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Synthesis and biological evaluation of scopoletin derivatives

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ABSTRACT

A series of new scopoletin derivatives were designed and synthesized. Their anti-proliferative effect was initially evaluated against various human cancer cell lines. Among the tested compounds, **A1**, **A2**, and **D6** showed significant anti-proliferative activities. Angiogenesis was detected by endothelial cell migration assay and tube formation study. The results showed that **A1**, **A2**, and **D6** inhibited the vascular endothelial growth factor (VEGF)-stimulated proliferation, migration, and tube formation of human umbilical vein endothelial cells in vitro. Moreover, they inhibited the vessel growth in the chorioallantoic membrane in vivo. This inhibition was correlated with a significant decrease in the VEGF-triggered phosphorylated forms of ERK1/2 and Akt. In summary, these findings strongly suggested that these scopoletin derivatives might be structurally novel angiogenesis inhibitors.

1. Introduction

Angiogenesis has gained increasing attention because of its crucial function in the initiation and progression of many diseases such as cancer and RA. Hence, anti-angiogenic intervention is becoming a promising approach for the treatment of these diseases. For example, Bevacizumab (Avastin, anti-VEGF monoclonal antibody) and Vatalanib (VEGFR inhibitor) have been clinically approved for treatment of many malignant tumors by anti-angiogenic pathways. Many TCMs that are often used for both cancer and RA treatment contain bioactive constituents with anti-angiogenic properties. For example, epigallocatechin-3-gallate (EGCG), which is a main polyphenolic constituent of green tea, functions as an angiogenesis inhibitor by modulating the protease activity during endothelial morphogenesis;[1] sinomenine, which is the main alkaloid constitute that is present in Sinomenium acutum and has been used to treat RA for over one thousand years in China, is able to alleviate angiogenesis in vitro and in vivo;[2] scopoletin, which is a coumarin compound with multiple bioactivities, is the main active constituent of *Erycibe obtusifolia* Benth stems that has long been used for RA treatment in TCM. Preliminary pharmacological experiments found that scopoletin can inhibit angiogenesis. Therefore, a series of new scopoletin derivatives was designed and synthesized.

Scopoletin (6-methoxy-7-hydroxycoumarin, Sco) is one of the main coumarin constituents that occur in the stems of *Erycibe obtusifolia* Benth, which is commonly used in traditional Chinese medicine (TCM) for treating various rheumatoid diseases. Sco possesses a wide range of biological activities such as anti-inflammatory, hypouricemic, and antioxidant activities.[3] Reports have indicated that several coumarin-type compounds block angiogenesis by inhibiting the endothelial cell growth.[4,5] Previous data also demonstrated that scopoletin and its derivatives have anti-tumor and anti-angiogenic activities.[6] These findings pave the way for future studies on the anti-angiogenic potential and related mechanisms of scopoletin derivatives.

2. Results and discussion

2.1. Chemistry

Scopoletin (6-methoxy-7-hydroxycoumarin) was synthesized according to previous method.[6] Briefly, 2,4-dihydroxy-5-methoxybenzaldehyde was obtained in a one-step reaction from 2,4,5-trimethoxybenzaldehyde by reaction with aluminium(III) chloride in dichloromethane, followed by acid hydrolysis. Treatment of 5 with malonic acid in pyridine for 24 h at room temperature (rt) using phenylamine as catalysts afforded 7-hydroxy-6-methoxy-2-oxo-2*H*-chromene-3-carboxylic acid in 86% yield. Then heating 7-hydroxy-6-methoxy-2-oxo-2*H*-chromene-3-carboxylic acid in a pyridine/ethylene glycol mixture (1:1.1) to reflux for 3 h gave scopoletin.

The design of class A and class B compounds: the reaction in the presence of scopoletin and alkyl or aromatic halides gave the products **A1**–**A7** or **B1**–**B6**. Dehydrohalogenation was happened under

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the conditions of potassium carbonate as acid binding agent, generating a series of derivative.

The design of class C compounds: the reaction in the presence of **B1** (7-(2-bromoethoxy)-6-methoxycoumarin) and different secondary amine gave the products **C1**–**C6**.

The design of class D compounds: the reaction in the presence of **B5** (7-(oxiran-2-methoxy)-6-methoxycoumarin) and different secondary amine gave the products **D1**–**D6**.

2.2. Effect of scopoletin and scopoletin derivatives on endothelial cell proliferation

The anti-proliferative activity of scopoletin and 25 scopoletin derivatives were tested with human umbilical vein endothelial cells (HUVEC) (Fig. 1). As shown in Table 1, the scopoletin derivatives **A1**, **A2**, **B5**, and **D6** exerted the strongest anti-proliferative effect with an IC_{50} of 16.5, 9.8, 4.8, and 24.2 μM. The anti-angiogenic activity of **A1**, **A2**, and **D6** were further studied, whereas **B5** was not used because of its instability.

2.3. Effect of scopoletin, A1, A2, and D6 against the proliferation of normal cells and tumor cells

We performed the cell proliferation assay to evaluate the cytotoxicity of scopoletin derivatives against both normal (peripheral blood mononuclear cells, PBMC) and tumor cell lines (human pancreatic carcinoma cell line PANC-1, human pancreatic adenocarcinoma cell line BxPC-3, human breast adenocarcinoma cell line MDA-MB-231, human breast ductal carcinoma cell line MDA-MB-435, human hepatic carcinoma HepG2, human colorectal carcinoma cell line HCT 116, human gastric carcinoma cell line MKN45, human lung carcinoma cell line A549, human epithelial carcinoma cell line A431, human glioma cell line SHG-44, human chronic myelogenous leukemia cell line K562, human promyelocytic leukemia cell line HL-60, and human T lymphoma cell line HuT 78). The concentration of the scopoletin derivatives with 50% inhibition (IC_{50}) on the cells was determined, and the results are summarized in Table 2. The results showed that **A2** possessed higher cytotoxicity to MDA-MB-231 cells while **A1** was the most effective on killing PANC-1 cells. **D6** showed higher cytotoxicity to many cancer cell lines than **A1** and **A2**.

2.4. A1, A2, and D6 reduced VEGF-stimulated HUVEC migration

The migration of the endothelial cells is a prerequisite for angiogenesis. VEGF is a potent stimulator for the migration of endothelial cell.[7] We determined the effect of **A1**, **A2**, and **D6** on HUVEC migration stimulated with $VEGF_{165}$ using the Boyden Chamber assay because scopoletin derivatives can inhibit HUVEC proliferation in a concentration-dependent manner.[6] After $VEGF_{165}$ stimulation for 20 h, a large number of cells migrated to the lower side of the Millicell chamber filter (Millipore, USA). However, the addition of scopoletin derivatives (25 μmol/L) to the top chamber significantly reduced the number of migrated cells (Fig. 2A). Comparatively, scopoletin did not show the same inhibitory effect at the same concentration.

2.5. A1, A2, and D6 suppressed tube formation of HUVECs

The maturation of the migrated endothelial cells into a tube-like structure is critical for the formation of functional vessels. We evaluated the effect of the scopoletin derivatives on the formation of the tube-like structures by plating HUVECs with $VEGF_{165}$ (10 ng/mL) on the matrigel. At 5 h of plating, the migrated endothelial cells rapidly aligned with one another, and formed into tube-like structures. This process required cell–matrix interaction, intercellular communication, and cell motility. However, the migration effect of $VEGF_{165}$ was significantly inhibited by **A1**, **A2**, and **D6**. Incomplete sprouting and branching or broken network between the tubes of HUVECs were observed (Fig. 2B). These results (the migration assay and tube formation) indicated that scopoletin derivatives (**A1**, **A2**, and **D6**) have the ability to block VEGF-induced in vitro angiogenesis far better than scopoletin (no inhibitory effect at the same concentration).

2.6. A1, A2, and D6 inhibited angiogenesis in vivo

The anti-angiogenic activity of the scopoletin derivatives on the chorioallantoic membrane (CAM) was examined using a chicken embryo model to investigate its responses to angiogenesis. Using this model, we additionally examined the potential in vivo anti-angiogenic activity of the scopoletin derivatives. New blood vessels formed on CAMs in the control group, whereas the addition of **A1**, **A2**, and **D6** at 5 nmol per egg incubated for 48 h showed a notable restraint. At 10 nmol per egg, the inhibition became more prominent (Fig. 3). These results demonstrate that **A1**, **A2**, and **D6** were able to suppress angiogenesis in the chicken embryos.

2.7. A1, A2 and D6 inhibited expression of MMPs

Gelatinases such as MMP-2 and MMP-9 have important functions in tumor invasion and cell migration.[8] In the present study, we detected the protein expression of these two MMPs in PANC-1 cells treated by scopoletin and scopoletin derivatives. In the control group, the cultured PANC-1 constitutively expressed the MMP-9 protein, whereas in the treated group, the protein expression of MMP-9 and MMP-2 were gradually down-regulated (Fig. 4).

2.8. Influence of A1, A2, and D6 on VEGF signaling pathways

The p44/42 mitogen-activated protein kinase (ERK1/2 MAPK) cascade is the main pathway responsible for the VEGF-induced

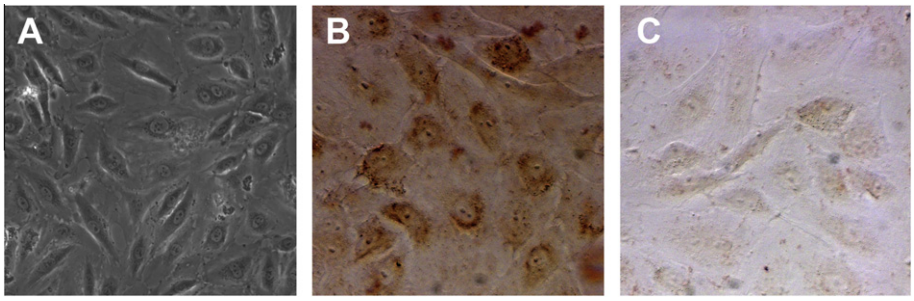
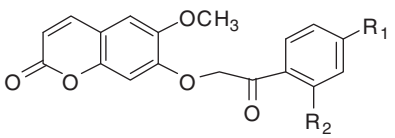


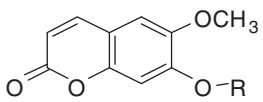
Figure 1. HUVEC. (A) photograph of HUVECs (200×); (B) characterization of HUVECs by assessing the Factor VIII Related Antigen (von Willebrand factor, vWF) expression (400×); (C) negative control group (immunocytochemical reaction with secondary antibody) (400×).

Table 1
Anti-proliferation activity of scopoletin and scopoletin derivatives (**A1**–**D6**) on HUVECs

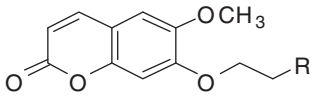
A



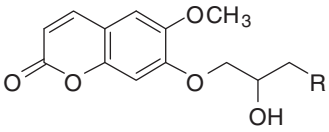
B



C



D



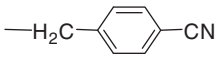
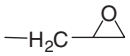
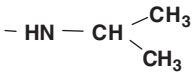
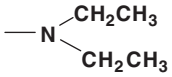
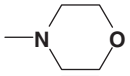
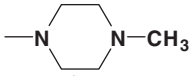
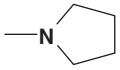
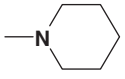
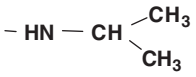
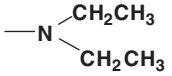
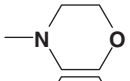
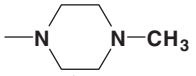
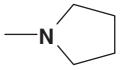
Compound	Structure	R1	R2	Inhibition rate (%)		IC_{50} (μM)
				At 10 μM	At 100 μM	
Scopoletin				0.0 ± 1.5	2.1 ± 0.2	/
A1	A	Br	H	13.2 ± 9.4	97.5 ± 0.7	16.8 ± 1.4
A2	A	Cl	H	62.6 ± 5.6	83.8 ± 1.6	10.0 ± 0.8
A3	A	NO_2	H	4.1 ± 2.3	27.2 ± 5.3	/
A4	A	OCH_3	H	7.6 ± 6.2	85.6 ± 0.2	37.5 ± 0.1
A5	A	F	H	3.6 ± 1.2	16.6 ± 2.2	/
A6	A	Cl	Cl	0.0 ± 4.6	42.8 ± 2.5	/
A7	A	H	H	3.4 ± 2.2	83.6 ± 0.2	62.4 ± 3.3
B1	B			4.9 ± 1.6	5.6 ± 1.1	/
B2	B	$-CH_2CH_2CH_2CH_2Br$		0.3 ± 4.4	3.3 ± 0.9	/
B3	B			0.1 ± 1.5	9.4 ± 1.0	/
B4	B	$-CH_2CH_2Br$		11.2 ± 0.9	16.9 ± 1.9	/
B5	B	$-CH_2CH_2Cl$		62.2 ± 0.1	95.5 ± 0.5	5.3 ± 0.5
B6	B	$-CH_2CH_2CH_2Cl$		1.2 ± 2.9	79.5 ± 0.4	42.4 ± 1.1
C1	C			0.1 ± 1.6	0.1 ± 4.0	/
C2	C			0.1 ± 4.9	3.1 ± 0.3	/
C3	C			0.0 ± 1.5	1.9 ± 0.5	/
C4	C			0.0 ± 2.5	12.0 ± 0.5	/
C5	C			1.9 ± 1.1	7.9 ± 0.7	/
C6	C			0.1 ± 2.9	6.1 ± 0.7	/
D1	D			0.1 ± 2.8	6.3 ± 0.7	/
D2	D			0.5 ± 2.3	0.3 ± 0.1	/
D3	D			0.4 ± 5.2	0.1 ± 0.1	/
D4	D			0.1 ± 1.4	3.4 ± 0.6	/
D5	D			0.5 ± 3.7	0.3 ± 2.6	/

Table 1 (*continued*)

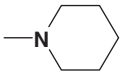
Compound	Structure	R1	R2	Inhibition rate (%)		IC_{50} (μM)
				At 10 μM	At 100 μM	
D6	D			31.4 ± 0.7	80.7 ± 2.3	24.2 ± 1.6

Table 2
IC_{50} cytotoxicity values (μM) of scopoletin derivatives against PBMC and tumor cell lines

Compound	PBMC	PANC-1	BxPC-3	MDA-MB-231	MDA-MB-435	HepG2	HCT 116	MKN45	A549	A431	SHG-44	K562	HL-60	HuT 78
Scopoletin	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
A1	>100	63.7 ± 1.6	>100	47.0 ± 1.4	>100	90.4 ± 1.1	>100	>100	>100	>100	>100	70.0 ± 1.2	90.4 ± 1.5	93.7 ± 2.4
A2	>100	19.8 ± 2.1	>100	>100	92.4 ± 2.2	56.3 ± 0.5	59.5 ± 1.3	99.2 ± 1.9	>100	>100	>100	52.5 ± 1.2	65.7 ± 0.7	62.1 ± 1.5
D6	>100	>100	35.0 ± 1.3	51.6 ± 1.2	63.0 ± 0.7	83.9 ± 1.4	26.1 ± 1.1	78.9 ± 1.7	42.7 ± 0.4	93.9 ± 0.9	42.8 ± 1.6	43.7 ± 1.6	30.2 ± 1.3	46.6 ± 1.0

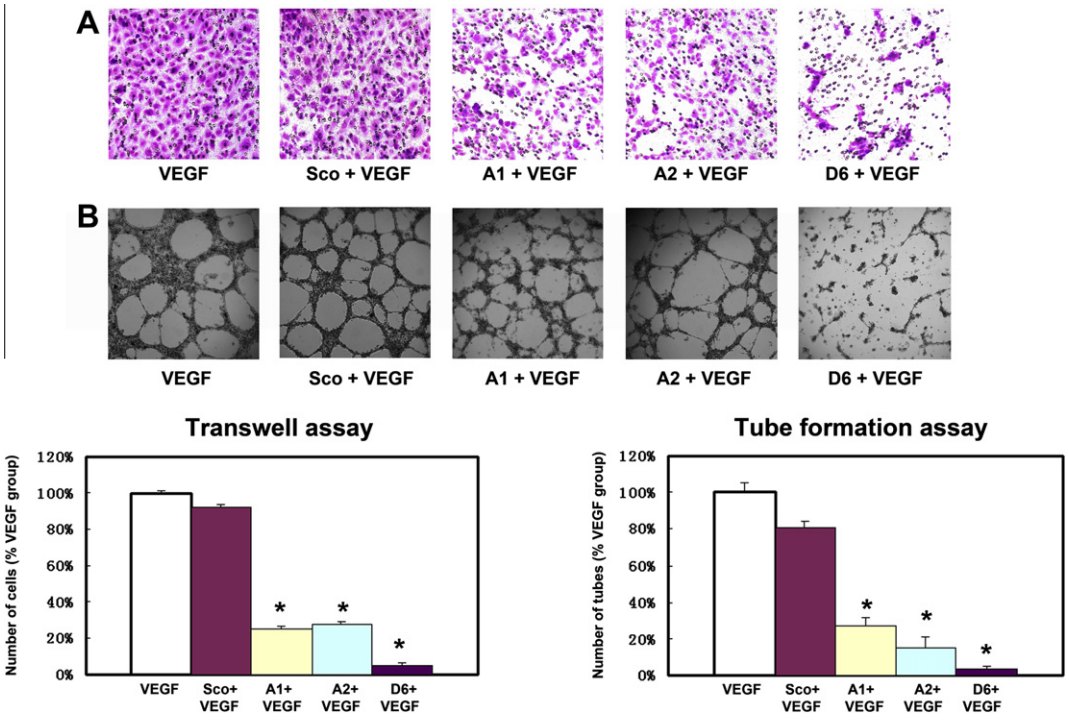


Figure 2. Scopoletin derivatives (**A1**, **A2**, and **D6**) inhibited VEGF-induced migration and tube formation on HUVECs. (A) HUVECs were plated onto 24-well Millicell chamber and stimulated with $VEGF_{165}$ in the presence or absence of scopoletin derivatives (**A1**, **A2**, and **D6**). After 20 h, the cells migrated into the bottom of the membrane and were photographed (100×); (B) HUVECs were seeded onto 96-well plate previously coated with matrigel and stimulated with $VEGF_{165}$ in the presence or absence of scopoletin derivatives (**A1**, **A2** and **D6**) for 5 h. The tube-like structure was photographed using a microscope (200×). Compared with the control group, $^{*}P$ <0.01.

proliferation of endothelial cells, whereas the stress-activated protein kinase-2 (p38 MAPK) pathway is principally responsible for conveying VEGF signals to microfilaments, which induces the actin cytoskeleton that regulates cell migration to rearrange. Both pathways are the key down-streams of VEGF signal transductions.[9,10] Our results showed that **A1**, **A2**, and **D6** antagonized the VEGF-induced angiogenesis in HUVECs. This result prompted us to investigate if the anti-angiogenic effect of the scopoletin derivatives results from its interaction with the VEGF signaling pathways. Therefore, we examined the effects of **A1**, **A2**, and **D6** on ERK1/2, Akt, src, and p38 MAPK. As shown in Figure 5, ERK1/2, Akt were phosphorylated by the addition of the exogenous VEGF to HUVECs. However, pre-treatment with **A1**, **A2**, or **D6** blocked the VEGF-induced phosphorylation of Akt without affecting the src and p38. The p-ERK1/2 was down-regulated by **A1** and **D6**, but had no effect on **A2**. In all cases, the total steady-state protein levels remained unchanged, which suggests that several VEGF receptors may be blocked by **A1**, **A2**, and **D6** and leads to the interruption of VEGF-triggered signaling.

3. Conclusions

Angiogenesis is a highly regulated process that involves a complex cascade of events.[11,12] VEGF is an endothelial cell-specific mitogenic and chemotactic agent,[13] and its inhibitory effects on endothelial cells should block the whole process of angiogenesis. Based on this target, a series of scopoletin and scopoletin derivatives were synthesized and tested their anti-angiogenesis activities.

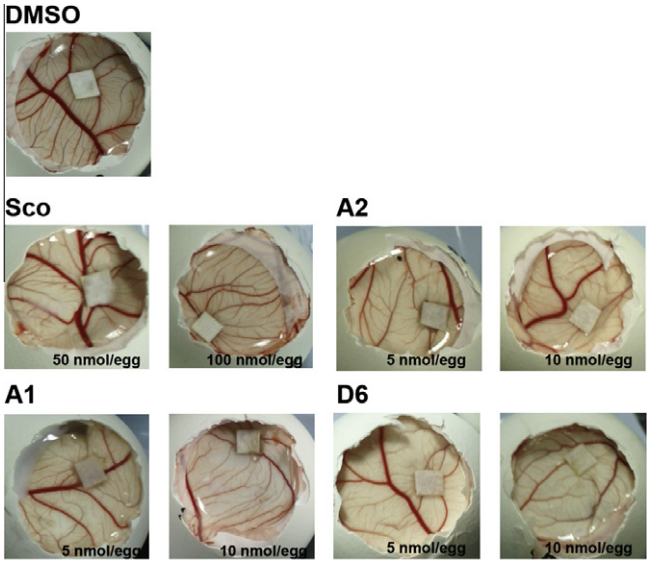


Figure 3. Scopoletin derivatives (**A1**, **A2**, and **D6**) inhibited angiogenesis in vivo. Photographs are representative pictures from three independent experiments.

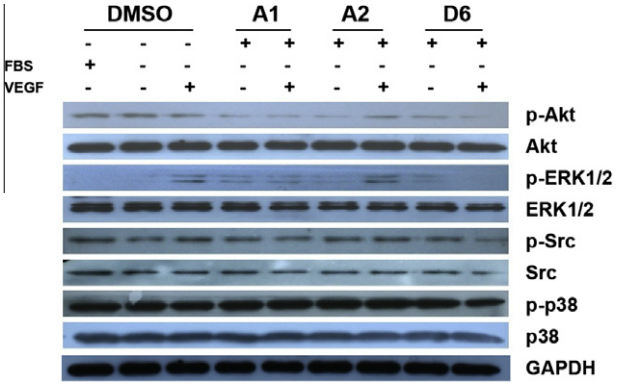


Figure 5. Effect of scopoletin derivatives (**A1**, **A2**, and **D6**) on VEGF-stimulated activation of related signaling pathways. Western blot assays were performed to examine the phosphorylation levels of src, ERK1/2, Akt, and p38 MAPK.

Cytotoxicity assay indicated that most of scopoletin derivatives showed potent cytotoxicity against both tumor cells and HUVEC compared to scopoletin, especially **A1**, **A2**, and **D6**. Compared with scopoletin, **A1**, **A2**, and **D6** exhibited significant inhibition of both proliferation and VEGF-induced migration and invasion at concentrations without evident cytotoxicity. In conclusion, the preliminary in vitro activities and in vivo activities of these compounds possess potential for design of better future molecules targeting tumor angiogenesis. Further studies on their mechanism of anti-angiogenesis activity are in progress.

4. Experimental protocols

4.1. Synthesis and characterization

4.1.1. General

All reagents were purchased from the Shanghai Chemical Reagent Company. Column chromatography: silica gel 60 (200–300 mesh). Thin-layer chromatography: silica gel 60 at F254 plates (250 mm; Qingdao Ocean Chemical Company, China). Melting point: capillary tube; uncorrected. IR spectra: Shimadzu FTIR-8400S spectrophotometer. NMR spectra: Bruker ACF-300 Q apparatus at 300 MHz for ^{1}H NMR (internal standard, TMS). Mass spectrometry: Hewlett-Packard 1100 LC/MSD spectrometer; in *m*/*z*. Elemental analyses: CHN–O-Rapid instrument.

4.1.2. Preparation of 2,4-dihydroxy-5-methoxybenzaldehyde

To a stirred suspension of aluminum(III) chloride (80 g, 0.60 mol) in dry dichloromethane (400 mL), a solution of 2,4,5-trimethoxybenzaldehyde (20 g, 0.1 mol) in dry dichloromethane (100 mL) was added dropwise, then the mixture was refluxed for 4 h. The reaction mixture was cooled and poured onto 500 g of ice to which 100 ml of concentrated hydrochloric acid was added. The organic layer was separated and the aqueous phase was extracted twice with dichloromethane (200 mL). The combined organic layer was washed with saturation salt solution, dried over magnesium sulfate, evaporated and recrystallized from toluene to give 2,4-dihydroxy-5-methoxybenzaldehyde as yellow solid (15.79 g, 92.12%), mp 152–153 °C.

^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 9.66 (s, 1H, CHO), 11.31 (s, 1H, 2-OH), 6.87 (s, 1H, 4-OH), 6.51 (s, 1H, H_6), 6.40 (s, 1H, H_3), 3.90 (s, 3H, OCH_3).

4.1.3. Preparation of 7-hydroxy-6-methoxy-2-oxo-2*H*-chromene-3-carboxylic acid

The flask was charged with 9.49 g (56 mmol) 2, 4-dihydroxy-5-methoxybenzaldehyde, 13.5 g, (130 mmol) malonic acid, 1 ml

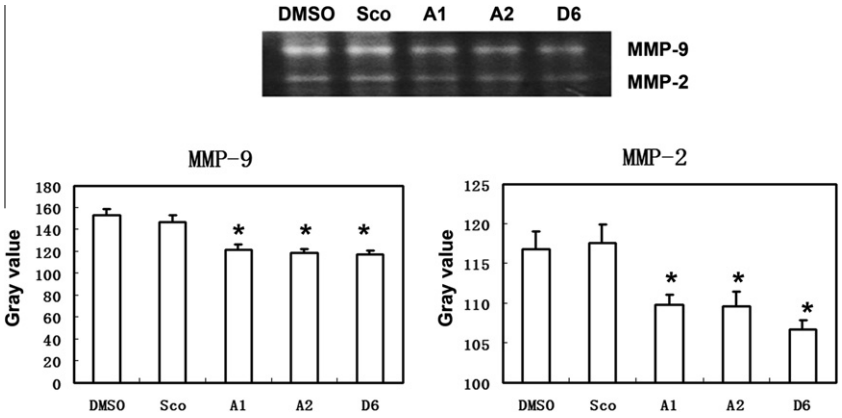


Figure 4. Gelatin zymography of MMP-9 and MMP-2. The bands indicated MMP-9 activity. MMP-9 and MMP-2 collected from the culture medium of the same number of PANC-1 cells cultured with scopoletin or scopoletin derivatives for 24 h. Compared with the control group, $^*P$ <0.01.

phenylamine and 30 mL pyridine. The resulting solution was stirred at room temperature for over 24 h and then acidified to pH 4 using dilute HCl. The precipitate was collected by suction filtration and followed by recrystallization from ethanol to give the pale yellow solid (11.49 g, 86.2%). Mp 231–232 °C.

^{1}H NMR (DMSO-d_6): δ: 3.80 (s, 3H, OCH_3), 4.04 (s, 1H, OH), 6.76 (s, 1H, C_5-H), 7.54 (s, 1H, C_8-H), 8.61 (s, 1H, C_4-H), 11.06 (s, 1H, COOH); EI-MS (m/z): 236 $[M]^+$.

4.1.4. Preparation of 7-hydroxy-6-methoxy-2*H*-chromen-2-one

3.5 g (14.8 mmol) 7-hydroxy-6-methoxy-2-oxo-2*H*-chromene-3-carboxylic acid was refluxed in 18 ml ethylene glycol and 20 ml pyridine for 3.5 h. After cooling the reaction, the mixture was acidified to pH 4 using a solution of HCl and extracted with CH_2Cl_2. The CH_2Cl_2 layers were pooled, dried (Na_2SO_4), and evaporated to give the product, followed by recrystallization from ethanol. Yellow solid (2.55 g, 89.5%); mp 201–202 °C.

^{1}H NMR (DMSO-d_6): δ: 3.79 (s, 3H, OCH_3), 6.16 (d, 1H, J = 9.4 Hz, C_3-H), 6.74 (s, 1H, C_5-H), 7.11 (s, 1H, C_8-H), 7.82 (1H, d, J = 9.4 Hz, C_4-H), 10.29 (s, 1H, OH); EI-MS (m/z): 193 $[M+H]^+$, 215 $[M+Na]^+$.

4.1.5. General procedures for the synthesis of the compounds A1–A7 and B1–B5

0.3 g (1.56 mmol) scopoletin, 1.87 mmol alkyl or aromatic halides and 0.32 g (2.34 mmol) K_2CO_3 were combined and refluxed in 20 mL aceton for 2 h, then removal of the solvent under reduced pressure. The crude product was washed with water, filtrered and dried, followed by recrystalliztion from petroleum ether and ethyl acetate.

4.1.5.1. Preparation of 7-[2-(4-bromo-phenyl)-2-oxo-ethoxy]-6-methoxy-2*H*-chromen-2-one (A1). Yield, 82.3%; white crystals, mp: 208–210 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.94 (s, 3H, OCH_3), 5.40 (s, 2H, $COCH_2O$), 6.31 (d, 1H, J = 9.3 Hz, C_3-H), 6.70 (s, 1H, C_5-H), 6.90 (s, 1H, C_8-H), 7.69 (d, 1H, J = 9.3 Hz, C_4-H), 7.66, 7.88 (dd, 4H, ArH); IR (KBr, ν): 3143, 3061, 1733, 1688, 1614, 1568, 1516, 1391, 1278, 1249, 1148 cm^{-1}; MS (ESI, m/z): $[M+Na]^+$ 413.

4.1.5.2. Preparation of 7-[2-(4-chloro-phenyl)-2-oxo-ethoxy]-6-methoxy-2*H*-chromen-2-one (A2). Yield, 87.2%; white crystals, mp: 218–220 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.95 (s, 3H, OCH_3), 5.41 (s, 2H, $COCH_2O$), 6.31 (d, 1H, J = 9.0 Hz, C_3-H), 6.71 (s, 1H, C_5-H), 6.91 (s, 1H, C_8-H), 7.64 (d, 1H, J = 9.0 Hz, C_4-H), 7.52, 7.96 (dd, 4H, ArH); IR (KBr, ν): 3414, 3059, 1732, 1695, 1614, 1568, 1518, 1388, 1278, 1250, 1150 cm^{-1}; MS (ESI, /z): $[M-H]^-$ 343.

4.1.5.3. Preparation of 6-methoxy-7-[2-(4-nitro-phenyl)-2-oxo-ethoxy]-2*H*-chromen-2-one (A3). Yield, 76.1%; white crystals, mp: 230–232 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.93 (s, 3H, OCH_3), 5.60 (s, 2H, $COCH_2O$), 6.38 (d, 1H, J = 9.6 Hz, C_3-H), 6.75 (s, 1H, C_5-H), 6.91 (s, 1H, C_8-H), 7.66 (d, 1H, J = 9.6 Hz, C_4-H), 8.17, 8.38 (dd, 4H, ArH); IR (KBr, ν): 3417, 3059, 1732, 1696, 1613, 1567, 1519, 1387, 1278, 1150 cm^{-1}; MS (ESI, m/z): $[M-H]^-$ 354.

4.1.5.4. Preparation of 6-methoxy-7-[2-(4-methoxy-phenyl)-2-oxo-ethoxy]-2*H*-chromen-2-one (A4). Yield, 58.8%; white crystals, mp: 199–201 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.94 (s, 3H, OCH_3), 5.41 (s, 2H, $COCH_2O$), 6.29 (d, 1H, J = 9.3 Hz, C_3-H), 6.69 (s, 1H, C_5-H), 6.90 (s, 1H, C_8-H), 7.62 (d, 1H, J = 9.3 Hz, C_4-H), 7.00, 7.99 (dd, 4H, ArH); IR (KBr, ν): 3414, 3030, 1711, 1684, 1601, 1566, 1515, 1390, 1273, 1240, 1147 cm^{-1}; MS (ESI, m/z): $[M-H]^-$ 339.

4.1.5.5. Preparation of 7-[2-(4-fluoro-phenyl)-2-oxo-ethoxy]-6-methoxy-2*H*-chromen-2-one (A5). Yield, 76.2%; white crystals, mp: 206–208 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): 3.94 (s, 3H, OCH_3), 5.41 (s, 2H, $COCH_2O$), 6.31 (d, 1H, J = 9.0 Hz, C_3-H), 6.70 (s, 1H, C_5-H), 6.90 (s, 1H, C_8-H), 7.63 (d, 1H, J = 9.0 Hz, C_4-H), 7.26, 8.03 (dd, 4H, ArH); IR (KBr, ν): 3415, 3061, 1732, 1601, 1568, 1388, 1279, 1237, 1147 cm^{-1}; MS (ESI, m/z): $[M-H]^-$ 327.

4.1.5.6. Preparation of 7-[2-(3,4-dichloro-phenyl)-2-oxo-ethoxy]-6-methoxy-2*H*-chromen-2-one (A6). Yield, 51.5%; white crystals, mp: 199–201 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.91 (s, 3H, OCH_3), 5.35 (s, 2H, $COCH_2O$), 6.32 (d, 1H, J = 9.6 Hz, C_3-H), 6.71 (s, 1H, C_5-H), 6.90 (s, 1H, C_8-H), 7.63 (d, 1H, J = 9.6 Hz, C_4-H), 7.40, 7.65 (dd, 4H, ArH); IR (KBr, ν): 3435, 1727, 1700, 1612, 1388, 1277, 1145 cm^{-1}; MS (ESI, m/z): $[M]^+$ 378.

4.1.5.7. Preparation of 6-methoxy-7-(2-oxo-2-phenyl-ethoxy)-2*H*-chromen-2-one (A7). Yield, 93.5%; white crystals, mp: 175–177 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.95 (s, 3H, OCH_3), 5.47 (s, 2H, $COCH_2O$), 6.30 (d, 1H, J = 9.3 Hz, C_3-H), 6.70 (s, 1H, C_5-H), 6.91 (s, 1H, C_8-H), 7.63 (d, 1H, J = 9.3 Hz, C_4-H), 7.56–7.97 (m, 5H, ArH); IR (KBr, ν): 3438, 3061, 1710, 1562, 1510, 1386, 1250, 1153 cm^{-1}; MS (ESI, m/z): $[M]^+$ 310.

4.1.5.8. Preparation of 7-(2-bromoethoxy)-6-methoxy-2*H*-chromen-2-one (B1). Yellow power, yield, 83.6%, mp 180–184 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.91 (3H, s, OCH_3), 6.32 (1H, d, J = 9.3 Hz, H3), 7.63 (1H, d, J = 9.6 Hz, H4), 6.84 (1H, s, H5), 6.89 (1H, s, H8), 4.23 (2H, t, OCH_2), 3.71 (2H, t, $BrCH_2$); IR (KBr, cm^{-1}): 3296, 3081, 2940, 1714, 1614, 1559, 1509, 1459, 1421, 1383, 1281, 1142, 1018; MS (EI, m/z): $[M]^+$ 298.

4.1.5.9. Preparation of 7-(2-chloro-ethoxy)-6-methoxy-2*H*-chromen-2-one (B2). Yield, 747%; yellow crystals, mp: 160–162 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.87 (t, 2H, $ClCH_2$), 3.91 (s, 3H, OCH_3), 4.33 (t, 2H, OCH_2), 6.32 (d, 1H, J = 9.3 Hz, C_3-H), 6.84 (s, 1H, C_5-H), 6.88 (s, 1H, C_8-H), 7.62 (d, 1H, J = 9.3 Hz, C_4-H); IR (KBr, ν): 3413, 2928, 1724, 1460, 1385, 1144, 1098 cm^{-1}; MS (ESI, m/z): $[M]^+$ 254.

4.1.5.10. Preparation of 7-(3-chloropropoxy)-6-methoxy-2*H*-chromen-2-one (B3). Yield, 65.2%; yellow crystals, mp: 118–120 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.89 (3H, s, OCH_3), 6.30 (1H, d, J = 9.6 Hz, H-3), 7.63 (1H, d, J = 9.6 Hz, H-4), 6.86 (1H, s, H-5), 6.90 (1H, s, H-8), 4.23 (2H, t, OCH_2), 2.36 (2H, m, C–CH_2–C), 3.78 (2H, t, $ClCH_2$); IR (KBr, cm^{-1}): 3414, 3067, 2926, 2857, 1716, 1613, 1560, 1509, 1383, 1257, 1141, 1091; MS (EI, m/z): $[M]^+$ 268.

4.1.5.11. Preparation of 7-(4-bromo-butoxy)-6-methoxy-2*H*-chromen-2-one (B4). Yield, 68.8%; white crystals, mp: 68–70 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 2.07 (S, 4H, CH_2CH_2), 2.36 (m, 2H, CCH_2C), 3.51 (t, 2H, $BrCH_2$), 3.89 (s, 3H, OCH_3), 4.23 (t, 2H, OCH_2), 6.30 (d, 1H, J = 9.3 Hz, C_3-H), 6.80 (s, 1H, C_5-H), 6.86 (s, 1H, C_8-H), 7.63 (d, 1H, J = 9.3 Hz, C_4-H); IR (KBr, ν): 3453, 2922, 2862, 1735, 1561, 1424, 1275, 1143, 1090 cm^{-1}; MS (ESI, m/z): $[M]^+$ 326.

4.1.5.12. Preparation of 6-methoxy-7-(oxiran-2-ylmethoxy)-2*H*-chromen-2-one (B5). Yield, 77.4%; white crystals, mp: 147–150 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.91 (3H, s, OCH_3), 6.31 (1H, d, J = 9.3 Hz, H_3), 7.63 (1H, d, J = 9.3 Hz, H_4), 6.87 (1H, s, H_5), 6.89 (1H, s, H_8), 2.93, 2.97 (2H, d, J = 3.9 Hz, CH_2), 3.42 (1H, m, CH), 4.06–4.39 (2H, d, J = 5.7 Hz, C–CH_2–O); IR (KBr, cm^{-1}): 3417, 3010, 1713, 1615, 1560, 1511, 1387, 1276, 1247, 1168; MS (EI, m/z): $[M]^+$ 248.

4.1.6. General procedures for the synthesis of the target compounds C1–C6

A mixture of 0.3 g (0.001 mmol) **B1**, 0.0012 mol amine were dissolved in 20 ml ethanol. And the mixture was heated under reflux for 24 h, after which it was cooled to room temperature. After evaporation of solvent, residual was subjected to silica gel column, and chromatographed with eluent (ethyl acetate/hexane 1:2) to yield the desired product.

4.1.6.1. Preparation of 7-(2-(diisopropylamino)ethoxy)-6-methoxy-2*H*-chromen-2-one (C1). Yield, 72.2%; white crystals, mp: 205–208 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.92 (3H, s, OCH_3), 6.30 (1H, d, J = 9.6 Hz, H_3), 7.61 (1H, d, J = 9.3 Hz, H_4), 6.84 (1H, s, H_5), 6.87 (1H, s, H_8), 1.21 (6H, d, J = 6.3 Hz, NCH $(CH_3)_2$), 3.03 (1H, m, NCH $(CH_3)_2$), 3.16 (2H, t, N–CH_2–C), 4.25 (2H, t, C–CH_2–O); IR (KBr, cm^{-1}): 3417, 3019, 2957, 2886, 1716, 1614, 1560, 1511, 1456, 1383, 1275, 1155, 1098; MS (ESI, *m*/*z*): $[M+H]^+$ 278.

4.1.6.2. Preparation of 7-(2-(diethylamino)ethoxy)-6-methoxy-2*H*-chromen-2-one (C2). Yield, 68.7%; yellowish crystals, mp: 233–235 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.81 (3H, s, OCH_3), 6.24 (1H, d, J = 9.3 Hz, H-3), 7.56 (1H, d, J = 9.6 Hz, H-4), 6.80 (1H, s, H-5), 6.80 (1H, s, H-8), 1.43 (6H, t, $2CH_3$), 3.30 (4H, m, 2 N–CH_2–CH_3), 3.42 (2H, t, N–CH_2–C), 4.56 (2H, t, C–CH_2–O); R (KBr, cm^{-1}): 3428, 3050, 2946, 1721, 1614, 1565, 1513, 1387, 1278, 1146; MS (ESI, *m*/*z*): $[M+H]^+$ 291.

4.1.6.3. Preparation of 6-methoxy-7-(2-morpholinoethoxy)-2*H*-chromen-2-one (C3). Yield, 81.97%; yellowish crystals, mp: 136–139 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.92 (3H, s, OCH_3), 6.25 (1H, d, J = 9.6 Hz, H-3), 7.56 (1H, d, J = 9.3 Hz, H-4), 6.99 (1H, s, H-5), 7.00 (1H, s, H-8), 2.96–3.76 (8H, m, morpholine), 3.20 (2H, t, N–CH_2), 4.24 (2H, t, CH_2–O); IR (KBr, cm^{-1}): 3494, 3050, 2946, 1721, 1614, 1565, 1513, 1387, 1278, 1146; MS (ESI, *m*/*z*): $[M+H]^+$ 306.

4.1.6.4. Preparation of 6-methoxy-7-(2-(4-methylpiperazin-1-yl)ethoxy)-2*H*-chromen-2-one (C4). Yield, 75.7%; yellow powder, mp: 198–201 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.82 (3H, s, OCH_3), 6.24 (1H, d, J = 9.6 Hz, H_3), 7.56 (1H, d, J = 9.3 Hz, H_4), 6.76 (1H, s, H_5), 6.78 (1H, s, H_8), 2.40 (3H, s, CH_3), 2.76–3.58 (8H, m, piperazine), 2.87 (2H, t, N–CH_2–C), 4.12 (2H, t, CH_2–O); IR (KBr, cm^{-1}): 3450, 1943, 2837, 1712, 1618, 1566, 1513, 1385, 1297, 1151; MS (ESI, *m*/*z*): $[M+H]^+$ 319.

4.1.6.5. Preparation of 6-methoxy-7-(2-(pyrrolidin-1-yl)ethoxy)-2*H*-chromen-2-one (C5). Yield, 69.2%; yellow powder, mp: 212–215 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.82 (3H, s, OCH_3), 6.21 (1H, d, J = 9.6 Hz, H_3), 7.54 (1H, d, J = 9.6 Hz, H_4), 6.79 (1H, s, H_5), 6.80 (1H, s, H_8), 1.93–2.99 (8H, m, pyrrolidine), 3.21 (2H, t, N–CH_2–C), 4.34 (2H, t, CH_2–O); IR (KBr, cm^{-1}): 3463, 3026, 2964, 2930, 1711, 1613, 1564, 1513, 1387, 1278, 1145; MS (ESI, *m*/*z*): $[M+H]^+$ 290.

4.1.6.6. Preparation of 6-methoxy-7-(2-(piperidin-1-yl)ethoxy)-2*H*-chromen-2-one (C6). Yield, 72.8%; yellow powder, mp: 115–117 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.82 (3H, s, OCH_3), 6.24 (1H, d, J = 9.6 Hz, H_3), 7.54 (1H, d, J = 9.6 Hz, H_4), 6.70 (1H, s, H-5), 6.79 (1H, s, H_8), 4.34 (2H, t, CH_2–O), 2.94 (2H, t, N–CH_2–C), 1.48–2.65 (10H, m, piperidin); IR (KBr, cm^{-1}): 3291, 3086, 2934, 1715, 1614, 1561, 1510, 1385, 1282, 1141; MS (ESI, *m*/*z*): $[M+H]^+$ 304.

4.1.7. General procedures for the synthesis of the target compounds D1–D6

A mixture of 0.248 g (0.001 mmol) of **B6** and 0.0012 mol amine were dissolved in 20 mL of ethanol. The mixture was heated in reflux for 24 h and cooled at room temperature. After the solvent evaporated, the residual mixture was subjected to silica gel column chromatography with an eluent (ethyl acetate/hexane 1:2) to obtain the desired product.

4.1.7.1. Preparation of 7-(2-hydroxy-3-(isopropylamino)-propoxy)-6-methoxy-2*H*-chromen-2-one (D1). Yield, 72.7%; yellowish powder, mp: 142–144 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.89 (3H, s, OCH_3), 6.30 (1H, d, J = 9.3 Hz, H-3), 7.63 (1H, d, 9.6 Hz, H_4), 6.86 (1H, s, H_5), 6.95 (1H, s, H-8), 1.20 (6H, d, $2CH_3$), 2.87 (1H, m, $(CH_3)_2CH$), 2.95–2.97 (2H, d, N–CH_2–C), 4.27 (1H, m, CH–OH), 4.12 (2H, d, J = 4.2 Hz, CH_2–O); IR (KBr, cm^{-1}): 3417, 3078, 2969, 2822, 1717, 1616, 1561, 1513, 1383, 1276, 1149; MS (ESI, *m*/*z*): $[M+H]^+$ 308.

4.1.7.2. Preparation of 7-(3-(diethylamino)-2-hydroxypropoxy)-6-methoxy-2*H*-chromen-2-one (D2). Yield, 97.7%; yellowish powder, mp: 82–84 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.89 (3H, s, OCH_3), 6.31 (1H, d, J = 9.6 Hz, H_3), 7.63 (1H, d, J = 9.6 Hz, H_4), 6.85 (1H, s, H_5), 6.88 (1H, s, H_8), 1.13 (6H, t, $2CH_3$), 2.70 (4H, m, 2N–CH_2–CH_3), 2.77 (2H, d, N–CH_2–C), 4.19 (1H, m, CH–OH), 4.07 (2H, d, J = 15.3 Hz, C–CH_2–O); IR (KBr, cm^{-1}): 3427, 3084, 2962, 2824, 1721, 1615, 1560, 1512, 1384, 1276, 1156, 1110; MS (ESI, *m*/*z*): $[M+H]^+$ 322.

4.1.7.3. Preparation of 7-(2-hydroxy-3-morpholinopropoxy)-6-methoxy-2*H*-chromen-2-one (D3). Yield, 74.6%; yellowish powder, mp: 120–122 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.97 (3H, s, OCH_3), 6.31 (1H, d, J = 9.3 Hz, H_3), 7.63 (1H, d, J = 9.6 Hz, H_4), 6.86 (1H, s, H_5), 6.89 (1H, s, H_8), 4.27 (2H, d, J = 3.9 Hz, C–CH_2–O), 4.52 (1H, m, CH–OH), 2.42 (2H, d, J = 6.3 Hz, N–CH_2–C), 2.50–3.57 (8H, m, morpholine); IR (KBr, cm^{-1}): 3421, 2929, 2854, 1721, 1651, 1560, 1509, 1385, 1276, 1115; MS (ESI, *m*/*z*): $[M+H]^+$ 336.

4.1.7.4. Preparation of 7-(2-hydroxy-3-(4-methylpiperazin-1-yl)propoxy)-6-methoxy-2*H*-chromen-2-one (D4). Yield, 50.4%; yellowish powder, mp: 98–100 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.89 (3H, s, OCH_3), 6.30 (1H, d, J = 9.3 Hz, H_3), 7.63 (1H, d, J = 9.6 Hz, H_4), 6.85 (1H, s, H_5), 6.88 (1H, s, H_8), 2.30 (3H, s, CH_3), 2.63–2.71 (8H, m, piperazine), 2.71 (2H, d, N–CH_2–C), 4.18 (1H, m, CH–OH), 4.07 (2H, d, J = 5.7 Hz, CH_2–O); IR (KBr, cm^{-1}): 3402, 3083, 2932, 2805, 1720, 1614, 1560, 1511, 1384, 1277, 1154; MS (ESI, *m*/*z*): $[M+H]^+$ 349.

4.1.7.5. Preparation of 7-(2-hydroxy-3-(pyrrolidin-1-yl)-propoxy)-6-methoxy-2*H*-chromen-2-one (D5). Yield, 94.0%; yellowish powder, mp: 105–108 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.89 (3H, s, OCH_3), 6.30 (1H, d, J = 9.3 Hz, H_3), 7.63 (1H, d, J = 9.6 Hz, H_4), 6.85 (1H, s, H_5), 6.88 (1H, s, H_8), 2.30 (3H, s, CH_3), 1.83–2.63 (8H, m, pyrrolidine), 4.20 (1H, m, CH–OH), 4.08 (2H, d, J = 4.2 Hz, CH_2–O), 2.77–2.84 (2H, d, N–CH_2–C); IR (KBr, cm^{-1}): 3402, 3081, 2955, 2806, 1719, 1613, 1562, 1512, 1389, 1276, 1142; MS (ESI, *m*/*z*): $[M+H]^+$ 320.

4.1.7.6. Preparation of 7-(2-hydroxy-3-(piperidin-1-yl)-propoxy)-6-methoxy-2*H*-chromen-2-one (D6). Yield, 90.4%; yellowish powder, mp: 158–160 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ, ppm: 3.78 (3H, s, OCH_3), 6.60 (1H, d, J = 9.6 Hz, H_3), 7.86 (1H, d, J = 9.3 Hz, H-4), 6.87 (1H, s, H-5), 6.95 (1H, s, H-8), 4.07 (2H, t, C–CH_2–O), 3.89 (1H, m, CH–OH), 2.58 (2H, d, J = 9.3 Hz, N–CH_2–C), 1.62–2.76 (10H, m, piperidine); IR (KBr, cm^{-1}): 3428, 3000, 2933,

2855, 1710, 1620, 1577, 1515, 1453, 1209, 1123; MS (ESI, *m*/*z*): $[M+H]^+$ 334.

4.2. Pharmacology

4.2.1. Materials

The scopoletin and its derivatives were kindly provided by Dr. Jinpei Zhou. The fertilized chicken eggs were purchased from QianYuanHao Biological Corporation Limited (Nanjing, China). The Medium 199 (M199), Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin streptomycin (10,000 units/mL penicillin; 10,000 ug/mL streptomycin) were obtained from Gibco (Grand Island, NY, USA). The endothelial cell growth supplement (ECGS) was purchased from the Upstate Biotechnology (Lake Placid, NY, USA). The recombinant human $VEGF_{165}$ was obtained from Pepro Tech Inc. (Rocky Hill, NJ, USA). Trypsin, EDTA, and Na_2 were obtained from Amresco (Solon, OH, USA). Heparin sodium was obtained from Wanbang (Jiangsu, China). Rabbit anti-Factor VIII Related Antigen antibody was purchased from Zhongshan Goldenbridge Biotechnology (Beijing, China). The antibodies p-ERK1/2, ERK1/2, p-p38, p38, p-Ake, Akt, p-Src, Src, and GAPDH were obtained from Cell Signaling Technology, USA.

4.2.2. Culture of tumor cell lines

The human pancreatic cell lines PANC-1 and BxPC-3, human breast cancer cell lines MDA-MB-231 and MDA-MB-435, human liver cancer cell line HepG2, human colon cancer cell line HCT 116, human stomach cancer cell line MKN45, human non-small cell lung cancer cell line A549, human epithelial carcinoma cell line A431, human glioma cell line SHG-44, human erythromyeloblastoid leukemia cell line K562, human promyelocytic leukemia cell line HL-60, and human T lymphoma cell line HuT 78 were all purchased from the Cell Bank of the Shanghai Institute of Biochemistry & Cell Biology, Shanghai Institute for Biological Sciences and Chinese Academy of Sciences. All cells lines were maintained in a DMEM or RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (all available from Invitrogen, Grand Island, NY, USA), and incubated in a humidified atmosphere of 95% air + 5% CO_2 at 37 °C.

4.2.3. Isolation and characterization of endothelial cell

The HUVECs were isolated from the human umbilical cord vein by trypsin treatment. The human umbilical vein obtained from healthy newborns of healthy mothers was treated with 0.1% trypsin for 12 min. The harvested endothelial cells were grown in M199 containing 15% FBS, 0.1 mg/mL heparin, 0.03 mg/mL ECGS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell characterization was evaluated by assessing Factor VIII Related Antigen (von Willebrand factor, vWF) expression, which was detected by immunocytochemical reaction with the use of the rabbit anti-Factor VIII Related Antigen antibody. Cell characteristic detection was achieved using DAB (Zhongshan Goldenbridge Biotechnology, Beijing, China). After 3–5 passages, HUVECs were collected, which were used in all experiments.

4.2.4. Endothelial cell proliferation assay

The HUVECs were seeded in 96-well plates consisting of 1×10^4 cells per well. The medium was replaced by a low-serum medium (1% fetal bovine serum in M199) containing scopoletin or derivatives. After 24 h of cell treatment, the viability of the HUVECs were analyzed by MTT [3-(4,5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide] assay as previously described.[14] The inhibition percentage of the treated cells was calculated using the formula: % inhibition = $1 - (A_{570nm} - A_{630nm})_{treated}/(A_{570nm} - A_{630nm})_{control} \times 100\%$. The IC_{50} of several scopoletin derivatives were further assayed.

4.2.5. Cytotoxicity assay

The cytotoxicity of the scopoletin derivatives, which have potent HUVEC inhibition ability, was analyzed by MTT assay with PBMC among the various cancer cell lines. The PBMCs were separated from 10 mL of heparinized human blood samples by centrifugation on a human lymphocyte separation medium (Tbdscience Biotech, Tianjin, China).

4.2.6. Endothelial cell migration assay

The migration ability of HUVECs was examined by Boyden Chamber assay. The HUVEC with 80% confluence was washed once with PBS and was serum-starved in the basal media (without serum and growth supplements) for 12 h. The harvested cells were counted, and reached a volume of 1×10^6 cells/mL. DMSO (control) and scopoletin or scopoletin derivatives (final concentration: 25 μmol/L) were added to the cell suspension for 30 min at room temperature with 500 μL of fresh M199 medium (1% FBS) containing 10 ng/mL $VEGF_{165}$ in the lower wells (24-well plate) (Corning, NY, USA). The cell migration Millicell chamber (8.0 μm/6.5 mm Billerico, USA) was inserted at an angle of 45°, gently pressed down to avoid generating bubbles, and 100 μL of the pre-treated cell suspension was added to each chambers. After 20 h of incubation, the cells were fixed and stained. Five randomly chosen fields were counted and photographed using a fluorescence microscope (Zeiss Axio Observer **A1**, Oberkochen, Germany).

4.2.7. Tube formation assay

In sterile conditions, the 96-well plates were coated with Matrigel at 50 μL/well (BD Biosciences, Bedford, MA, USA) without introducing air bubbles, and set at 37 °C for 30 min to allow gelling. The HUVEC was pre-incubated in M199 containing 1% FBS for 6 h, and then harvested after trypsin treatment and was suspended in M199 containing 1% FBS and 10 ng/mL $VEGF_{165}$ at 5×10^5 cells/mL. DMSO (control group) and scopoletin or scopoletin derivatives (final concentration: 25 μmol/L) were added to the cells for 30 min before plating onto the Matrigel. After 5 h, the HUVECs differentiated and formed into capillary-like structures on the Matrigel. The enclosed networks of complete tubes from five randomly chosen fields were counted and photographed using a microscope (100×).

4.2.8. Chicken chorioallantoic membrane (CAM) assay

The fertilized chicken eggs were incubated for seven days at a relative humidity of ~55% at 37.5 °C. The eggs were arranged with the rounded portion positioned upwards at 45° to prevent microbial growth, and were regularly turned (at least two times per day). On the experiment day, the eggs were inspected using an egg candler and only live, fertilized eggs were randomly divided with ten eggs per group. A 1.5 × 1.5 cm 'window' was created on the rounded portion of the egg where the air sac was located. The air sac membrane was carefully punctured using an injection needle to avoid breaking the blood vessel. About 50 μL of sterilized water was injected between the air sac membrane and the CAM so that the air sac membrane could be easily peeled off. Sterilized filter paper (5 × 5 mm) saturated with DMSO (control group) and scopoletin (50 and 100 nmol/egg) or scopoletin derivatives (5 and 10 nmol/egg) were air-dried and then placed on the CAMs. The eggs were then covered with parafilm and were placed back to the incubator. After three days, 20% fat emulsion (Chia-tai Tianqing Pharmaceutical Co., Jiangsu, China) was injected into the CAM, and the blood vessels were photographed using a Sony α100 camera (Sony, Japan).

4.2.9. Zymography

The HUVECs were treated with DMSO (control group) and scopoletin (100 μmol/L) or **A1** (10, 25, and 50 μmol/L) for 12 h. The suspension was collected and centrifuged to detect the activity of MMP-2 and MMP-9 enzymes. The samples were prepared in the standard SDS–PAGE treatment buffer but without boiling and without a reducing agent. For the substrate, 1 mg/mL gelatin was pre-polymerized on a 10% polyacrylamide gel. After electrophoresis, the SDS was removed from the gel and was incubated in a raising buffer (2.5% Triton X-100, 50 mmol/L Tris–HCl, 0.05% NaN_3, 5 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, and pH 7.6), followed by incubation in an appropriate digestion buffer (50 mmol/L Tris–HCl, 0.05% NaN_3, 5 mmol/L $CaCl_2$, and 1 μmol/L $ZnCl_2$, pH7.6) for 14 h at 37 °C. The zymogram was subsequently stained with coomassie brilliant blue. The clear bands against the darkly stained background were photographed using the Gel/Chemi Doc System (Bio-rad, CA, USA).

4.2.10. Western blot

The HUVECs were pre-treated with DMSO (control group) and scopoletin (100 μmol/L) or **A1** (10, 25, and 50 μmol/L) for 30 min and were stimulated with 40 ng/mL $VEGF_{165}$ for 5 min (for p-ERK1/2, p-p38 protein detection) or 30 min (for p-Akt, p-Src protein detection). After stimulation, the cells were collected and lysed on ice. The lysate was centrifuged at 13,000×*g* for 5 min at 4 °C. The protein concentration in the supernatants was detected using a Nanodrop 1000 Spectrophotometer (Thermo, NH, USA). An equal amount of protein was separated on 13% SDS–polyacrylamide gels (SDS–PAGE) and transferred onto the PVDF membranes (Millipore, MA, USA). The proteins were detected using specific primary antibodies (p-ERK1/2, ERK1/2, p-p38, p38, p-Ake, Akt, p-Src, Src, and GAPDH) for 2 h at room temperature, followed by HRP-conjugated secondary antibodies for 1 h at room temperature. The detection of the developed proteins was performed using enhanced chemiluminescence (Luminata Crescendo Western HRP substrate, Millipore, USA). The membranes were then exposed to the film.

5. Statistical analysis

All results shown are expressed as means ± SEM from triplicate experiments that were performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. All comparisons were made relative to the untreated control group, and the significance of difference was indicated as $^{*}P$ <0.01.

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