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Original article

Flavonoids from the stems of *Millettia pachyloba* Drake mediate cytotoxic activity through apoptosis and autophagy in cancer cells

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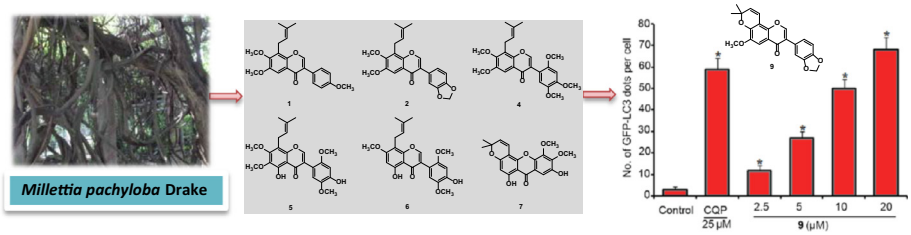
HIGHLIGHTS

- Six new natural compounds were isolated from *Millettia pachyloba* Drake.
- The cytotoxic activities of these new compounds were evaluated.
- Ten (**3**–**5**, **9**, **12**, **17**–**19**, **24**, and **25**) of 28 isolated compounds showed cytotoxicity.
- The ten cytotoxic compounds induced autophagy in cancer cells.
- Compound **9** induced apoptosis and autophagy, suggesting it could be a potential anticancer drug candidate.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, systematic separation and subsequent pharmacological activity studies were carried out to identify cytotoxic natural products from the dried stems of *Millettia pachyloba* Drake. Five previously undescribed isoflavones, pachyvones A–E; one previously undescribed xanthone, pachythone A; and twenty-two known compounds were obtained. The structures of these compounds were assigned on the basis of 1D/2D NMR data and high-resolution electrospray ionization mass spectroscopy analysis. Preliminary activity screening with HeLa and MCF-7 cells showed that ten compounds (**3**–**5**, **9**, **12**, **17**–**19**, **24**, and **25**) had potential cytotoxicity. Further in-depth activity studies with five cancer cell lines (HeLa, HepG2, MCF-7, Hct116, and MDA-MB-231) and one normal cell line (HUVEC) revealed that these ten compounds showed specific cytotoxicity in cancer cells, with IC_{50} values ranging from 5 to 40 μM, while they had no effect on normal cell lines. To investigate whether the cytotoxicity of these ten compounds was associated with autophagy, their autophagic effects were evaluated in GFP-LC3-HeLa cells. The results demonstrated that compound **9** (durmillone) significantly induced autophagy in a concentration-dependent manner and had the best activity as an autophagy inducer among all of the compounds. Therefore, compound **9** was selected for further study. The PI/Annexin V double staining assay and Western blotting results revealed that compound **9** also induced obvious apoptosis in HeLa and MCF-7 cells, which suggests that it mediates cytotoxic activity through activation of both apoptosis and autophagy. Taken together, this study identified ten natural cytotoxic products from the dried stems

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of *Millettia pachyloba* Drake, of which compound **9** induced apoptosis and autophagy and could be an anticancer drug candidate.

Introduction

Millettia (Leguminosae) is a genus with approximately 200 species that are primarily distributed in tropical and subtropical regions, such as Africa, Asia, America, and Australia [1]. Historically, *Millettia* is a traditional medicine used in the treatment of gynecological diseases, dysentery, cardiovascular diseases, intestinal pain, rheumatic arthritis, and skin diseases [2,3]. Previous phytochemical investigations of this genus have demonstrated the presence of steroids, alkaloids, triterpenoids, and flavonoids [4–7].

Millettia pachyloba Drake (*M. pachyloba*) belongs to the Leguminosae family and is a type of semi-evergreen perennial woody vine plant primarily distributed in the Guangdong, Hainan, Guangxi, and Yunnan Provinces of China. The stems of *M. pachyloba* are often used by locals as a herbal medicine for the treatment of tumors, rheumatic arthritis and removing edema from patients. To date, only three studies have focused on the phytochemical study of *M. pachyloba* [8–10], which is far from providing a deep understanding of *M. pachyloba*. Thus, further intensive phytochemical study of *M. pachyloba* is needed.

Autophagy is the primary cellular process for protecting cells and organisms from natural stressors such as ER-stress as well as nutrient deficiency. In addition to its function in normal physiology, autophagy also plays a role in cancer [11]. Recently, it was established as a tumor suppression mechanism; loss of autophagy function was required for the initiation of cancer [12]. Because previous research reported that isolated flavonoids from *M. pachyloba* exhibited initial cytotoxicity against KB cells [8], it was worthwhile screening the autophagy inducer in *M. pachyloba* and further testing the underlying mechanism. Durmillone is an isoflavone with a dimethyl pyran moiety connected to C6 and C7. It is widespread in the genus of *Millettia* [13,14] and *Lonchocarpus* [15]. However, there is no research reporting its cytotoxic mechanism of action.

This study carried out intensive phytochemical study of *M. pachyloba*. In addition, the initial cytotoxic mechanism of action of the compounds separated form *M. pachyloba* were investigated.

Material and methods

General experimental procedures

The following equipment and methods were used in the present study: silica gel column chromatography (200–300 mesh, Qingdao Makall Group Co., Qingdao, China), Sephadex LH-20 column chromatography (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), high-performance liquid chromatography (HPLC, Waters, Milford, USA), a Sunfire C_{18} column (5 μm, 4.6 mm × 150 mm; Waters, Milford, USA), a semipreparative HPLC instrument (SP-HPLC, NovaSep, Miramas, France), a digital polarimeter for optical rotation measurements (Jasco P-1020, Tokyo, Japan), a UV-2100 spectrophotometer for ultraviolet absorbance detection (Shimadzu, Kyoto, Japan), a Nicolet-6700 FT-IR spectrometer for IR spectral detection (Thermo Scientific, Waltham, USA), an Aviv Model 400 CD spectrometer (Aviv Biomedical, Lakewood, USA), an Avance-400 spectrometer for NMR spectral detection (Bruker, Billerica, USA), and a Q-TOF Premier mass spectrometer coupled with an ESI source (Waters, Milford, USA).

Plant material

Researcher Hua Peng (Kunming Institute of Botany, Chinese Academy of Sciences) collected and identified the stems of *M. pachyloba* at Pingbian, Yunan, China, in September 2015. A voucher specimen (SKLB-201509) was deposited in the Lab of Natural Product Drugs and Cancer Biotherapy, Sichuan University.

Extraction and isolation

Air-dried stems of *M. pachyloba* (10 kg) were ground into powder (approximately 20-mesh). The powder was extracted with 60 L 95% aqueous EtOH three times. The EtOH extracts were combined and evaporated to dryness *in vacuo* to produce 712 g crude sample. It was then suspended in 5 L deionized H_2O and successively exhausted with 5 L petroleum ether and 5 L CH_2Cl_2 to give dried petroleum ether (48 g) and CH_2Cl_2 (77 g) extracts, respectively, for further separation.

The petroleum ether extract was subjected to silica gel column chromatography (petroleum ether/EtOAc from 100/1 to 1/1, *v/v*) for rough separation, and eleven fractions (Fr. A1–Fr. A11) were collected. Fr. A5 (1.3 g) was subjected to SP-HPLC (MeOH/H_2O, 75/25, *v/v*) to yield 7.8 mg of compound **18**. Fr. A7 (1.7 g) was also subjected to SP-HPLC (MeOH/H_2O, 85/15, *v/v*) to yield 15.7 mg of compound **1**. Fr. A8 (3.9 g) was subjected to silica gel column chromatography (petroleum ether/EtOAc from 20/1 to 1/5, *v/v*) and produced five subfractions (Fr. A8.1–Fr. A8.5). Fr. A8.2 was subjected to SP-HPLC (MeOH/H_2O, 80/20, *v/v*) to produce 135.5 mg compound **6**, 5.1 mg compound **7**, and 15.4 mg compound **9**. Fr. A 8.3 was subjected to Sephadex LH-20 column chromatography (CH_2Cl_2/MeOH from 10/1 to 1/5, *v/v*) to yield 83.4 mg compound **22** and 112.7 mg compound **23**. Fr. A9 (2.8 g) was subjected to silica gel column chromatography (petroleum ether/EtOAc, from 20/1 to 1/5, *v/v*) and further purified by SP-HPLC (MeOH/H_2O, 80/20 and 85/15, *v/v*, respectively) to yield 8.7 mg compound **3** and 19.6 mg compound **5**.

The CH_2Cl_2 extract was subjected to silica gel column chromatography (petroleum ether/EtOAc, from 50/1 to 1/5, *v/v*), and 15 fractions (Fr. B1–Fr. B15) were produced. Fr. B3 (1.3 g) was subjected to SP-HPLC (MeOH/H_2O, 85/15, *v/v*) to yield 5.9 mg compound **19**. Fr. B5 (4.6 g) was subjected to silica gel column chromatography using petroleum ether/EtOAc (from 20/1 to 1/5, *v/v*), and six subfractions were obtained (Fr. B5.1–Fr. B5.6). Fr. B5.3 and Fr. B5.5 were subjected to SP-HPLC (MeOH/H_2O, 85/15, and 70/30, respectively) to produce 12.5 mg compound **2** and 16.3 mg compound **25**. Fr. B6 (1.7 g) was purified using SP-HPLC (MeOH/H_2O, 65/35, *v/v*) to obtain 61.4 mg compound **20**. Fr. B7 (4.8 g) was subjected to silica gel column chromatography (petroleum ether/EtOAc from 20/1 to 1/5, *v/v*) and further purified using Sephadex LH-20 column chromatography (CH_2Cl_2/MeOH, from 10/1 to 1/5, *v/v*) to produce 9.3 mg compound **8**, 23.1 mg compound **13** and 17.2 mg compound **21**. Fr. B8 (2.4 g) was subjected to SP-HPLC (MeOH/H_2O, 75/25, *v/v*) to yield 7.1 mg compound **10**. Fr. B9 (3.6 g) was also subjected to SP-HPLC (MeOH/H_2O, 80/20, and 75/25, respectively) to yield 38.5 mg compound **4** and 21.5 mg compound **11**. Fr. B10 (4.1 g) was separated on a silica gel column (petroleum ether/EtOAc, from 20/1 to 1/5, *v/v*) and by SP-HPLC (MeOH/H_2O, 75/25, and 65/35, respectively) to yield

47.8 mg compound **12** and 13.7 mg compound **15**. Fr. B11 (4.5 g) was purified in the same manner as Fr. B10 to produce 10.2 mg compound **14**, 36.3 mg compound **24** and 38.1 mg compound **27**. Fr. B12 (2.3 g) underwent Sephadex LH-20 column chromatography (CH_2Cl_2/MeOH, from 10/1 to 1/5, *v/v*) to yield 18.5 mg compound **16** and 16.1 mg compound **17**. Fr. B13 (3.7 g) underwent Sephadex LH-20 column chromatography (H_2O/MeOH, from 10/1 to 1/5, *v/v*) to yield 48.3 mg compound **26** and 53.9 mg compound **28**. In total, 28 compounds with purities>98% analyzed by HPLC (Waters, Milford, USA) were isolated from the ethanol extract of the stems of *M. pachyloba*.

Pachyvone A (1)

White powder; ultraviolet (UV) (MeOH) λ_{max} (log ε) 262 (4.18), 326 (2.97) nm; Infrared (IR) (KBr) ν_{max} 3028, 2910, 1673, 1456, 834 cm^{-1}. Both ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR data are shown in Table 1; high-resolution electrospray ionization mass spectroscopy (HRESIMS) *m/z* 381.1707 $[M+H]^+$ (calcd for $C_{23}H_{25}O_5$, 381.1702).

*Pachyvone **B** (2)*

White powder; UV (MeOH) λ_{max} (log ε) 263 (3.64), 326 (3.07) nm; IR (KBr) ν_{max} 3019, 2917, 1682, 1462, 863, 845 cm^{-1}. Both ^1H NMR and ^{13}C NMR data are shown in Table 1; HRESIMS *m/z* 395.1506 $[M+H]^+$ (calcd for $C_{23}H_{23}O_6$, 395.1495).

*Pachyvone **C** (4)*

White powder; UV (MeOH) λ_{max} (log ε) 255 (3.38), 298 (2.87) nm; IR (KBr) ν_{max} 3025, 2921, 1679, 1443, 865 cm^{-1}. Both ^1H NMR and ^{13}C NMR data are shown in Table 1; HRESIMS *m/z* 441.1914 $[M+H]^+$ (calcd for $C_{25}H_{29}O_7$, 441.1913).

*Pachyvone **D** (5)*

Yellowish powder; UV (MeOH) λ_{max} (log ε) 266 (3.76) nm; IR (KBr) ν_{max} 3593, 3016, 2934, 1688, 1459, 827 cm^{-1}. Both ^1H NMR and ^{13}C NMR data are shown in Table 1; HRESIMS *m/z* 443.1705 $[M+H]^+$ (calcd for $C_{24}H_{27}O_8$, 443.1706).

*Pachyvone **E** (6)*

Yellowish powder; UV (MeOH) λ_{max} (log ε) 264 (3.58), 294 (2.92) nm; IR (KBr) ν_{max} 3598, 3023, 2928, 1663, 1466, 831 cm^{-1}. Both ^1H NMR and ^{13}C NMR data are shown in Table 1; HRESIMS *m/z* 413.1605 $[M+H]^+$ (calcd for $C_{23}H_{25}O_7$, 413.1600).

*Pachythone **A** (7)*

Yellow powder; UV (MeOH) λ_{max} (log ε) 287 (4.18), 340 (2.17) nm; IR (KBr) ν_{max} 3604, 2894, 1664, 1456, 848, 715 cm^{-1}. Both ^1H NMR and ^{13}C NMR data are shown in Table 2; HRESIMS *m/z* 371.1137 $[M+H]^+$ (calcd for $C_{20}H_{19}O_7$, 371.1131).

Cell culture and transfection

GFP-LC3-HeLa (HeLa cells stably expressing GFP-LC3) were established as previously reported [16,17]. HeLa, HepG2, MCF-7, Hct116, MDA-MB-231, and HUVECs were obtained from KeyGEN Biotech Co. (Nanjing, China) and cultured with Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured at 37 °C in a humidified atmosphere, and the concentration of CO_2 was set at 5%.

Cytotoxicity assay

The cytotoxic effects of the isolated compounds were investigated using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Briefly, cells were plated in 96-well plates with 1×10^4 cells per well. Cells were cultured for 24 h before treatment with different compounds at various concentrations (0–40 μM) for 72 h. Then, 20 μL MTT solution (5 mg/mL) was added to each well and incubated for another 4 h. The supernatants were discarded, and 150 μL dimethyl sulfoxide (DMSO) was added to each well and incubated for 10 min. The absorbance

Table 1
^1H and ^{13}C NMR spectroscopic data for compounds **1**, **2**, **4–6**[a] (400 and 100 M*Hz* for ^1H and ^{13}C NMR, $CDCl_3$).

Position	**1**		**2**		**4**		**5**		**6**	
	δ_C	δ_H (*J* in *Hz*)	δ_C	δ_H (*J* in *Hz*)	δ_C	δ_H (*J* in *Hz*)	δ_C	δ_H (*J* in *Hz*)	δ_C	δ_H (*J* in *Hz*)
2	152.3	8.02, s	152.5	8.01, s	154.5	8.05, s	155.4	7.97, s	155.2	7.94, s
3	123.9		124.1		120.9		119.8		119.9	
4	176.1		175.9		176.0		181.8		181.4	
4a	120.7		120.7		120.8		108.6		105.8	
5	103.8	7.60, s	103.9	7.59, s	103.9	7.59, s	152.5		160.9	
6	151.1		151.2		151.0		136.6		95.1	6.41, s
7	151.9		151.9		151.8		157.1		162.7	
8	124.7		124.7		124.7		113.0		107.8	
8a	150.0		149.9		149.9		150.4		154.6	
1′	124.5		125.9		112.5		110.0		109.9	
2′	130.1	7.52, d (8.8)	122.3	7.00, dd (8.0, 1.6)	151.9		152.4		152.5	
3′	113.9	6.98, d (8.8)	108.4	6.87, d (8.0)	98.3	6.63, s	100.0	6.67, s	100.0	6.66, s
4′	159.5		147.6		149.8		146.9		146.8	
5′	113.9	6.98, d (8.8)	147.6		143.1		140.4		140.4	
6′	130.1	7.52, d (8.8)	109.8	7.12, d (1.6)	115.3	6.96, s	114.4	6.87, s	114.4	6.88, s
7′			101.1	5.99, s						
1″	22.9	3.59, d (7.2)	22.9	3.59, d (7.2)	23.0	3.60, d (7.2)	22.2	3.46, d (7.2)	21.5	3.42, d (7.2)
2″	121.5	5.21, t (7.2)	121.5	5.21, t (7.2)	121.6	5.23, t (7.2)	122.2	5.18, t (7.2)	122.1	5.17, t (7.2)
3″	132.7		132.7		132.6		132.1		131.9	
4″	17.9	1.84, s	17.9	1.84, s	17.9	1.84, s	17.8	1.81, s	17.8	1.79, s
5″	25.8	1.69, s	25.8	1.69, s	25.8	1.70, s	25.8	1.70, s	25.8	1.68, s
6-OCH_3	56.0	3.96, s	56.1	3.96, s	56.0	3.96, s	60.7	3.93, s		
7-OCH_3	61.1	3.93, s	61.1	3.93, s	61.1	3.93, s	61.4	4.01, s	56.1	3.90, s
2′-OCH_3					56.9	3.79, s	56.5	3.74, s	56.5	3.75, s
4′-OCH_3	55.3	3.84, s			56.2	3.94, s				
5′-OCH_3					56.6	3.86, s	56.7	3.86, s	56.7	3.86, s

[a] Chemical shifts are given in ppm. *J* values (*Hz*) are given in parentheses. Assignments were made based on the analysis of ^1H–^1H COSY, HSQC, and HMBC data.

Table 2
^{1}H and ^{13}C NMR spectroscopic data for compound **7**[a] (400 and 100 MHz for ^{1}H and ^{13}C NMR, $CDCl_3$).

position	**7**	
	δ_C	δ_H
1	156.9	
2	95.1	6.40, s
3	160.5	
4	104.7	
4a	157.5	
5	139.8	
6	145.6	
7	145.5	
8	103.6	7.47, s
8a	116.5	
9	180.1	
9a	103.3	
10a	145.1	
4′	115.5	6.73, d (10.0)
5′	127.5	5.60, d (10.0)
6′	78.2	
7′	28.4	1.48, s
8′	28.4	1.48, s
5-OCH_3	61.9	4.05, s
6-OCH_3	61.5	4.16, s

[a] Chemical shifts are given in ppm. *J* values (Hz) are given in parentheses. Assignments were made based on the analysis of ^{1}H–^{1}H COSY, HSQC, and HMBC data.

at 570 nm was detected using a microplate reader (BioTek, Winooski, USA). The cytotoxicity (IC_{50}, half maximal inhibitory concentration) of each compound was calculated using GraphPad Prism 5.

Autophagy detection using GFP-LC3 expression in HeLa cells

The effects of compound-induced autophagy were determined in GFP-LC3-HeLa cells [16,17]. The GFP-LC3-HeLa cells were plated in 24-well plates and incubated with the tested compounds at various concentrations. Chloroquine phosphate treatment-induced LC3 dots were used as an observation control. Plates were incubated for 24 h, and the GFP-LC3 puncta were detected and imaged under a fluorescence microscope (Olympus, Tokyo, Japan) with Olympus Stream software.

Detection of apoptotic cells using flow cytometry

HeLa and MCF-7 cells seeded on six-well plates for 24 h were incubated with 0, 2.5, 5, 10, and 20 μM compound **9** or colchicine (positive control) for 48 h. Cells were collected, digested with ethylenediaminetetraacetic acid-free trypsin for 3 min and centrifuged (170 × g, 3 min) before being washed with phosphate buffered saline (PBS) buffer twice and centrifuged (170 × g, 3 min). Then, the cells were resuspended and stained with reagents from the Annexin V/propidium iodide (PI) Apoptosis Detection Kit (Invitrogen) for approximately 30 min according to the manufacturer's instructions. The stained cells were subjected to flow cytometry (Attune NxT, Life Technology, Waltham, USA) for analysis. Data and image analyses were conducted using FlowJo 7.6 software. The PI−/Annexin V−, PI+/Annexin V−, PI−/Annexin V+, and PI+/Annexin V+ cells were considered viable cells, necrotic cells, early apoptotic cells, and late apoptotic cells, respectively. In this study, the early apoptotic cells and late apoptotic cells were combined and counted as the total number of apoptotic cells.

Western blotting analysis

HeLa cells were collected and washed twice with PBS before being lysed with protein lysis radioimmunoprecipitation assay buffer for 30 min at 4 °C. Samples were subjected to centrifugation at 18894*g* for 30 min at 4 °C. The supernatants were collected, and the protein concentration was determined using a bicinchoninic acid assay (Thermo Scientific, Waltham, USA). Proteins were denatured in 1 × loading buffer in boiling water for 10 min. Equal amounts (20 μg) of samples were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis for the ionophoretic separation of proteins for 1 h using a constant voltage of 120 V. Proteins in the gel were transferred to polyvinylidene difluoride (PVDF) membranes using 260 mA constant current for 2 h. Transferred PVDF membranes were blocked with a 5% milk solution (in 1 × PBST buffer (0.1% Tween® 20 in PBS buffer)) for 1 h at room temperature before incubation with primary antibodies at 4 °C overnight. PVDF membranes were washed three times (10 min each) with 1 × PBST buffer. Secondary antibodies were incubated with PVDF membranes for 45 min at room temperature, and the membranes were washed three times (10 min each) with 1 × PBST. The membranes were stained with enhanced chemiluminescence reagents (Millipore, Burlington, USA) and imaged using a chemiluminescence image analysis system (Tianneng, Shanghai, China) with Tanon-5200 Multi software (Tianneng, Shanghai, China).

Results and discussion

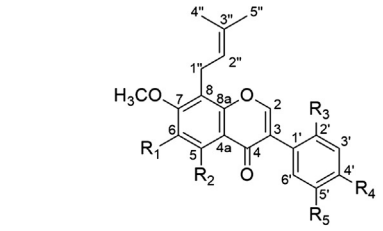
Isolation of compounds 1–28.

Approximately 10 kg dry stems of *M. pachyloba* were shattered into powder (approximately 20-mesh) and extracted with 95% aqueous EtOH three times. The EtOH extracts were combined, evaporated to dryness, suspended in H_2O and successively extracted with petroleum ether and CH_2Cl_2. The petroleum ether and CH_2Cl_2 extracts were further separated using column chromatography (silica gel and Sephadex LH-20) as well as reverse-phase SP-HPLC to obtain compounds **1**–**28** (see Fig. 1).

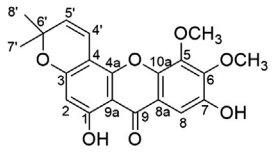
Chemical structure identification of the isolated compounds.

Compound **1** was obtained as a white powder and assigned a molecular formula of $C_{23}H_{24}O_5$ by HRESIMS at *m*/*z* 381.1707 ([M+H]$^+$, calcd for 381.1702), indicating twelve double bond equivalents. The UV spectrum absorption at 256 and 326.4 nm, ^{1}H (δ_H 8.02 for H-2) and ^{13}C (δ_C 152.3 for C-2) NMR spectra and ^{1}H detected heteronuclear multiple bond correlation (HMBC) correlations (Fig. 2) of H-2 (δ_H 8.02) to C-3 (δ_C 123.9), C-8a (δ_C 150.0) and C-4 (δ_C 176.1) showed this compound to be an isoflavone-type skeleton [18,19]. Analysis of the ^{1}H NMR (Table 1) and homonuclear chemical shift correlation spectroscopy (COSY) correlations revealed a *γ*, *γ*-dimethylallyl unit [δ_H 3.59 (2H, d, *J* = 7.2 *Hz*), 5.21 (1H, t, *J* = 7.2 *Hz*), 1.84 (3H, s), 1.69 (3H, s)], a 1,4-disubstituted benzene ring [δ_H 7.52 (2H, d, *J* = 8.8 *Hz*), 6.98 (2H, d, *J* = 8.8 *Hz*)], three methoxy groups [δ_H 3.96 (3H, s), 3.93 (3H, s), 3.84 (3H, s)], an aromatic proton [δ_H 7.60 (1H, s)] and a vinyl proton [δ_H 8.02 (1H, s)]. The ^{13}C NMR spectra of **1** indicated 23 signals, including three methoxy groups [δ_C 55.3, 56.0 and 61.1] and one *γ*, *γ*-dimethylallyl unit [δ_C 17.9, 22.9, 25.8, 121.5 and 132.7]. Comparison of the NMR data of **1** and millesianin H [2] revealed similar carbon and proton resonances, except that **1** contained one more methoxy group. A further HMBC correlation study (Fig. 2) showed that this methoxy group (δ_H 3.93) was attached to C-7 (δ_C 151.9). Therefore, the chemical structure of compound **1** was identified as 8-(*γ*, *γ*-dimethylallyl)-6,7,4′-trimethoxyisoflavone, and it was named pachyvone A.

Compound **2** was isolated as a white powder. Its formula of $C_{23}H_{22}O_6$ was deduced by HRESIMS at *m*/*z* 395.1506 ([M+H]$^+$, calcd for 395.1495). In the ^{1}H and ^{13}C NMR spectra of **2**, an olefinic



1 $R_1 = OCH_3$, $R_2 = H$, $R_3 = H$, $R_4 = OCH_3$, $R_5 = H$
2 $R_1 = OCH_3$, $R_2 = H$, $R_3 = H$, R_4, $R_5 = -OCH_2O-$
3 $R_1 = OCH_3$, $R_2 = H$, $R_3 = OCH_3$, R_4, $R_5 = -OCH_2O-$
4 $R_1 = OCH_3$, $R_2 = H$, $R_3 = R_4 = R_5 = OCH_3$
5 $R_1 = OCH_3$, $R_2 = OH$, $R_3 = OCH_3$, $R_4 = OH$, $R_5 = OCH_3$
6 $R_1 = H$, $R_2 = OH$, $R_3 = OCH_3$, $R_4 = OH$, $R_5 = OCH_3$



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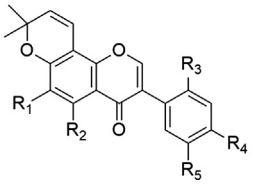
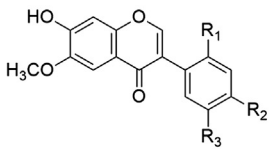
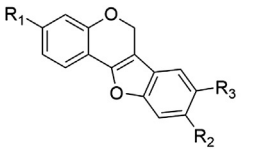


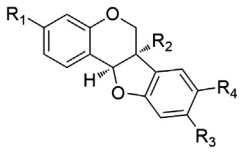
8 $R_1 = OCH_3$, $R_2 = H$, $R_3 = H$, $R_3 = H$, $R_4 = OCH_3$, $R_5 = H$
9 $R_1 = OCH_3$, $R_2 = H$, $R_3 = H$, R_4, $R_5 = -OCH_2O-$
10 $R_1 = OCH_3$, $R_2 = H$, $R_3 = H$, $R_4 = R_5 = OCH_3$
11 $R_1 = OCH_3$, $R_2 = H$, $R_3 = OCH_3$, R_4, $R_5 = -OCH_2O-$
12 $R_1 = OCH_3$, $R_2 = H$, $R_3 = R_4 = R_5 = OCH_3$
13 $R_1 = H$, $R_2 = OH$, $R_3 = R_4 = R_5 = OCH_3$



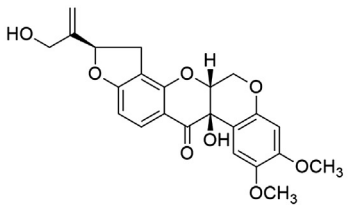
14 $R_1 = H$, $R_2 = R_3 = OCH_3$
15 $R_1 = OCH_3$, R_2, $R_3 = -OCH_2O-$
16 $R_1 = R_2 = R_3 = OCH_3$



17 $R_1 = OH$, $R_2 = OH$, $R_3 = H$
18 $R_1 = OH$, R_2, $R_3 = -OCH_2O-$
19 $R_1 = OCH_3$, R_2, $R_3 = -OCH_2O-$



20 $R_1 = OH$, $R_2 = H$, $R_3 = OCH_3$, $R_4 = H$
21 $R_1 = OH$, $R_2 = H$, R_3, $R_4 = -OCH_2O-$
22 $R_1 = OCH_3$, $R_2 = OH$, $R_3 = OCH_3$, $R_4 = H$
23 $R_1 = OCH_3$, $R_2 = OH$, R_3, $R_4 = -OCH_2O-$



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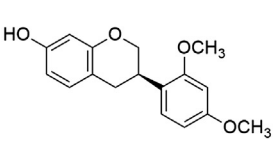

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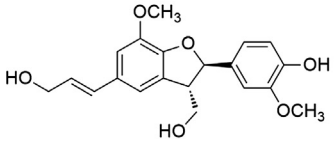

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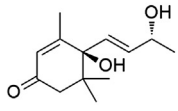

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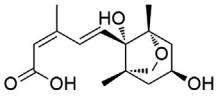
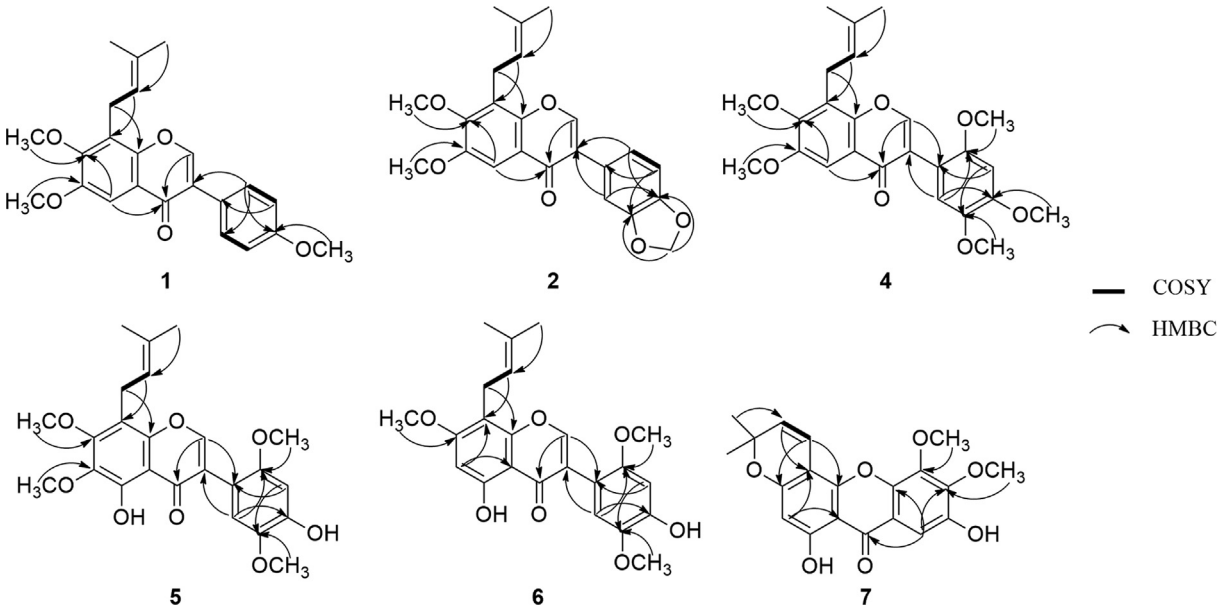


28

Fig. 1. Structures of compounds **1**–**28**.



1 **2** **4**

5 **6** **7**

Fig. 2. Key COSY and HMBC correlations of compounds **1**, **2**, and **4–7**.

proton at δ_H 8.01 (s, H-2), an oxygenated carbon resonance at δ_C 152.5 (C-2) and a carbonyl carbon resonance at δ_H 175.9 (C-4) suggested an isoflavone skeleton [18,19]. The ^{1}H NMR spectrum showed (Table 1) the presence of a γ, γ-dimethylallyl unit [δ_H 3.59 (2H, d, J = 7.2 *Hz*), 5.21 (1H, t, J = 7.2 *Hz*), 1.84 (3H, s), 1.69 (3H, s)], an ABX-type benzene ring [δ_H 6.87 (1H, d, J = 8.0 *Hz*), 7.00 (1H, dd, J = 8.0 *Hz*, J = 1.6 *Hz*), 7.12 (1H, d, J = 1.6 *Hz*)], two methoxy groups [δ_H 3.96 (3H, s), 3.93 (3H, s)], a methylenedioxy group [δ_H 5.99 (2H, s)], an aromatic proton [δ_H 7.59 (1H, s)] and a vinyl proton [δ_H 8.01 (1H, s)]. The ^{13}C NMR spectra of **2** indicated 23 signals, including two methoxy groups [δ_C 56.1 and 61.1], one γ, γ-dimethylallyl unit [δ_C 17.9, 22.9, 25.8, 121.5, and 132.7] and a methylenedioxy functionality [δ_C 101.1]. These signals were similar to the resonances of predurmillone [20], except that the hydroxyl group in predurmillone was replaced by a methoxyl group (δ_H 3.93) in **2**. These results were further directly supported by the HMBC correlation (Fig. 2) from the proton signal at δ_H 3.93 to C-7 (δ_C 151.96) and indirectly demonstrated by the HMBC correlations (Fig. 2) from the proton signals at δ_H 7.59 (s) to C-7 (δ_C 151.96) and C-4 (δ_C 175.9) because this proton was not substituted and the methoxyl group (δ_H 3.93) should be attached to C-7. Therefore, the structure of compound **2** was identified as 8-(γ, γ-dimethylallyl)-6,7-dimethoxy-4′,5′-methylenedioxyisoflavone, and it was named pachyvone B.

Compound **4** was isolated as a white powder and assigned the molecular formula $C_{25}H_{28}O_7$, as indicated by the HRESIMS at m/z 441.1914 ([M+H]$^+$, calcd for 441.1913), which suggested twelve double bond equivalents. A singlet at δ_H 8.05 (H-2) in the ^{1}H NMR spectrum and the ^{13}C NMR signals at δ_C 154.5 (C-2), 120.9 (C-3), and 176.0 (C-4) were consistent with an isoflavone core structure that was further corroborated by its UV spectrum (λ_{max} at 255.4 and 297.9 nm) [18,19]. Compound **4** and millesianin I [2] had similar ^{1}H and ^{13}C NMR data (Table 1) because both compounds contained a γ, γ-dimethylallyl unit, a 1,2,4,5-tetrasubstituted benzene ring, four methoxy groups, an aromatic proton and a vinyl proton, except that compound **4** contained one additional methoxy group signal at δ_C 61.11 and δ_H 3.93. HMBC correlation (Fig. 2) of the proton resonance at δ_H 3.93 with C-7 (δ_C 151.81) demonstrated that the additional methoxy group was attached to C-7. Accordingly, the structure of compound **4** was established as 8-(γ, γ-dimethylallyl)-6,7,2′,4′,5′-pentamethoxyisoflavone, and it was named pachyvone C.

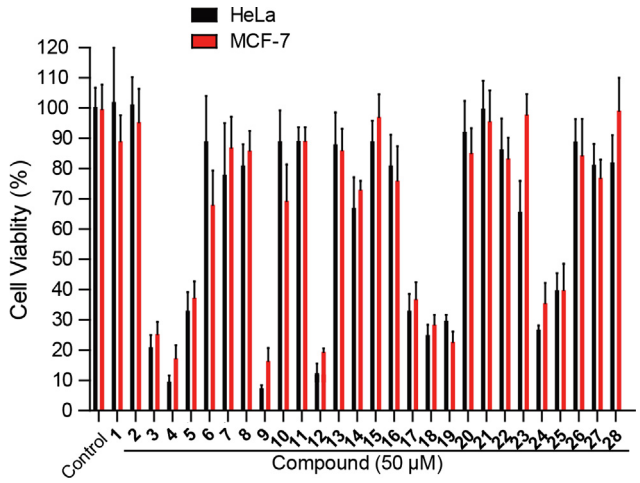


Fig. 3. Preliminary screening of active compounds on HeLa and MCF-7 cells. HeLa and MCF-7 cells were treated with 50 μM for 72 h, and then cell viability was tested by MTT assay.

Compound **5** was isolated as a yellowish powder. Its molecular formula was determined to be $C_{24}H_{26}O_8$ by HRESIMS at m/z 443.1705 ([M+H]$^+$, calcd for 443.1706), suggesting twelve double bond equivalents. The UV maxima at λ_{max} 266.0 and 300.0 nm as well as the specific proton signal at δ_H 7.97 (1H, s, H-2) that was correlated with δ_C 155.4 (C-2), as shown by the heteronuclear single quantum coherence spectrum, suggested that compound **5** possessed an isoflavone-type skeleton [18,19]. The ^{1}H NMR (Table 1) and COSY correlations of compound **5** revealed signals for a γ, γ-dimethylallyl unit [δ_H 3.46 (2H, d, J = 7.2 Hz), 5.18 (1H, t, J = 7.2 Hz), 1.81 (3H, s), 1.70 (3H, s)], a 1,2,4,5-tetrasubstituted benzene ring [δ_H 6.67 (1H, s), 6.87 (1H, s)], four methoxy groups [δ_H 4.01 (3H, s), 3.93 (3H, s), 3.86 (3H, s), 3.74 (3H, s)] and a hydroxyl group [δ_H 12.88 (1H, s)]. The ^{13}C NMR spectrum of **5** indicated 23 signals, including four methoxy groups [δ_C 56.5, 56.7, 60.7 and 61.4] and one γ, γ-dimethylallyl unit [δ_C 17.8, 22.2, 25.8, 122.2 and 132.1]. Compound **5** exhibited NMR data very similar to those of compound **4.** However, **5** had a chelated hydroxyl group at δ_H 12.88 (1H, s, OH-5), which is absent in **4**. Moreover, there is one more methoxyl group in **5** compared to **4**. The HMBC correlations of the hydroxyl group (δ_H 12.88) with C-4a (δ_C 108.60), C-5 (δ_C 152.47) and C-6 (δ_C 136.64) suggested a hydroxy group at the C-5 position. HMBC correlations from the proton signals of four methoxy groups [δ_H 4.01 (3H, s), 3.93 (3H, s), 3.86 (3H, s), 3.74 (3H, s)] demonstrated that these four methoxy groups were attached to C-7, C-6, C-5′, and C-2′, respectively. The chemical shift of C-4′ (δ_C 146.89) combined with the molecular formula $C_{24}H_{26}O_8$ showed that compound **5** had a hydroxy group at the C-4′ position. The key HMBC correlations are shown in Fig. 2. On the basis of the evidence obtained, the structure of compound **5** was determined to be 8-(γ, γ-dimethylallyl)-5,4′-dihydroxy-6,7,2′,5′-tetramethoxyisoflavone, and it was named pachyvone D.

Compound **6** was obtained as a yellowish powder with the molecular formula $C_{23}H_{24}O_7$ deduced by HRESIMS at m/z

Table 3
Cytotoxicity of selected compounds against five cancer cell lines and a normal cell lines (HUVEC).[a]

Compound	IC$_{50}$ (μM)					
	HeLa	HepG2	MCF-7	HCT-116	MDA-MB-231	HUVEC
3	14.56 ± 0.54	15.97 ± 0.67	19.61 ± 0.66	23.21 ± 1.22	20.78 ± 2.35	>50
4	7.86 ± 1.21	8.74 ± 0.83	18.46 ± 0.51	8.61 ± 0.72	15.85 ± 2.15	>50
5	35.67 ± 3.91	31.61 ± 2.06	35.05 ± 1.44	25.91 ± 0.85	27.64 ± 4.54	>50
9	6.09 ± 1.09	17.85 ± 1.60	11.08 ± 0.68	15.14 ± 0.61	12.89 ± 3.10	>50
12	14.82 ± 2.12	8.05 ± 0.90	14.37 ± 1.84	19.78 ± 1.29	11.09 ± 0.91	>50
17	36.15 ± 7.34	34.25 ± 1.87	30.34 ± 1.32	39.66 ± 2.06	36.78 ± 5.61	>50
18	22.50 ± 1.09	13.39 ± 1.41	21.21 ± 0.93	21.90 ± 1.73	25.45 ± 2.09	>50
19	30.19 ± 0.54	25.38 ± 1.92	21.10 ± 1.65	27.03 ± 1.64	22.76 ± 3.54	>50
24	19.89 ± 2.09	32.61 ± 1.84	33.12 ± 1.93	16.65 ± 0.82	27.16 ± 3.25	>50
25	40.12 ± 4.32	28.61 ± 2.90	36.42 ± 2.08	31.90 ± 1.52	33.45 ± 2.33	>50
Doxorubicin	0.03 ± 0.001	0.02 ± 0.002	0.02 ± 0.003	0.03 ± 0.003	0.03 ± 0.002	0.04 ± 0.005

[a] Results are presented as means ± SEM (n = 3).

413.1605 (([M+H]$^+$, calcd for 413.1600)). The UV maxima at λ_{max} 263.7 and 294.4 nm along with the IR absorptions (ν_{max}) at 1663 and 1466 cm^{-1} showed this compound to be an isoflavonoid, as supported by the characteristic ^{1}H and ^{13}C NMR resonances at $\delta_{H\text{-}2}$ 7.94 and $\delta_{C\text{-}2}$ 155.2 for this type of natural product [18,19]. The NMR data (Table 1) were very similar to those of compound **5**, except for the loss of one methoxy group signal and the appearance of one additional aromatic proton signal at δ_C 95.12 and δ_H 6.41, which indicates that one methoxy group of compound **5** may be replaced by an aromatic proton to obtain compound **6**. The HMBC correlations (Fig. 2) from the aromatic proton signal at δ_H 6.41 to C-4a (δ_C 105.83), C-5 (δ_C 160.93), C-7 (δ_C 162.72) and C-8 (δ_C 107.82) suggested that the aromatic proton was attached to C-6. Therefore, compound **6** was determined to be 8-(γ, γ-dimethylallyl)-5,4′-dihydroxy-7,2′,5′-trimethoxyisoflavone, and it was named pachyvone E.

The physical nature of isoflavones has a close relationship with their structure. In these newly isolated isoflavones, compounds **1**, **2,** and **4** were reported as white powders, while compounds **5** and **6** were reported as yellow powders. Careful investigation of the differences in structures revealed that the presence of a 4-OH group in the yellow-colored compounds (**5** and **6**) could verify the extended conjugation system, while this 4-OH moiety is absent or replaced in the white-colored compounds.

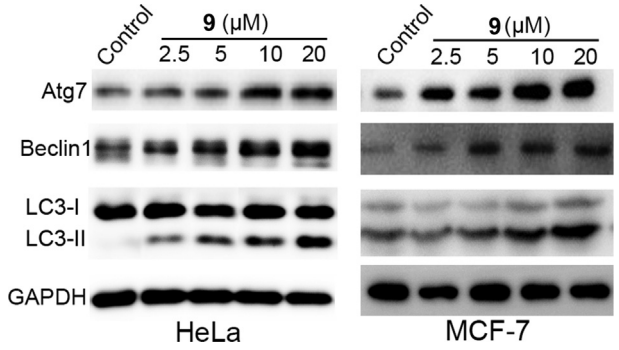


Fig. 5. Compound **9** induced autophagy in HeLa and MCF-7 cells. HeLa and MCF-7 cells were treated with the indicated concentrations of compound **9** for 24 h. Western blottings were used to measure the protein levels of LC3, Beclin1, and Atg7. GADPH was used as a loading control.

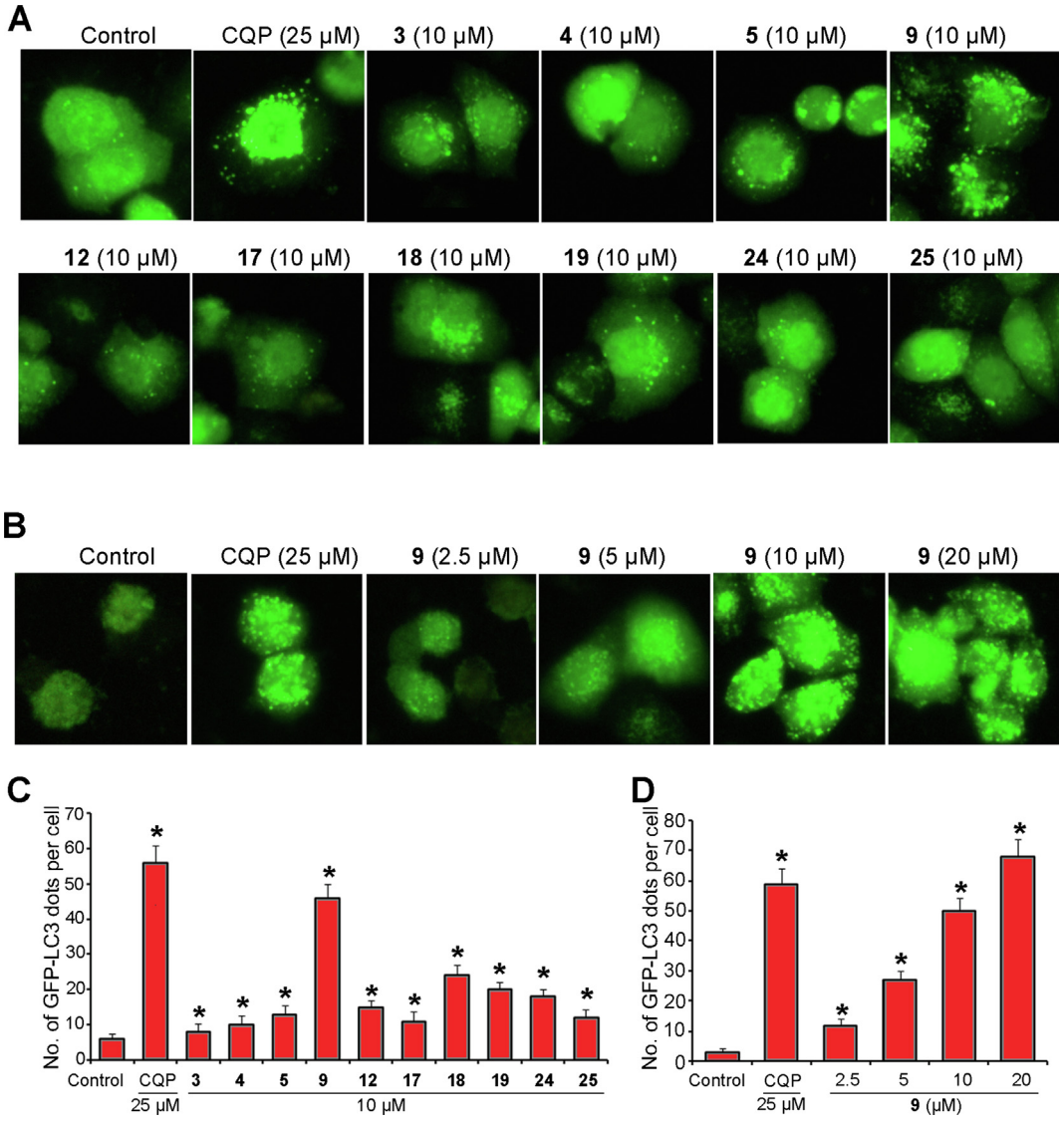


Fig. 4. Selected compounds isolated from *M. pachyloba* induced autophagy. A. HeLa cells stably expressing GFP-LC3 (GFP-LC3-HeLa) were treated with ten compounds (**3**–**5**, **9**, **12**, **17**–**19**, **24**, and **25**) at 10 μM or with chloroquine phosphate (CQP) at 25 μM for 24 h. B. GFP-LC3-HeLa cells were treated with compound **9** at 2.5, 5, 10, and 20 μM or chloroquine phosphate at 25 μM for 24 h. C and D. The number of GFP-LC3 dots/cell was quantified.

Compound **7** was obtained as a yellow powder, and its molecular formula was assigned as $C_{20}H_{18}O_7$ from the positive ion peak at *m/z* 371.1137 ([M+H]$^+$, calcd for 371.1131) in the HRESIMS, which corresponded to twelve double bond equivalents. The ^{1}H NMR spectrum (Table 2) showed similar signals to nigrolineaxanthone F [21]: two aromatic protons [δ_H 6.40 (1H, s) and 7.47 (1H, s)] and dimethylchromene protons [δ_H 5.60 (1H, d, *J* = 10.0 *Hz*), 6.73 (1H, d, *J* = 10.0 *Hz*) and 1.48 (6H, s)]. These protons were located at the same positions as nigrolineaxanthone F according to their HMBC correlations. The major difference between compound **7** and nigrolineaxanthone F was that compound **7** exhibited two more methoxy group signals at δ_H 4.05 and 4.16 and nigrolineax-

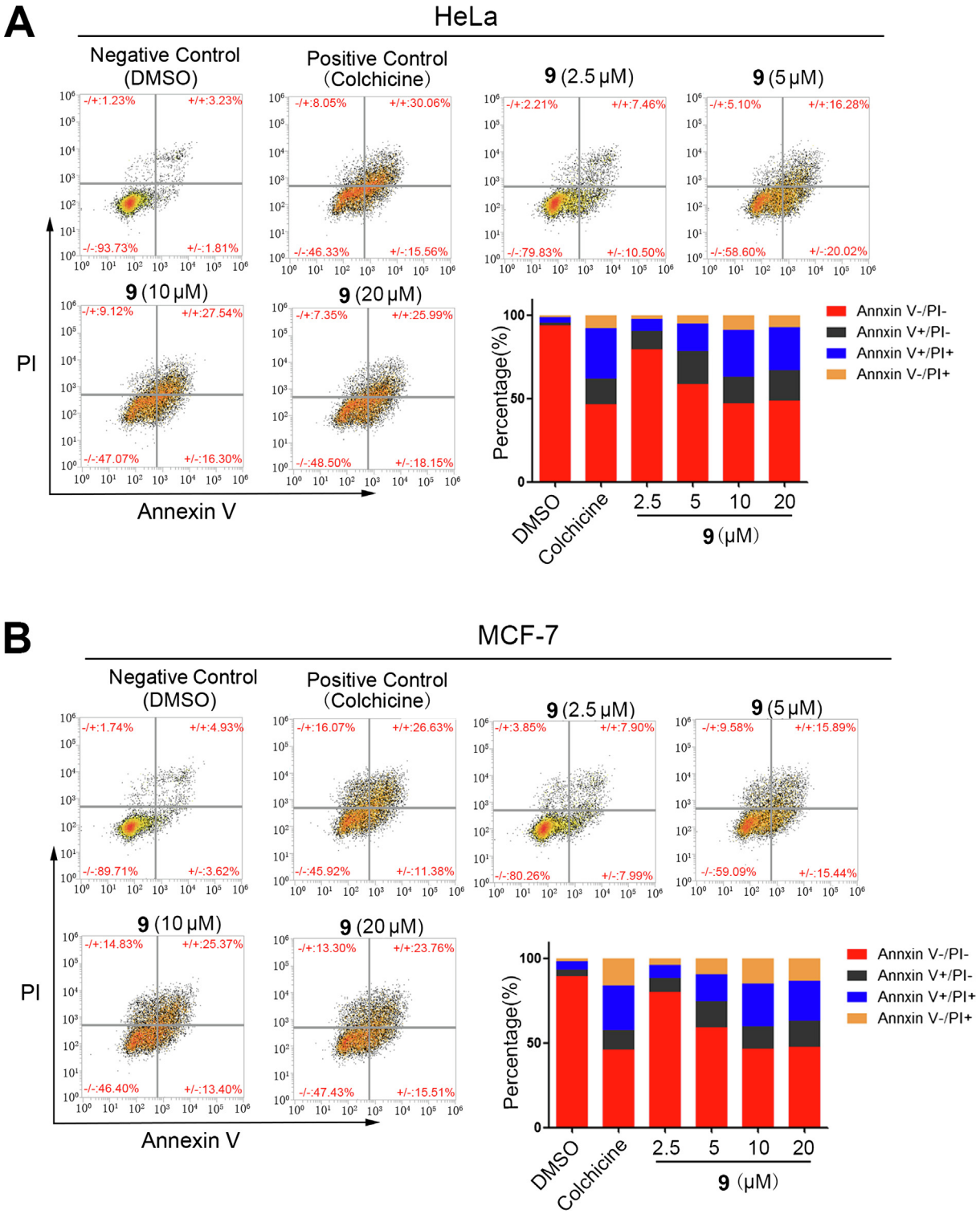


Fig. 6. Quantitative analysis of apoptosis using the Annexin V/PI double-staining assay and flow cytometry calculations. (A) HeLa cells were treated with compound **9** at different concentrations (0, 2.5, 5.0, 10.0, and 20.0 μM) or 1 μM colchicine for 48 h; the histogram shows the percentages of viable cells (PI−/Annexin V−), necrotic cells (PI+/Annexin V−), early apoptotic cells (PI−/Annexin V+), and late apoptotic cells (PI+/Annexin V+). (B) MCF-7 cells were treated with compound **9** at different concentrations (0, 2.5, 5.0, 10.0, and 20.0 μM) or 1 μM colchicine for 48 h; the histogram shows the percentages of viable cells (PI−/Annexin V−), necrotic cells (PI+/Annexin V−), early apoptotic cells (PI−/Annexin V+), and late apoptotic cells (PI+/Annexin V+).

anthone F had two more vinyl signals at δ_H 7.39 (1H, d, *J* = 8.5 *Hz*) and 7.28 (1H, dd, *J* = 8.5 *Hz*, *J* = 3.0 *Hz*), which suggests that the vinyl protons were substituted by these two methoxy groups. This result was corroborated by the HMBC correlations (Fig. 2) from the proton resonance at δ_H 7.47 (H-8) to C-6 (δ_C 145.6). Therefore, the structure of compound **7** was determined to be 1,7-dihydroxy-5,6-dimethoxy-6′,6′-dimethylpyrano (2′,3′:3,4) xanthone, and it was named pachythone A.

Based on the spectroscopic data and comparisons with the data found in the literature, the known compounds were identified as 8-prenylmilldurone (**3**) [22], 6-methoxycalpogonium isoflavone A (**8**) [23], durmillone (**9**) [24], durallone (**10**) [25], ichthynone (**11**) [8], millesianin C (**12**) [26], toxicarol isoflavone (**13**) [27], cladrastin (**14**) [28], dalpatein (**15**) [29], 7-hydroxy-2′,4′,5′,6-tetramethoxy-isoflavone (**16**) [30], 3,9-dihydroxypterocarp-6a-en (**17**) [31], dehydromaackiain (**18**) [32], flemichapparin B (**19**) [33], (−)-medicarpin (**20**) [34], (−)-maackiain (**21**) [35], (−)-variabilin (**22**) [36], (−)-pisatin (**23**) [37], dalbinol (**32**) [38], (−)-sativin (**25**) [39], (−)-dehydrodiconiferyl alcohol (**26**) [40], (+)-vomifoliol (**27**) [41], and dihydrophaseic acid (**28**) [42].

Primary screening for cytotoxic compounds

Flavones have shown cytotoxic activity toward cancer cells such as HeLa and MCF-7 cell lines [43,44]. Therefore, the primary cytotoxic activities of 28 compounds were tested on HeLa and MCF-7 cells by MTT assay. As shown in Fig. 3, ten compounds (**3**–**5**, **9**, **12**, **17**–**19**, **24**, and **25**) showed growth inhibition of HeLa and MCF-7 cells at a 50 μM concentration, while the other compounds possessed no activity. Notably, compounds **4**, **9**, and **12** are the most active compounds.

Cytotoxic activities of selected compounds on cancer cells and normal cells

Isoflavones are known to be phytoestrogens, and thus, an estrogen receptor-positive cell line (MCF-7) and estrogen receptor-negative cell line (MDA-MB-231) together with other cancer cell lines were used in this study. The cytotoxic activities of the ten active compounds were evaluated in five cancer cell lines (HeLa, HepG2, MCF-7, Hct116, and MDA-MB-231) and one normal cell line (HUVEC) using doxorubicin as a positive control. Cancer cells were treated with increasing concentrations of the compounds (0, 2.5, 5, 10, 20, and 40 μM), and normal cells were treated with the compounds at 50 μM for 72 h. Cell viability was examined by the MTT assay. The IC_{50} values of the ten compounds were calculated and are presented in Table 3. Compounds **4**, **9**, and **12** showed better anticancer activities than the other compounds. These compounds showed no selectivity on estrogen receptor-negative and estrogen receptor-positive cells, implying that these compounds exhibit no activity on estrogen receptors. Notably, all of these compounds had no activity against normal cells, suggesting that these compounds are safe anticancer candidate compounds.

Compound 9 induced autophagy in HeLa and MCF-7 cells

Numerous flavonoids mediate cell death using an autophagy-dependent pathway [45–47]. Here, a GFP-LC3-HeLa cell line was used to investigate whether the cytotoxicity of the ten compounds was associated with autophagy. LC3 is an autophagy marker protein that forms autophagosomes during autophagy induction [48]. Autophagosome dots, indicating aggregated LC3 protein, were directly observed in GFP-LC3-HeLa cells stably expressing GFP-labeled LC3 proteins using a fluorescence microscope. GFP-LC3-HeLa cells were treated with the ten compounds for 24 h at 10 μM. Chloroquine phosphate treatment-induced LC3 dots were used as the observation control. Fig. 4 shows that chloroquine phosphate induced an obvious increase in the GFP-LC3 dots, which indicates the appearance of autophagosomes. All the tested compounds produced an increase in GFP-LC3 dots, and compound **9** (durmillone) showed the best activity. Durmillone (**9**) induced GFP-LC3 punctation in a dose-dependent manner. These results suggest that the ten compounds induce autophagy and that compound **9** exhibits the best activity.

The expression of autophagy-associated proteins, such as LC3-II, Beclin1, and Atg7 [48], was detected in HeLa and MCF-7 cells treated with compound **9** using Western blotting analysis, further verifying this result. The results indicated that compound **9** remarkably and dose-dependently upregulated the expression levels of LC3-II, Beclin1, and Atg7 in HeLa and MCF-7 cells (Fig. 5). Taken together, these results demonstrate that compound **9** induced obvious autophagy in HeLa cells. As compound **9** exhibited the best activity in inducing autophagy, it was chosen for further study.

Compound 9 induced apoptosis in HeLa and MCF-7 cells.

Plasma membrane surface Annexin V is a marker of apoptosis, and propidium iodide (PI) is used to detect late apoptotic cells, so the combined PI/Annexin V double staining method is a classic method of apoptosis detection. In the present study, the PI/Annexin V double staining flow cytometric assay was used to further investigate compound **9** induction of apoptosis in cancer cells. Colchicine, a tubulin inhibitor, was employed as a positive control. The results revealed that compound **9** induced apoptosis in HeLa and MCF-7 cells in a concentration-dependent manner. The apoptosis rates were 5.04%, 45.62%, 17.96%, 36.30%, 43.84%, and 44.14% for HeLa cells treated with the negative control (DMSO), positive control (colchicine), and 2.5, 5, 10 and 20 μM compound **9**, respectively. Additionally, the apoptosis rates in MCF-7 cells were 8.55%, 38.01%, 15.89%, 31.33%, 38.77%, and 39.27% for the negative control (DMSO), positive control (colchicine), and 2.5, 5, 10 and 20 μM compound **9**, respectively (Fig. 6).

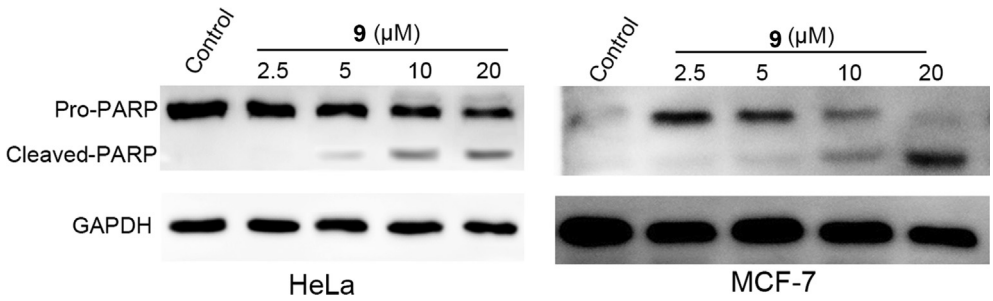


Fig. 7. Compound **9** induced apoptosis in HeLa and MCF-7 cells. HeLa and MCF-7 cells were treated with the indicated concentrations of compound **9** for 48 h. Western blottings were used to measure protein levels of PARP. GADPH was used as a loading control.

To further verify the induction of apoptotic events by compound **9** in cancer cells, the level of poly ADP-ribose polymerase (PARP) cleavage, which is a marker of late apoptotic events, was determined using Western blotting in HeLa and MCF-7 cells. Cells treated with 2.5, 5, 10 and 20 μM compound **9** for 48 h induced obvious PARP cleavage in a concentration-dependent manner (Fig. 7), which further demonstrates that compound **9** also induces apoptosis in cancer cells.

In summary, the results of the present study suggest that compound **9** mediates cytotoxic activity through the combined action of apoptosis and autophagy.

Conclusions

In this study, systematic separation and subsequent pharmacological activity studies were carried out to obtain cytotoxic natural products from the dried stems of *M. pachyloba*. Ten cytotoxic natural products (**3**–**5**, **9**, **12**, **17**–**19**, **24**, and **25**) from the dried stems of *Millettia pachyloba* Drake were obtained, and compound **9** exhibited the highest cytotoxic activity through the combined action of apoptosis and autophagy.

Phytochemical investigation of the stems of *Millettia pachyloba* led to the isolation of five previously undescribed isoflavones (**1**, **2**, and **4**–**6**), one previously undescribed xanthone (**7**), and twenty-two known compounds. These findings enrich the diversity of chemical components of the genus *Millettia*. Biological assays to examine the cytotoxic effects of ten compounds (**3**–**5**, **9**, **12**, **17**–**19**, **24**, and **25**) showed that these compounds produced cytotoxic effects in HepG2, MCF-7, and HeLa cell, with IC_{50} values ranging from 5 to 40 μM. Notably, durmillone (**9**) induced cytotoxicity through the combined action of apoptosis and autophagy in HeLa cells, which suggests that flavonoids are responsible for the cytotoxicity of *M. pachyloba*.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2019.06.002.

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