions of the world, such as Africa, America, Borneo, India, Sri Lanka and Asia. [49,50] It is an annual or short-lived herb that is 40-60 cm tall, grown in damp area[51] and has low rate of germination or poor vegetative propagation.[50] The flowers arranged in head inflorescence, yellow flower head and have pungent taste accompanied by tingling and numbness on the tongue.[51]

Figure 23. *Spilanthes acmella*
(*http://* aoki2.si.gunma-u.ac.jp)

The plant species has been used commonly as a folk remedy, e.g. for toothache and skin diesease,[52] rheumatism and fever,[51] dysentery, a snake bite remedy, stammering in children,[50] antiseptic, antibacterial, antifungal, antimalarial, flu, cough, rabies diseases, and tuberculosis.[53,54]

This plant is very popular among the ancient tribal community. Special food item is prepared it in religious festival. The poor people offered it along with the ―Ajeng Dues" in Dobur Uie.[55] The flowers are crushed and applied at the site of toothache, particularly in ―Irula tribe of Hasanur hills in Erode district of Tamilnadu" where it is known by the local name ―Mandal Poo Chedi".[56] Apart from Tamil Nadu, root paste of the plant is used in throat problems in Chindwara and Betul district of Madhya Pradesh.[57] It is also known to be used as panacea (Sumatra, Indonesia), stimulant of toothache (Sudan), stomatitis (Java, Indonesia), and wound healing (India).[58] In Cameroon, it is used as a snakebite remedy and articular rheumatism.[59] It is supposed to be useful in cases of tuberculosis.[54] Leaves and flowers are also used to treat leucorrhoea in females among people of tribes in Bangladesh.[58] The whole plant is also used as poisonous sting in Chittagong hill tracts of Bangladesh where it is also known as Jhummosak.[60]

The main constituents from the whole aerial parts, flower heads and roots of this plant yield –spilanthol” and –acmellonate”, they are sometimes used to reduce the pain associated with toothaches, induce saliva secretion[52,53,61] and is a powerful insecticide and local anaesthetic.[50,62,63] In addition, it also has an important source of highly valuable bioactive compounds such as phenolics, coumarin (scopoletin) and triterpenoids.[64] It uses as a spice, antiseptic, anti-bacterial, antifungal, antimalarial, flu, cough and tuberculosis.[65]

The antipyretic activity of this plant is attributed to the presence of flavonoids which are predominant inhibitors of either cyclo-oxygenase or lipo-oxygenase.[66] The aqueous extract of this plant also showed the analgesic activity by using acetic acid induced abdominal constriction and tail flick method. The presence of flavonoids which are potent inhibitors of prostaglandins at later stages of acute inflammation.

Ethanolic extracts of the flowers of *Spilanthes acmella* are demonstrated to inhibit pancreatic lipase activity (40% at 2 mg/mL concentration in vitro).[67] Significantly, the ethyl acetate extract exhibited immediate vasorelaxation in nanogram levels and is the most potent antioxidant in the diphenylpicryl hydrazine assay. The chloroform extract displays the highest vasorelaxation with the highest antioxidant concentration. Hexane and chloroform extracts were found to suppress nitric oxide production in stimulated macrophages at 80 μg/mL by 72% and 85%, respectively. Spilanthol demonstrated dose-dependent prevention of macrophage activation with 60% and 20% production of nitric oxide at 90 and 360 μM, respectively.[68]

Ethanol extract of Spilanthol from the flower heads of *Spilanthes acmella* was found to be active against *P. Xylostella*[69] and shown a potent ovicidal, insecticidal, and pupacidal activity at dose of 7.5 ppm with 100% of Anopheles, Culex, and Aedes mosquito.[70] The hexane extract of dried flower buds of *Spilanthes acmella* (spilanthol, undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide and undeca-2*E*-en-8,10-diynoic acid isobutylamide) was found active against *Aedes aegypti* larvae. Ethanolic extracts of the whole plants of *Spilanthes acmella* were screened against early 4^{th} instar larvae of Culex quinquefasciatus, spilanthol was shown to be toxic against adults of *P. americana*.[71]

In the previous study, the *n*-hexane, ethyl acetate, butanol and water layer from *Spilanthes acmella* were evaluated the stimulative activity on alkaline phosphatase (ALP) of

MC3T3-E1 osteoblast cells. Among the tested, the butanol and water layers showed the stimulatory activity to 126% and 127% respectively.[72)]

Further investigation of the butanol layers of the same plant has demonstrated the present of a new methyl threonolactone glycoside (**16**), a new methyl threonolactone fructofuranoside (**17**), two new pyroglutamates (**18** – **19**), 2-*C*-methyl-D-threono-1,4 lactone (**20**), 2-deoxy-D-ribono-1,4 lactone (**21**), methyl pyroglutamate (**22**), dendranthemoside A (**23**), dendranthemoside B (**24**), ampelopsisionoside (**25**), icariside B2 (**26**), benzyl-*α*-L-arabinopyranosyl (1→6)-*β*-D-glucopyranoside (**27**), chicoriin (**28**) and uridine (**29**) (Fig. 25). They were isolated by various chromatographic techniques such as silica gel, ODS, HPLC and determined as follows by spectrometric analysis (UV, IR, HR-ESI-MS, 1D and 2D NMR). We report here the isolation and structural elucidation of four new compounds. Two of them were found to be glycosylated with the different disaccharide, consisting D-glucose and L-fructose of **16** and **17**, respectively.

2.2. Extraction and Isolation of Chemical Constituents

The air-dried plants (2.0 kg) were extracted with methanol (10 L × 3). The methanol solution was concentrated and adjusted to 95% aqueous methanol by the addition of water and then partitioned with *n*-hexane (1.0 L × 3, 23.5 g). The remaining aqueous methanol layer was evaporated and re-suspended in 0.5 L of water and then partitioned with ethyl acetate (1.0 L × 3, 40.4 g) and butanol (1.0 L × 3, 47.5 g), successively.

The butanol layer (40.0 g) was proceeded on silica gel (300 g) CC with increasing amounts of methanol in chloroform [*n*-hexane – chloroform (1:1), 4l, chloroform – methanol (50:1, 40:1, 30:1, 20:1, 15:1, 10:1, 7:1, 5:1, 3:1, 2:1, 2l), 500 ml fractions being collected], yielding 19 fractions (Fr. Sab1 – Sab12). The fraction Sab5 (710 mg) was subjected by open reversed phase (ODS) column chromatography in 10% aq. methanol (400 ml) – 100% methanol (400 ml), linear gradient, lead 10 fractions (Fr. Sab5-1 – Sab5-10). The residue of fraction Sab5-1 (483 mg) was purified by HPLC, 100% aq. (YMC column) to give **18** (7.80 mg), **19** (4.21 mg) and **22** (methyl pyroglutamate, 6.63 mg), respectively. We also found **19** (5.80 mg) from the residue of fraction Sab4-2 (193 mg).

The mixture of fraction Sab6, Sab7, Sab8 and Sab9 (2.06 g) were subjected by open reversed phase (ODS) column chromatography in 10% aq. methanol (400 ml) – 100% methanol (400 ml), linear gradient, lead 10 fractions (Fr. Sab6-9-1 – Sab6-9-10). The residue of fraction Sab6-9-1 (340 mg) was purified by HPLC, 100% aq. (YMC column) to give **21** (2–deoxy–D–ribono–1,4 lactone, 6.01 mg). The other fraction Sab6-9-4 (114 mg) was purified by HPLC, 40% aq. methanol to give **25** (ampelosisinoside, 5.43 mg).

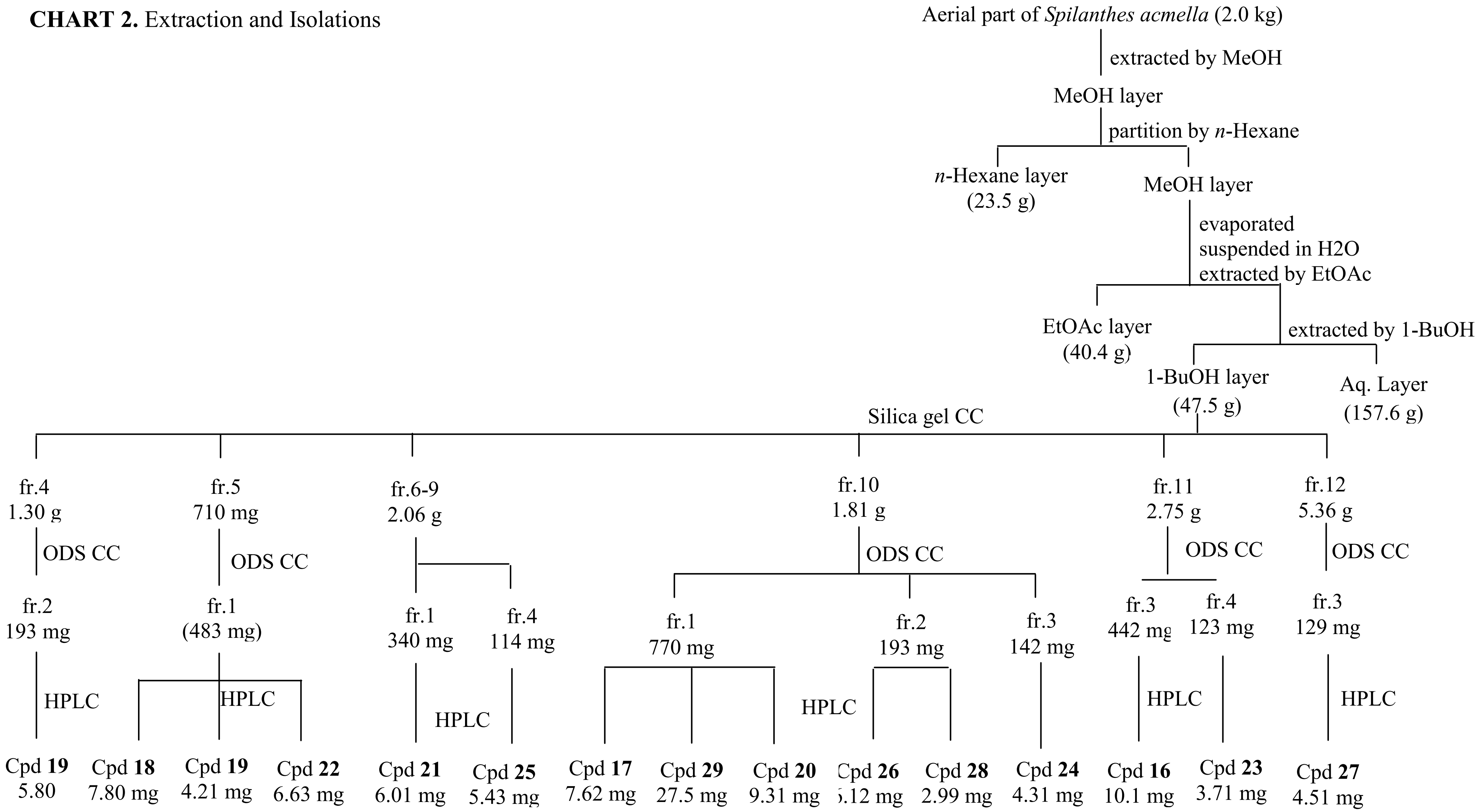
The fraction Sab10 (1.81 g) was subjected by open reversed phase (ODS) column chromatography in 10% aq. methanol (400 ml) – 100% methanol (400 ml), linear gradient, lead 7 fractions (Fr. Sab10-1 – Sab10-7). The residue of fraction Sab10-1 (770 mg) also was purified by HPLC, 100% aq (YMC column). Three peaks which appeared at 5, 18 and 35 minute were collected to give **17** (7.62 mg), **20** (2-*C*-methyl-D-threono-1,4-lactone, 9.31 mg), and **29** (uridine, 27.5 mg). The other residue of fraction Sab10-2 (193 mg) was purified by HPLC, 20% aq. methanol to give **26** (icariside B2, 6.12 mg) and **28** (cichoriin, 2.99 mg). The residue of fraction Sab10-3 (142 mg) was purified by HPLC, 35% aq. methanol to give **24** (dendranthemoside B, 4.31 mg).

The fraction Sab11 (2.75 g) was subjected by open reversed phase (ODS) column chromatography in 10% aq. methanol (400 ml) – 100% methanol (400 ml), linear gradient, lead 10 fractions (Fr. Sab11-1 – Sab11-10). The residue of fraction Sab11-3 (442 mg) and

Sab11-4 (123 mg) were purified by 35% aq. methanol of HPLC to get **16** (10.1 mg) and **23** (dendranthemoside A, 3.71 mg), respectively.

The fraction Sab12 (5.36 g) was subjected by open reversed phase (ODS) column chromatography in 10% aq. methanol (400 ml) – 100% methanol (400 ml), linear gradient, lead 10 fractions (Fr. Sab12-1 – Sab12-10). The residue of fraction Sab12-3 (129 mg) was purified by HPLC, 35% aq. methanol to get **27** (benzyl-α-L-arabinopyranosyl (1→6)-β-D-glucopyranoside, 4.51 mg).

CHART 2. Extraction and Isolations



2.3. Structural Elucidations of Chemical Constituents

The 1-BuOH layers from methanol extract of aerial part of *Spilanthes acmella* was fractionated by silica gel and ODS column chromatography, then futher purified by HPLC to afford fourteen compounds (**16** – **29**) (Fig. 25).

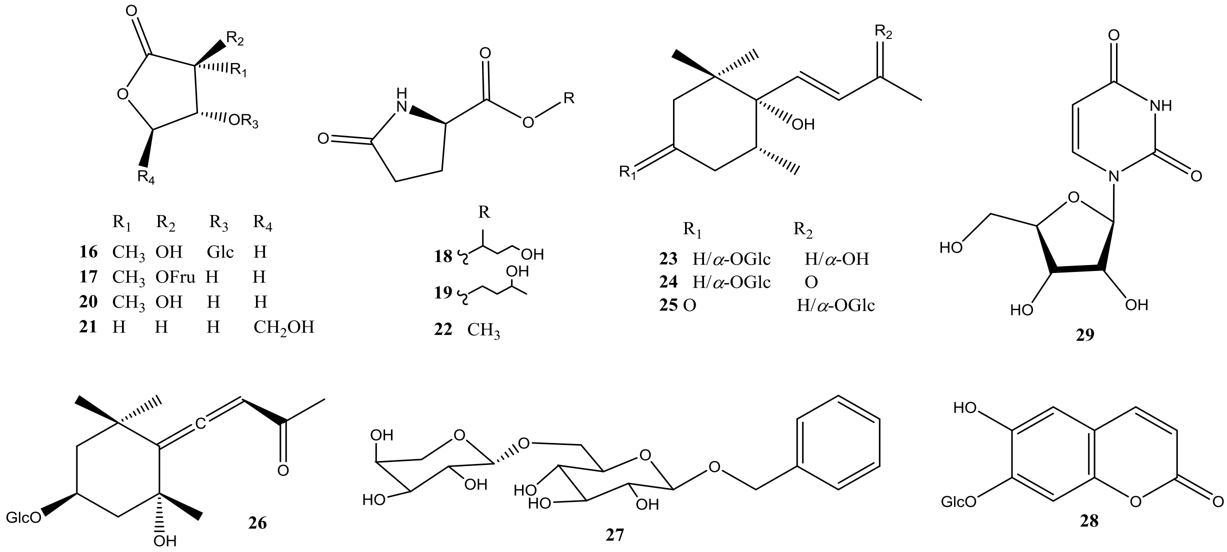


Figure 25. Isolated compounds of *Spilanthes acmella*

New compounds **16**, 2-*C*-methyl-D-threono-1,4-lactone-*β*-D-glucopyranoside, was obtained as colorless powder with molecular formula of $C_{11}H_{18}O_9$ as determined by HR-ESI-MS at *m/z* 317.0845 $[M]^+$(calcd. for $C_{11}H_{18}O_9Na$: 317.0843). The presence of a saturated *γ*-lactone moiety was observed in the IR spectrum by the carbonyl group signal at 1777 cm^{-1} and a broad carbonyl stretching band in the region 1100–1600 cm^{-1} susgested as a glycosidic nature.

Table 6

^{13}C NMR spectroscopic data of **16** – **19**

Position	**16**	**17**	**18**	**19**
2	179.4	180.2	181.2	181.3
3	75.7	76.0	30.5	30.6
4	83.4	57.7	26.0	26.3
5	69.6	73.0	57.1	57.4
6	19.2	17.8	174.1	176.2
7			66.0	60.5
8			38.8	42.6
9			65.5	66.3
10			23.9	23.9
1′	104.0	60.7		
2′	74.9	109.4		
3′	78.2	82.7		
4′	72.2	79.1		
5′	78.1	84.8		
6′	63.2	63.0		

Recorded at 600 MHz in CD_3OD. Chemical shifts (*δ*) are expressed in ppm

The ^{13}C NMR spectrum (Table 6) of **16** showed 11 carbon resonances that classified by analysis of its chemical shift values and its HSQC spectrum as a methyl carbon (δ_C 19.2), two oxygenated methylene carbons (δ_C 63.2, 69.6), five oxygenated methine carbons (δ_C 72.2, 74.9, 78.1, 78.2, 83.4), a quartenary carbon (δ_C 75.7), an anomeric carbon (δ_C 104.0) and a carbonyl carbon at δ_C 179.4.

The ^{1}H NMR spectrum (Table 7) displayed signals due to a methyl proton at δ_H 1.41 (s), five oxygenated methine protons at δ_H 3.24 (dd, J = 9, 7 Hz), 3.26 (dd, J = 10, 9 Hz), 3.34 (ddd, J = 10, 7, 3 Hz), 3.36 (t-like, J = 9 Hz) and 4.43 (t-like, J = 6 Hz), four oxygenated methylene protons at δ_H 3.60 (dd, J = 12, 7 Hz), 3.91 (dd, J = 12, 3 Hz), 4.10 (dd, J = 9, 6 Hz) and 4.52 (dd, J = 9, 6 Hz), and an anomeric proton at δ_H 4.37 (d, J = 7 Hz). The lactone moiety was clearly evidenced by the chemical shifts and coupling constants of an AMX system corresponding to H-5α (4.52, dd, J = 9, 6 Hz), H-5β (4.10, dd, J = 9, 6 Hz) and H-4 (4.43, t-like, J = 6 Hz).

Table 7

^{1}H NMR spectroscopic data of **16 – 19**

Position	**16**	**17**	**18**	**19**
2	-	-	-	-
3	-	-	2.24 – 2.30 m	2.24 – 2.30 m
4	4.43 t-like (6)	4.18 dd (5, 4)	2.40 m	2.40 m
			2.09 m	2.09 m
5α	4.52 dd (9, 6)	4.49 dd (9, 5)	4.22 t (5)	4.16 t (6)
β	4.10 dd (9, 6)	3.97 dd (9, 4)		
6	1.41 s	1.35 s	-	-
7			4.19 dd (6, 3)	3.57 m
8			1.67 – 1.73 m	1.54 – 1.61 m
9			3.79 m	3.82 m
10			1.13 d (6)	1.10 d (6)
1′	4.37 d (7)	3.71 d (12)		
		3.63 d (12)		
2′	3.24 dd (9, 7)	-		
3′	3.36 t-like (9)	4.04 d (4)		
4′	3.26 dd (10, 9)	3.90 dd (6, 4)		
5′	3.34 ddd (10, 7, 3)	3.85 ddd (6, 4, 3)		
6′	3.91 dd (12, 3)	3.78 dd (12, 3)		
	3.60 dd (12, 7)	3.64 dd (12, 6)		

Recorded at 125 MHz in CD_3OD. Chemical shifts (δ) are expressed in ppm and J values are presented in Hz in parenthesis

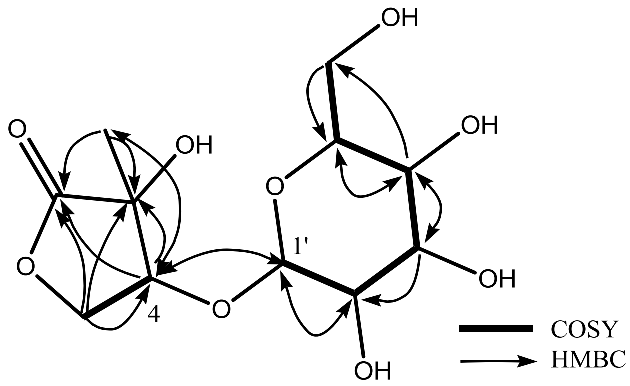


Figure 26. HMBC and COSY correlations of **16**

The NMR spectroscopic data of **16** closely resembled that of 2-*C*-methyl-D-threono-1,4 lactone (**20**),[73)] except for some differences in the chemical shift values at C-4. The deshielded proton at δ_H 4.43 and carbon at δ_C 83.4 of **16** suggested that glucoside group was attached to C-4. This was confirmed in the HMBC experiment by the correlation between H-4 (δ_H 4.43) and carbon signal at δ_C 104.0 (Fig. 26). While five signals were corresponded to C1-C5 of *β*-D-glucopyranose, the anomeric carbon signal, resonating at δ_C 104.0, was indicative of the participation of this carbon in an ester linkage. The highly deshielded chemical shift of an anomeric proton signal (δ_H 4.37) was in accordance with this assumptiom and its coupling constant (J = 7 Hz) recommended the *β* mode of linkage. Its relative configuration of **16** was established by NOESY analysis. The correlation of H-5/Me-6 suggested as *α* orientation. Another correlations were observed between H-5/H-4 which suggested as *β*-oriented (Fig. 27).

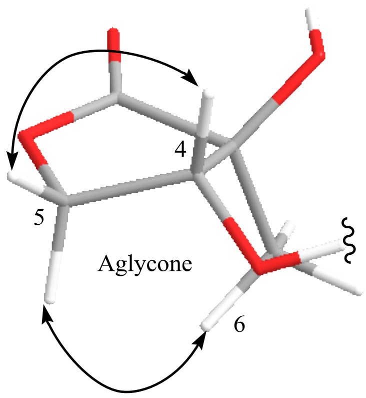


Figure 27. Key NOESY correlations of **16**

Mild acid hydrolysis of **16** with 1N HCl showed initial removal of a 2-*C*-methyl-D-threono-1,4 lactone (**20**) and D-glucose unit (Fig. 28). Based on the above NMR data and chemical reactions, the structure of **16** was determined as 2-*C*-methyl-D-threono-1,4-lactone-*β*-D-glucopyranoside.

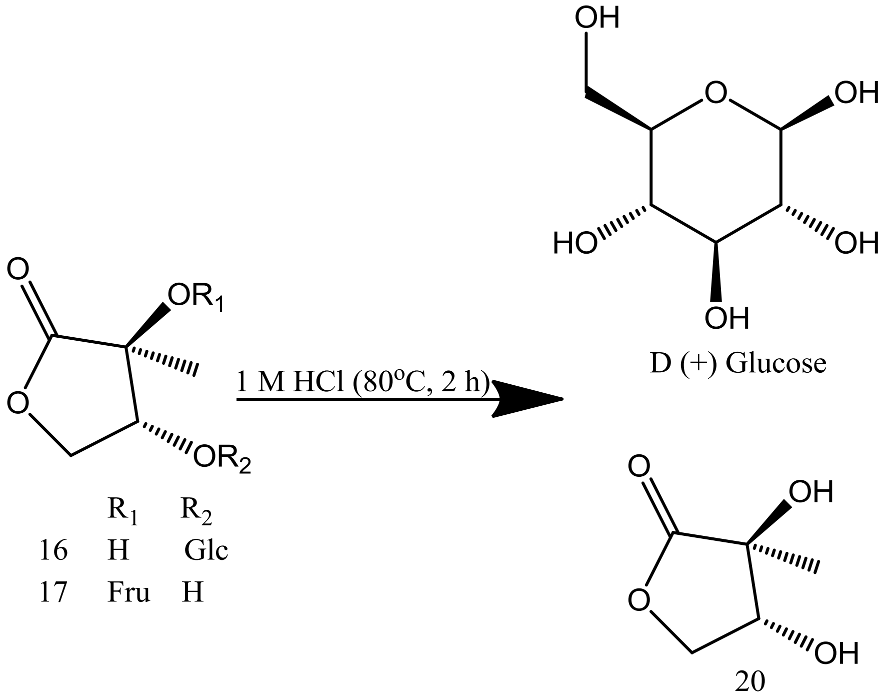


Figure 28. Acid hydrolysis reaction of **16**

The 2-*C*-methyl-D-threono-1,4-lactone-*α*-D-fructofuranoside (**17**) was also lactone moeity. It was obtained as pale yellow solid, whose molecular formula was determined to be $C_{11}H_{18}O_9$ from its positive-ion mode HR-ESI-MS data at *m/z* 317.0844 $[M+Na]^+$ (calcd. 317.0843). The ^1H and ^{13}C NMR spectrum (Table 6 and 7) of **17** were very similar to those of 2-*C*-methyl-D-threono-1,4 lactone (**20**). The ^{13}C NMR were also showed two secondary carbons at δ_C 60.7 and 63.0, three tertiary carbons at δ_C 79.1, 82.7 and 84.8 and a quartenary carbon at δ_C 109.4, they indicated as fructofuranose moiety. The diastereomer of 2-*C* methyl group was distiquished by ^{13}C NMR data of Me-6, therefore its chemical shift is at δ_C 19, it indicated as *cis*-erythrono but its is δ_C 17 that indicated as *trans*-threono.[74)]

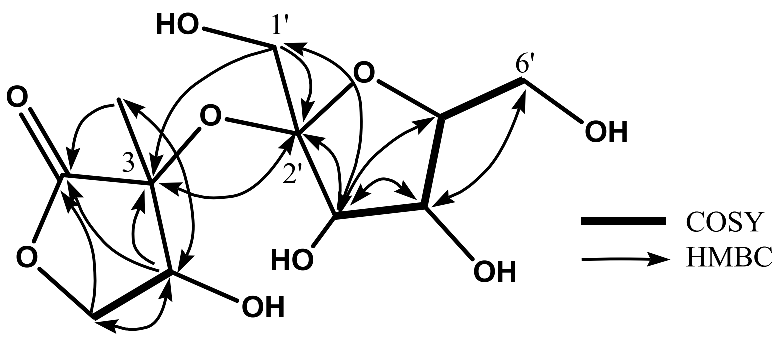


Figure 29. HMBC and COSY correlations of **17**

The position of fructofuranose moeity were deduced to be at C-3 by analysis of the HMBC data showing correlations of H-1′ and H-2′ to carbons at δ_C 76.0 (Fig. 29). Its relative configuration of **17** was established by NOESY analysis. The correlation of H-5/Me-6 suggested as *α* orientation. Another correlations were observed between H-5/H-4 which suggested as *β*-oriented (Fig. 30). Zheng et al. (2009) described that *α* and *β* orientation of D-fructofuranose were distiguished by *J* value of position 3 and chemical shift of C–2 of fructofuranose moeity. The *J* = 3 – 4 Hz and C–2 > 2 – 4 ppm, 107-109 is *α* orientation, while the *J* = 7 – 9 Hz and C–2 < 2 – 4 ppm, 103 – 106 is *β* orientation.[75] Based on that reference, **17** was *α*-D-fructofuranose. The acid hydrolysis of **17** yielded the aglycone (2-*C*-methyl-D-threono-1,4-lactone-) and D-fructofuranose (Fig. 28), thus the structure of **17** was determined as 2-*C*-methyl-D-threono-1,4-lactone-*α*-D-fructofuranoside.

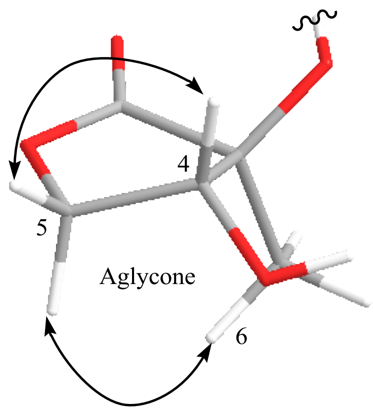


Figure 30. Key NOESY correlations of **17**

The 2-butanol pyroglutamate (**18**) was also colorless solid and displayed an $[M+Na]^+$ ion peak at *m/z* 224.0890 (calcd. 224.0893) corresponding to a molecular formula of $C_9H_{15}O_4$. The IR spectrum showed strong absorbantion band for hydroxy (3331 cm^{-1}) and carbonyl (1735 cm^{-1}). The ^1H and ^{13}C NMR spectra of **18** showed signals assignable to two methylenes [δ_H 2.24 – 2.30 (2H, m); δ_C 30.5 (C-3); δ_H 2.09 and 2.40 (each 1H, m); δ_C 26.0 (C-4)], a methine [δ_H 4.22 (1H, t, *J* = 5 Hz); δ_C 57.1 (C-5)], and a carbonyl [δ_C 181.2 (C-6)] (Table 6 and 7). The chemical shift values and coupling patterns of these signals suggested that there was a methyl pyroglutamate moeity in **18**.[76] In addition, the ^1H and ^{13}C NMR spectra also revealed a 2-butanol framework, with a methyls [δ_H/δ_C 1.13 (d, *J* = 6 Hz)/23.9 (Me-10)], a methylene [δ_H/δ_C 1.67 – 1.73 (m)/38.8 (C-8)], an olefinic methine [δ_H 3.79 (m); δ_C 65.5 (C-9)], and an olefinic methylene [δ_H 4.19 (dd, *J* = 6, 3 Hz); δ_C 66.0 (C-7)]. This was confirmed by 2D NMR experiments of **18**.

The ^1H-^1H COSY spectrum displayed correlation between H-3, H-4 and H-5, and also H-7 and H-8, which in turn correlated with H-9, while the HMBC spectrum demonstrated correlations of C-2 with H-4 and H-3, and C-6 with H5 and H-4. Furthermore, in the HMBC spectrum strong correlation from H-7 to C-6 established that the 2-butanol moiety was located at the C-6 (Fig. 31). Therefore, the structure of **18** was deduced as 2-butanol pyroglutamate.

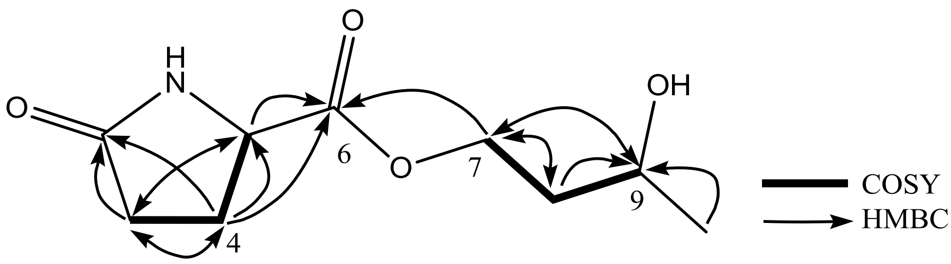


Figure 31. HMBC and COSY correlations of **18**

The 1-butanol pyroglutamate (**19**) was also colorless solid. The NMR spectra together with molecular ion at *m/z* 224.0893 $[M+Na]^+$ (calcd. for $C_9H_{15}O_4NNa$: 224.0893) in HR-ESI-MS indicated that **19** was closely related to **18** except for the position of hydroxyl group of butanol. The shielded of H-7 [δ_H 3.57 (1H, m)] and C-7 (δ_C 60.5) and deshielded of H-9 [δ_H 3.82 (1H, m)] and C-8 (δ_C 66.3) suggested that the hydroxyl position of butanol was changed from C-9 to C-7 in **19**. That was further supported by a correlation between the proton at δ_H 1.10 (H-10) to carbon signal at δ_C 60.5 (C-7) and 42.6 (C-8) (Fig. 32) in the HMBC spectrum. Accordingly the structure of **20** was determined as 1-butanol pyroglutamate.

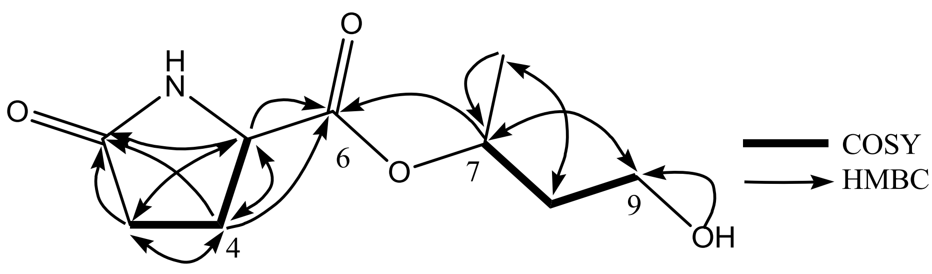


Figure 32. HMBC and COSY correlations of **19**

2.4. Bioassay of chemical constituents

Osteoporosis, a disease caused by reduction in skeleton mass, occurs due to a decrease in bone formation by osteoblasts and an increase in bone resorption by osteoclasts. Treatment methods for osteoporosis include inhibition of osteoclast activities or stimulation of the osteoblastic lineage proliferation and induction of osteoblast differentiation.

Osteoblasts are specialized fibroblasts that secrete and mineralize the bone matrix. They develope from mesenchymal precusors. The mineralized extracellular matrix is mainly composed of type I collagen and smaller but significant amounts of osteocalcin (OC), matrix gla protein, osteopontin (OPN), bone sialoprotein (BSP), BMPs, TGF-β, and the inorganic mineral hydroxylapatite.[77)]

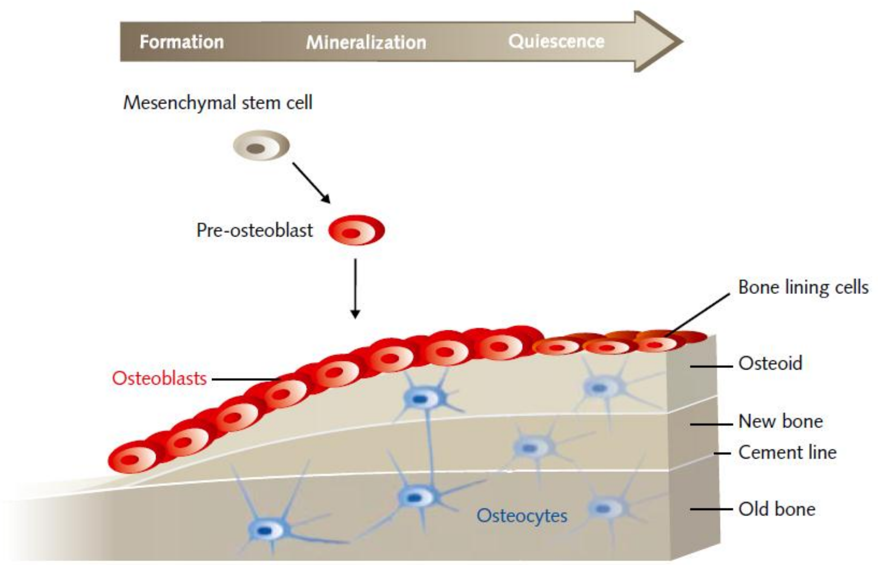


Figure 33. Bone remodeling

Osteoblast differentiation *in vitro* and *in vivo* can be characterized in three stages: (a) cell proliferation. (b) matrix maturation, and (c) matrix mineralization. *In vitro*, matrix maturation and mineralization are usually enhanced by growing the cells to complete confluency and by adding specific osteogenic factor.[78)] During proliferation, several extracellular matrix protein (procollagen I, TGF-β, and fibronectin) can be detected. The matrix maturation phase (b) is characterized by maximal expression of alkaline phosphatase (ALP). Finally, at the beginning of matrix mineralization, genes for proteins such as OC, BSP, and OPN are expressed and once mineralization is completed, calcium deposition can be visualized using adequate staining methods.

Analysis of bone cell-specific markers like AP, OC and collagen type I or detection of functional mineralization is frequently used to characterize osteoblast *in vitro*.[78)] The mineralization process of osteoblast in *in vitro* culture has also been used as a model for testing the effects of drug treatments and mechanical loading on bone cell differentiation and bone formation.[79)]

Recently, many osteoporotic patients have already lost a substantial amount of bone at the time of diagnosis, bone mass must be increased by stimulating the osteoblastic lineage proliferation and inducing the differentiation of osteoblasts. However, as a commercially available drugs used to treat osteoporosis are mostly osteoclast inhibitors that contain drugs such as estrogen, estrogen receptor derivatives, calcitonin, and bisphosphonates, their effects on increasing or recovering bone mass are relatively small.[80)] As potential complications such as breast cancer, uterine bleeding, and cardiovascular disease have also been reported in the use of these drugs, there is major interest in finding new agents that can enhance osteoblast differentiation activity and increase skeletal bone formation. In our study, we screened several Indonesian medicinal plants to find active compounds that have capability to differentiate MC3T3-E1 osteoblastic cells.

MC3T3-E1 cells, an osteoblast-like cell line, have been reported to retain their capacity to differentiate into osteoblasts, and may provide very useful information about the effect of phytochemicals on the differentiation of osteoblasts. During differentiation, osteoblasts exhibits various characteristic in time dependent-manner: increase in alkaline phosphatase (ALP) activity followed by extracellular matrix (ECM synthesis) and result in mineralization. Therefore, ALP activity and mineralization are major osteoblast differentiation markers. And *in vitro* studies, when a phytochemical shown increase the growth of MC3T3-E1 cell and also significantly increase ALP activity and mineralization that would be concluded it stimulates proliferation and differentiation of osteoblast MC3T3-E1 cell and hence increase bone formation.

2.4.1. Alkaline Phosphatase Stimulation Activity of Chemical Constituents

Osteoblasts are the most important cells in bone tissues and are critical for bone formation through proliferation and differentiation. During osteoblast differentiation, bone morphogenetic protein (BMP) induces the expression of osteoblastic markers such as alkaline phosphatase (ALP). Proliferating osteoblasts show alkaline phosphatase (ALP) activity, which is greatly enhanced during *in vitro* bone formation. ALP is a membrane bound enzyme that is often used as marker for osteogenic differentiation.

To evaluate the effects of **16 – 29** on osteoblast function, ALP activity, which is related to the osteosid and initiates the deposition of minerals, was determined. In this study, it was found that **19**, **21**, **25** and **27** stimulated the ALP activity that markedly increased osteoblasts growth and differentiation in osteoblastic MC3T3-E1 cells. At concentrations of 25 μM of **25** and **27** stimulated the ALP activity up to 112 %, compared to that of the control and stronger than the positive control, 17*β*-estradiol (Fig. 34).

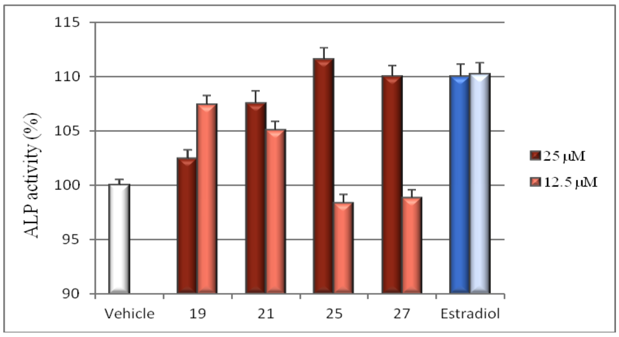


Figure 34. ALP activity of **16–29** toward MC3T3-E1 cell lines

2.4.2 Mineralization Stimulation Activity of Chemical constituents

Osteoblasts can be induced to produce vast extracellular calcium deposits *in vitro*. This process is called mineralization. Calcium deposits are an indication of successful *in vitro* bone formation and can specifically be stained bright orange-red using Alizarin Red S. The effect of **16 – 29** were then examined by measuring the calcium deposition by Alizarin Red Staining. As was found for the ALP activity study, **19**, **21**, **25** and **27** showed stimulatory effects on mineralization. Compound **25** and **27** stimulated the mineralization to 112 %, compared to that of the control at a concentration of 25 μM (Fig. 35).

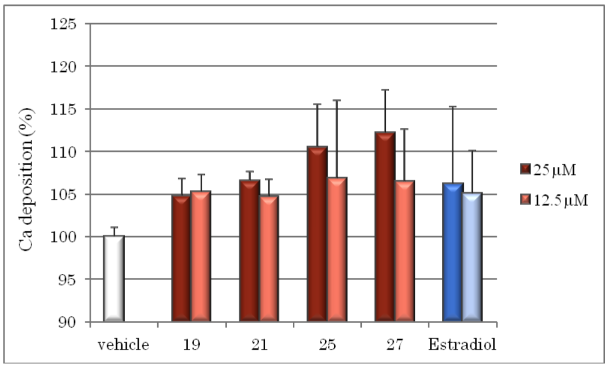


Figure 35. Calcium deposition of **16–29** toward MC3T3-E1 cell lines

In bone formation, osteoblasts are key cell in bone matrix formation and calcification. Osteogenesis starts with osteoblast producing and secreting type I collagen, which makes about 90% of the organic bone matrix or the osteoid. Osteoblast also become high in alkaline phosphatase, a phosphate-splitting enzyme. Alkaline phosphatase is released into the osteoid to initiate the deposit of minerals. After mineralization, the complete bone becomes hard and rigid with necessary mechanical properties to withstand the external forces to support the body and protect the internal organs. Our study demonstrates that **19**, **21**, **25** and **27** stimulated ALP activity and calcium deposition in osteoblastic MC3T3-E1 cell *in vitro*. These studies suggest that compound **19**, **21**, **25** and **27** may be able to stimulate osteoblastic bone formation and play an important role in bone remodeling.

2.5. Experimental

2.5.1 General Experimental Procedures

^{1}H and ^{13}C-NMR spectra were taken on a Bruker Ultrashield 600 spectrometer at 600 MHz and 150 MHz, respectively, with TMS as an internal standard. IR and UV spectra were measured on a HORIBA FT-720 and JASCO V-520 UV/Vis spectrophotometer, respectively. Optical rotations and CD spectra were measured on a JASCO P-1030 digital polarimeter and a JASCO J-720 spectropolarimeter, respectively. Positive ion HR-ESI-MS was performed with an Applied Biosystems QSTAR XL NanoSprayTM System. Silica gel open column chromatography (CC) and reversed phase [octadecyl silylated silica gel (ODS)] CC were performed on silica gel 60 (E. Merck, Darmstadt, Germany), and Cosmosil 75C18-OPN (Nacalai Tesque, Kyoto, Japan; Φ = 35 mm, L = 350 mm), respectively. HPLC was performed on an ODS column (Inertsil ODS-3, GL Science, Tokyo, Japan; Φ = 6 mm, L = 250 mm) o r YMC column (Triart C18, YMC co., Ltd., 250 x 4.6 mml. D. S-5 μm. 12 nm)and the eluate was monitored with a JASCO RI-930 intelligent detector and a JASCO PU-1580 intelligent pump.

2.5.2 Plant Material

Whole plants of *Spilanthes acmella* were collected in late June 2007 in Kebun Raya Purwodadi, Malang, Indonesia, and a voucher specimen was deposited at the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University.

2.5.3 Isolated Compounds

*2-C-methyl-D-threono-1,4-lactone-β-D-glucopyranoside (**16**)*

Colorless solid; $[\alpha]_D^{26.7}$ -28.6 (c = 0.78, MeOH); IR (film) ν_{max} cm^{-1}: 3392, 2924, 1777, 1713, 1650, 1557, 1456, 1391, 1210, 1078; ^{1}H NMR and ^{13}C NMR, see Tables 6 and 7; positive HR-ESI-MS *m/z* 317.0845 $[M]^+$(calcd. for $C_{11}H_{18}O_9Na$: 317.0843)

*2-C-methyl-D-threono-1,4-lactone-α-D-fructofuranoside (**17**)*

Pale yellow solid; $[\alpha]_D^{26.9}$ -10.4 (c = 0.74, MeOH); IR (film) ν_{max} cm^{-1}: 3386, 2938, 1774, 1732, 1651, 1540, 1456, 1339, 1206, 1073; ^{1}H NMR and ^{13}C NMR, see Tables 6 and 7; positive HR-ESI-MS *m/z* 317.0844 $[M+Na]^+$(calcd. for $C_{11}H_{18}O_9Na$: 317.0843).

*2-butanol pyroglutamate (**18**)*

Colorless solid; $[\alpha]_D^{27.7}$ -0.86 (c = 0.42, MeOH); IR (film) ν_{max} cm^{-1}: 3331, 2926, 1735, 1684, 1557, 1457, 1338, 1207, 1052; ^1H NMR and ^{13}C NMR, see Tables 6 and 7; positive HR-ESI-MS m/z 224.0890 $[M]^+$(calcd. for $C_9H_{15}O_4NNa$: 224.0893).

*1-butanol pyroglutamate (**19**)*

Colorless solid; $[\alpha]_D^{27.1}$ +1.40 (c = 0.41, MeOH); IR (film) ν_{max} cm^{-1}: 3314, 2931, 1735, 1683, 1557, 1457, 1338, 1229, 1054; ^1H NMR and ^{13}C NMR, see Tables 6 and 7; positive HR-ESI-MS m/z 224.0893 $[M]^+$(calcd. for $C_9H_{15}O_4NNa$: 224.0893).

*2-C-methyl-D-threono-1,4-lactone (**20**)*[73)]

Colorless solid; $[\alpha]_D^{27.1}$ +1.40 (c = 0.41, MeOH); IR (film) ν_{max} cm^{-1}: 3314, 2931, 1735, 1683, 1557, 1457, 1338, 1229, 1054; ^1H NMR (methanol-d_4, δ): 1.34 (3H, s, H-6), 3.96 (1H, dd, J = 9, 4 Hz, H-5α), 4.17 (1H, dd, J = 5, 4 Hz, H-4), 4.48 (1H, dd, J = 9, 5 Hz, H-5β) and ^{13}C NMR (methanol-d_4, δ): 17.7 (CH_3-6), 73.0 (CH_2-5), 75.7 (C-3), 76.0 (C-4), 180.2 (C-2); positive HR-ESI-MS m/z 155.0310 $[M]^+$(calcd. for $C_5H_8O_4Na$: 155.0315).

*2–deoxy–D–ribono–1,4 lactone (**21**)*[81)]

Colorless solid; ^1H NMR (pyridine-d_5, δ): 2.86 (1H, dd, J = 18, 3 Hz, H-3α), 3.32 (1H, dd, J = 18, 6 Hz H-3β), 4.03 (1H, dd, J = 12, 3 Hz, H-6α), 4.13 (1H, dd, J = 12, 3 Hz, H-6β), 4.91 (1H, dd, J = 6, 3 Hz, H5), 5.00 (1H, ddd, J = 7, 4, 2 Hz, H-4) and ^{13}C NMR (pyridine-d_5, δ): 39.9 (CH_2-3), 62.5 (CH_2-6), 69.7 (CH-4), 90.2 (CH-5), 177.5 (C-2).

*Methyl pyroglutamate (**22**)*[76)]

Colorless solid; $[\alpha]_D^{27.4}$ -3.48 (c = 0.66, MeOH); IR (film) ν_{max} cm^{-1}: 3343, 2958, 2930, 1738, 1697, 1557, 1456, 1338, 1221, 1043, 670; ^1H NMR (methanol-d_4, δ): 2.08 (1H, m, H-4α), 2.25 (2H, m, H_2-3), 2.40 (1H, m, H-4β), 3.69 (3H, s, H_3-7), 4.23 (1H, dd, J = 12, 6 Hz, H-5) and ^{13}C NMR (methanol-d_4, δ): 26.0 (CH_2-4), 30.4 (CH_2-3), 53.0 (CH_3-7), 57.2 (CH-5), 174.6 (C-6), 181.2 (C-2); positive HR-ESI-MS (positive) m/z : 166.0474 $[M]^+$(calcd. for $C_6H_9O_3NNa$: 166.0475).

*Dendranthemoside A (**23**)*[82)]

Yellow powder; ^1H NMR (methanol-d_4, δ): 0.84 (3H, d, J = 7 Hz, H_3-13), 0.88 (3H, s, H_3-12), 0.96 (3H, s, H_3-11), 1.24 (3H, d, J = 7 Hz, H_3-10), 1.49 (1H, dd, J = 12, 7 Hz, H-4β), 1.56 (1H, ddd, J = 12, 5, 2 Hz, H-2β), 1.67 (1H, dd, J = 12, 7 Hz, H-2α), 1.82 (1H, m, H-4α), 1.95 (1H, m, H-5), 3.13 (1H, dd, J = 8, 7 Hz, H-2′), 3.26 (1H, t, J = 7 Hz, H-5′), 3.27 (1H, t, J = 7 Hz, H-4′), 3.34 (1H, d, J = 7 Hz, H-3′), 3.65 (1H, m, H-6′α), 3.86 (1H, m, H-6′β), 3.95 (1H, m, H-3), 4.29 (1H, d, J = 7 Hz, H-9), 4.35 (1H, t, J = 7 Hz, H-1′), 5.55 (1H, dd, J = 16, 7

Hz, H-7), 5.73 (1H, dd, J = 16, 7 Hz, H-8) and ^{13}C NMR (methanol-d_4, δ): 16.6 (CH_3-13), 24.3 (CH_3-10), 25.3 (CH_3-12), 26.0 (CH_3-11), 35.7 (CH-5), 38.3 (CH_2-4), 40.6 (C-1), 42.7 (CH_2-2), 63.0 (CH_2-6′), 69.4 (CH-9), 71.9 (CH-4′), 75.3 (CH-2′), 75.8 (CH-3), 78.0 (CH-5′), 78.2 (CH-3′), 78.4 (C-6), 102.8 (CH-1′), 133.9 (CH-7), 135.7 (CH-8); positive HR-ESI-MS *m/z* 337.0684 $[M+Na]^+$(calcd. for $C_{17}H_{14}O_6Na$: 337.0683).

*Dendranthemoside B (**24**)*[82)]

Yellow powder; ^{1}H NMR (methanol-d_4, δ): 0.77 (3H, d, J = 7 Hz, H_3-13), 0.83 (3H, d, J = 7 Hz, H_3-11), 1.00 (3H, d, J = 7 Hz, H_3-12), 1.47 (1H, dd, J = 12, 7 Hz, H-4β), 1.55 (1H, ddd, J = 12, 5, 2 Hz, H-2β), 1.67 (1H, dd, J = 12, 7 Hz, H-2α), 1.83 (1H, m, H-4α), 2.08 (1H, m, H-5), 2.23 (3H, d, J = 7 Hz, H_3-10), 3.10 (1H, dd, J = 8, 7 Hz, H-2′), 3.23 (2H, t, J = 7 Hz, H-4′, 5′), 3.30 (1H, d, J = 7 Hz, H-3′), 3.62 (1H, m, H-6′α), 3.83 (1H, m, H-6′β), 3.95 (1H, m, H-3), 4.33 (1H, t, J = 7 Hz, H-1′), 6.31 (1H, dd, J = 16, 7 Hz, H-8), 6.85 (1H, dd, J = 16, 7 Hz, H-7) and ^{13}C NMR (methanol-d_4, δ): 16.6 (CH_3-13), 25.2 (CH_3-12), 26.1 (CH_3-11), 27.5 (CH_3-10), 35.5 (CH-5), 38.0 (CH_2-4), 41.1 (C-1), 42.6 (CH_2-2), 63.0 (CH_2-6′), 71.9 (CH-4′), 75.3 (CH-2′), 75.6 (CH-3), 78.0 (CH-5′), 78.2 (CH-3′), 79.2 (C-6), 102.9 (CH-1′), 131.7 (CH-8), 154.4 (CH-7), 201.0 (C-9); positive HR-ESI-MS *m/z* : 411.1991 $[M+Na]^+$(calcd. for $C_{19}H_{32}O_6Na$: 411.1989).

*Ampelosisinoside (**25**)*[83)]

Yellow powder; ^{1}H NMR (methanol-d_4, δ): 0.90 (3H, d, J = 6 Hz, H_3-13), 0.93 (3H, s, H_3-12), 0.99 (3H, s, H_3-11), 1.32 (3H, d, J = 6 Hz, H_3-10), 1.82 (1H, d, J = 14 Hz, H-2β), 2.11 (1H, dd, J = 13, 2 Hz, H-4β), 2.28 (1H, m, H-5), 2.44 (1H, t, J = 13 Hz, H-4α), 2.87 (1H, dd, J = 14, 3 Hz, H-2α), 3.12 (1H, t, J = 8 Hz, H-5′), 3.18 (2H, dd, J = 9, 7 Hz, H-2′), 3.29 (1H, m, H-4′), 3.35 (1H, t, J = 4 Hz, H-3′), 3.65 (1H, dd, J = 11, 5 Hz, H-6′α), 3.84 (1H, m, H-6′β), 4.35 (1H, d, J = 7 Hz, H-1′), 4.44 (1H, q, H-9), 5.73 (1H, d, J = 16 Hz, H-7), 5.91 (1H, dd, J = 16, 7 Hz, H-8) and ^{13}C NMR (methanol-d_4, δ): 16.5 (CH_3-13), 21.5 (CH_3-10), 25.0 (CH_3-11), 25.4 (CH_3-12), 37.8 (CH-5), 43.9 (C-1), 46.4 (CH_2-4), 52.5 (CH_2-2), 62.7 (CH_2-6′), 71.6 (CH-4′), 75.4 (CH-2′), 77.8 (C-6, 9), 78.1 (CH-5′), 78.2 (CH-3′), 102.6 (CH-1′), 134.0 (CH-7), 134.9 (CH-8), 214.9 (C-3); positive HR-ESI-MS *m/z* : 411.1990 $[M+Na]^+$(calcd. for $C_{19}H_{32}O_6Na$: 411.1989).

*Icariside B2 (**26**)*[84)]

Colorless solid; ^{1}H NMR (pyridine-d_6, δ): 1.10 (3H, s, H_3-12), 1.52 (3H, s, H_3-11), 1.53 (3H, s, H_3-13), 1.67 (1H, dd, J = 12, 2 Hz, H-4β), 2.21 (3H, s, H-10), 2.39 (1H, ddd, J = 12, 4, 2 Hz, H-2α), 2.88 (1H, ddd, J = 12, 4, 2 Hz, H-4α), 3.92 (1H, m, H-5′), 4.10 (1H, t, J = 8 Hz, H-2′), 4.28 (1H, t, J = 9 Hz, H-3′), 4.31 (1H, t, J = 9 Hz, H-4′), 4.42 (1H, dd, J = 12, 2 Hz, H-6′β), 4.54 (1H, dd, J = 12, 5 Hz, H-6′α), 4.98 (1H, m, H-3), 5.12 (1H, d, J = 7 Hz, H-1′), 5.91 (1H, s, H-8) and ^{13}C NMR (pyridine-d$_6$, δ): 26.9 (CH_3-10), 29.6 (CH_3-11), 31.5 (CH_3-13), 32.4 (CH_3-12), 36.7 (C-1), 47.5 (CH_2-4), 48.5 (CH_2-2), 63.2 (CH_2-6′), 71.7 (C-5), 72.2 (CH-4′), 72.3 (CH-3), 75.8 (CH-2′), 78.8 (CH-5′), 79.1 (CH-3′), 100.9 (CH-8), 103.5 (CH-1′), 120.3 (CH-6), 198.2 (C-7), 210.1 (C-9); positive HR-ESI-MS *m/z* : 409.1836 [M+Na]$^+$(calcd. for $C_{19}H_{30}O_8Na$: 409.1833).

*Benzyl-α-L-arabinopyranosyl (1→6)-β-D-glucopyranoside (**27**)*[85)]

Colorless solid; ^{1}H NMR (methanol-d$_4$, δ): 3.24 (1H, dd, J = 9, 8 Hz, H-2′), 3.28 (1H, m, H-3′), 3.29 (2H, m, H-4′, 5′), 3.44 (1H, dd, J = 6, 3 Hz, H-3″), 3.46 (1H, dd, J = 8, 2 Hz, H-5″α), 3.52 (1H, dd, J = 9, 7 Hz, H-2″), 3.68 (1H, dd, J = 12, 6 Hz, H-6′α), 3.74 (1H, m, H-4″), 3.79 (1H, dd, J = 12, 3 Hz, H-5″), 4.05 (1H, dd, J = 12, 2 Hz, H-6′β), 4.28 (1H, d, J = 7 Hz, H-1″), 4.35 (1H, d, J = 8 Hz, H-1′), 4.66 (1H, d, J = 12 Hz, H-7α), 4.81 (1H, d, J = 12 Hz, H-7β), 7.26 (1H, t, J = 7 Hz, H-4), 7.32 (2H, t, J = 7 Hz, H-3, 5), 7.43 (2H, d, J = 7 Hz, H-2, 6) and ^{13}C NMR (methanol-d$_4$, δ): 66.8 (CH_2-5″), 69.6 (CH-4″), 69.7 (CH_2-6′), 71.9 (CH-4′), 72.1 (CH_2-7), 72.6 (CH-2″), 74.4 (CH-3″), 75.3 (CH-2′), 77.2 (CH-5′), 78.1 (CH-3′), 103.6 (CH-1′), 105.4 (CH-1″), 128.8 (CH-4), 129.3 (CH-2, 6), 129.4 (CH-3, 5), 139.3 (C-1). Positive HR-ESI-MS *m/z* : 425.1414 [M]$^+$(calcd. for $C_{18}H_{26}O_{10}Na$: 425.1418).

*Cichoriin (**28**)*[86)]

Colorless solid; ^{1}H NMR (pyridine-d$_6$, δ): 4.13 (1H, ddd, J = 7, 6, 2 Hz, H-3′), 4.28 (1H, d, J = 7 Hz, H-2′), 4.30 (1H, d, J = 9 Hz, H-4′), 4.36 (1H, d, J = 9 Hz, H-5′), 4.39 (1H, dd, J = 11, 5 Hz, H-6′α), 4.58 (1H, dd, J = 11, 2 Hz, H-6′β), 5.61 (1H, d, J = 8 Hz, H-1′), 6.24 (1H, d, J = 9 Hz, H-3), 7.13 (1H, s H-8), 7.60 (1H, br s, H-4), 7.69 (1H, s, H-5) and ^{13}C NMR (pyridine-d$_6$, δ): 62.9 (CH_2-6′), 71.7 (CH-4′), 75.4 (CH-2′), 78.9 (CH-5′), 79.7 (CH-3′), 104.7 (CH-1′), 104.9 (CH-8), 111.9 (C-10), 113.0 (CH-3), 116.9 (CH-5), 144.6 (C-7), 144.7 (C-7), 152.5 (C-6), 154.3 (C-9), 161.8 (C-2). Positive HR-ESI-MS *m/z* : 363.0686 [M+Na]$^+$(calcd. for $C_{15}H_{16}O_9Na$: 363.0687).

Uridine (29)[87)]

Yellow powder; ^{1}H NMR (pyridine-d$_6$, δ): 4.20 (1H, dd, J = 12, 2 Hz, H-5′β), 4.31 (1H, dd, J = 12, 2 Hz, H-5′α), 4.66 (1H, m, H-4′), 4.92 (2H, d, m, H-2′, 3′), 5.80 (1H, d, J = 8 Hz, H-5), 6.83 (1H, d, J = 4 Hz, H-1′), 8.54 (1H, d, J = 8 Hz, H-6) and ^{13}C NMR (pyridine-d$_6$, δ): 62.1 (CH_2-5′), 71.6 (CH-3′), 76.5 (CH-2′), 86.7 (CH-4′), 90.8 (CH-1′), 102.8 (CH-5), 141.5

(CH-6), 152.7 (C-2), 164.8 (C-4). Positive HR-ESI-MS *m/z* : 267.0587 $[M+Na]^+$(calcd. for $C_9H_{12}O_6N_2Na$: 267.0587).

2.5.4 Acid hydrolysis to identification of sugar moeity of **16** and **17**

A solution of each **16** and **17** (@ 1 mg) in 1 N HCl (0.1 ml) were heated under conditions of reflux for 2 h. The mixture was neutralized by addition of amberlite IRA400 (OH^- form) and the resin was removed by filtration. Then, the filtrates were extracted with EtOAc. The aqueous layers were subjected to HPLC analysis [column: Shodex Asahipak NH 2P-50 4E, 250 x 4.6 mm i.d.; mobile phase: 75% CH_3CN in aq; detection: oprical rotation (JASCO 2090Plus Chiral); flow rate: 1.0 ml/min] to identify D-fructose (**16**) and D-glucose (**17**), which were identified by comparison of their retention times with those of authentic samples; t_R: 5.11 (D-fructose, positive optical rotation) and t_R: 6.10 (D-glucose, positive optical rotation).

2.5.5 Alkaline phosphatase (ALP) activity

The cell were treated, at 90% confluence, with culture medium containing 10 mM *β*-glycerophosphate and 50 μg/ml ascorbic acid, to initiate in vitro mineralization. The medium was changed every 2–3 d. After 6 days, the cells were cultured with medium containing 0.3% bovine serum and isolated compounds (**16** – **29**) individually for 3 days. On harvesting, the medium was removed and the cell monolayer gently washed twice with phosphate buffered saline. The cells were lysed with 0.2% triton X-100, with the lysate centrifuged at 14000 x g for 5 min. The clear supernatant was used to measure ALP activity, which was determined using an ALP activity assay kit.[88)]

2.5.6 Mineralization of MC3T3-E1

The cell were treated, at 90% confluence, with culture medium containing 10 mM *β*-glycerophosphate and 50 μg/ml ascorbic acid, to initiate in vitro mineralization. After 12 days, the cells were cultured with medium containing 0.3% bovine serum and isolated compounds (**16** – **29**) individually for 2 days. On harvesting, the cells were fixed with 70% ethanol for 1 hour and then stained with 40 mM Alizarin Red S for 10 min with gentle shaking. To quantify the bound dye, the stain was solubilized with 10% cetylpyridium chloride by shaking for 15 min. The absorbance of the solubilized stain was measured at 561 nm.[88)]

CONCLUSION

Chemical investigation of non-polar fraction of methanol extract of *Linaria japonica* led to the isolation of fifteen compounds (**1** – **15**), including five new diterpenoids (**1** – **5**), three new flavonoid glycosides (**6** – **8**) and eight known compounds (**9** – **15**). These isolated compounds were examined their inhibitory activity toward A549 cytotoxic cell lines and *L. major* parasites. Linarenone C (**3**) showed moderate inhibitory activity toward A549 cell lines and linarenone A (**1**), linarienone (**9**), and desacetyl-linarienone (**10**) showed moderate inhibitory activities of *L. major*. Their flavonoids didn't show A549 and *L. major* inhibitory activity, therefore they were evaluated their AGEs formation and collagenase inhibitory activities. Isolinariin C (**6**), isolinariin D (**7**), isolinariin E (**8**) and luteolin (**15**) showed stronger AGEs inhibition. Linariin (**11**) and pectolinarin (**12**) showed moderate inhibitory while luteolin (**15**) showed stronger inhibition of collagenase activity without any cytotoxicity, which indicated that these compounds and crude extract of *L. japonica* may become an useful remedy for the AGEs associated diseases and skin deterioration.

Chemical investigation of the butanol layer of methanol extract of *Spilanthes acmella* obtain fourteen compounds (**16** – **29**) including two new methyl threono lactones (**16** – **17**), and two new pyroglutamate (**18** – **19**). The isolated compounds had evaluated ALP and mineralization stimulatory activity. Our study demonstrates that 1-butanol pyroglutamate (**19**), 2–deoxy–D–ribono–1,4 lactone (**21**), ampelosisinoside (**25**) and benzyl-α-L-arabinopyranosyl (1→6)-β-D-glucopyranoside (**27**) stimulated ALP activity and calcium deposition in osteoblastic MC3T3-E1 cell *in vitro*. These studies suggest that compound **19**, **21**, **25** and **27** may be able to stimulate osteoblastic bone formation and play an important role in bone remodeling.

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